Copper-Binding Amyloid Precursor Protein Undergoes a Site-Specific Fragmentation in the Reduction of Hydrogen Peroxide†

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ABSTRACT: The extracellular domain of transmembrane Aβ amyloid precursor protein (APP) has a Cu(II) reducing activity upon Cu(II) binding associated with the formation of a new disulfide bridge. The complete assignment of the disulfide bond revealed the involvement of cysteines 144 and 158 around copper-binding histidine residues. The vulnerability of APP−Cu(I) complexes to reactive oxygen species was elaborated as a site-specific and random fragmentation of APP in a time-dependent manner and at low concentrations of H_2O_2. Analysis of the specific reaction revealed the generation of C-terminal polypeptides, containing the Aβ domain. APP catalyzed the reduction of H_2O_2 and oxidation of Cu(I) to Cu(II) in a "peroxidative" reaction in vitro. The resulting bound copper−hydroxyl radical intermediate [APP−Cu(II)(·OH)] then likely participated in a Fenton type of reaction with radical formation as a prerequisite for protein degradation. Evidence from two observations suggests that the reaction takes place in two phases. Bathocuproine, a trapping agent for Cu(I), abolished the initial fragmentation, and chelation of Cu(II) by DTPA (diethylenetriaminepentaacetic acid) interrupted the reaction cascade induced by H_2O_2 at later stages. Consequently, the results suggest that a cytotoxic gain-of-function of APP−Cu(I) complexes might result in a perturbation of free radical homeostasis. What significance such a perturbation may have for the pathogenesis of Alzheimer's disease remains to be determined.

The common denominator at least in familial AD (FAD)[1] is the processing of the amyloid precursor protein (APP), leading to an elevated extracellular and intracellular concentration of the soluble amyloidogenic 42 or 43 amino acid Aβ peptide 1−42(43) which is selectively deposited in AD and Down's Syndrome brains [for a review, see (*1*)]. The proposed function of the holoprotein includes roles in cell growth and neurite length regulation, cell−cell and cell−matrix adhesion, and copper and zinc transport (*2*−*9*). Consistent with its latter role, incubation with Zn(II) increased binding of APP to heparin and has been shown to potentiate the inhibition of coagulation factor XIa (FXIa) by APP isoforms possessing the Kunitz-type inhibitory domain (KPI) (*10*−*13*).

Whereas Zn(II) exclusively exists in one oxidation state and is assumed to play a purely structural role, we found that APP binds Cu(II) in the region of APP135−155 (*14*−*16*). This binding includes the reduction of Cu(II) to Cu(I). Accordingly, in vitro APP has a function in electron transfer to Cu(II) with the copper-binding site in APP remaining intact even after the redox reaction (*15*, *16*).

Although copper is an important component of various enzymes, copper ion mediated oxidative damage to proteins through reactive oxygen radicals can be an important process in vivo (*17*, *18*). Thus, a metal ion catalyzed oxidation of APP might be the missing link for an understanding of the increased level of protein oxidation observed in Alzheimer's disease and normal aging (*19*) and, in general explain, how oxidation could be important in a variety of neurodegenerative diseases [reviewed in (*20*)]. But the most convincing evidence so far for a link between neurological disorders and oxygen radical formation is the strong association found between familial amyotrophic lateral sclerosis (FALS) and

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[1] Abbreviations: Aβ, amyloid Aβ peptide; AD, Alzheimer's disease; FAD, familial Alzheimer's disease; FALS, familial amyotrophic lateral sclerosis; APP, amyloid precursor protein; SOD, superoxide dismutase; EPR, electronic paramagnetic resonance; ESI-MS, electrospray ionization mass spectrometry; BC, bathocuproine disulfonate; DTPA, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide.

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mutations in the Cu/Zn superoxide dismutase (SOD) gene, implicating that oxygen radicals might be responsible for the selective degeneration of motor neurons occurring in FALS (*21*−*23*).

In the present study, we investigated the site-specificity of metal ion catalyzed modification of cysteine residues in APP of Alzheimer's disease. Utilization of hydrogen peroxide led to C-terminal fragmentation of APP in a "peroxidative" reaction of APP−Cu(I) complexes. Thus, we propose a possible mechanism for the relationship between the oxidation site and the higher order structure of APP.

Consequently, the results suggest that a cytotoxic gain-of-function of APP−Cu(I) complexes in the redox reaction with H_2O_2 might result in a perturbation of free radical homeostasis and/or lead to an accumulation of oxidized protein which is known to be associated with a number of diseases, including Alzheimer's disease (*24*).

EXPERIMENTAL PROCEDURES

All the reagents were of highest purity and obtained from Sigma. Hydrogen peroxide was obtained from Merck (Perhydrol Suprapur, 30%).

Construction of Expression Vectors and Purification of Proteins. Full-length (APP_{770}) and a C-terminally truncated recombinant form of APP (APP_{N262}) were prepared and purified by methods essentially as described (*9*, *14*, *15*). Protein determinations were made according to Bradford (*25*), by using the Protein Assay Reagent (Bio-Rad), employing BSA as a standard.

Identification of -SH Groups of APP Involved in Oxidation by Cu(II). Recombinant APP_{N262} was separated from salts and SDS by gel filtration on Excellulose GF-5 columns (Pierce) and renatured in 10 mM Tris-HCl, pH 7.5. The protein (0.1 mg/mL) was oxidized in the presence of 10 μM Cu(II) in phosphate-buffered saline (PBS) at 37 °C for 30 min. The oxidized APP_{N262} and the control [without Cu(II) treatment] were then carboxymethylated to modify remaining free cysteines. The reaction was carried out with final concentrations of urea and iodoacetamide of 3 M and 100 mM, respectively. The samples were desalted over a GF-5 column equilibrated with 50 mM sodium phosphate buffer (pH 8.0).

Nonoxidized (30 μg) and Cu(II)-oxidized (30 μg) APP_{N262} were then digested with 1 μg of endoproteinase Asp-N (Boehringer) at 37 °C for 48 h. The copper-binding fragment was isolated by affinity chromatography on copper-loaded chelating Sepharose as described previously (*14*) except that all buffers contained 4 M urea. The eluate was injected onto a RP-HPLC column (Aquapore RP-300, Applied Biosystems); the peptides were eluted using a linear gradient (buffer A, 0.1% TFA; buffer B, 70% acetonitrile in 0.1% TFA) from 0% to 70% buffer B at a flow rate of 200 μL/min, sequenced (Applied Biosystems 477A), and analyzed by LC-ESI MS as described (*15*, *16*).

Analytical Electrophoresis and Western Immunoblotting. The electrophoresis was carried out either overnight at a constant voltage of 55 V or for 4 h at 200 V by using 16.5%/3% Tris−Tricine or 15% Tris−glycine gels. Proteins were silver-stained by using the Silver Stain Kit (Bio-Rad, 161-0443) according to Merril et al. (*26*).

The proteins were transferred onto nitrocellulose membranes at 350 mA for 3 h (*27*). The membranes were blocked with 10% skim milk in PBS overnight at 4 °C. The primary antibody W0-2 (anti Aβ 1−16) reacting with APP and Aβ (*28*) was diluted in PBS (1.5 μg/mL) and incubated for 2 h at room temperature. The immunoblot was developed by exposing to the horseradish peroxidase-conjugated anti-mouse Ig (Amersham Corp.) (1:2500 in PBS) and using the ECL detection system (Amersham Corp.). All gels and immunoblots shown represent the results of at least three experiments from different protein preparations.

Oxidation of Purified APP by Copper. Recombinant APP_{770} (0.1−0.2 μg/mL, 1.17−2.34 nM) was diluted in PBS, and the solution was adjusted to 5 mM EDTA, incubated at 37 °C for 15 min to remove traces of bivalent ions, and separated from excess EDTA by GF-5 columns. Protein-containing eluate fractions were pooled, and oxidative reactions were started by addition of $CuCl_2$ to 10 μM. Incubations were conducted in a water bath at 37 °C as previously described (*15*). Samples were concentrated by vacuum centrifugation (SpeedVac) and subjected to gel filtration as described above, here to remove free copper(II). Copper-untreated APP samples were similarly treated and incubated.

Proteolytic Susceptibility of H_2O_2-Modified APP. The proteolytic susceptibility of control and H_2O_2-treated APP_{770}−Cu(I) complexes was determined in PBS buffer containing 100 μM to 1mM H_2O_2. In a typical experiment, 2.2 μg of APP_{770}−Cu(I) was incubated with 100 μM H_2O_2 in 10 mM Tris-HCl (pH 7.5) in a volume of 400 μL at 37 °C. At appropriate times, samples were removed, and the reaction was stopped by lyophilization before redissolving the protein in SDS sample buffer to analyze the proteolytic susceptibility of APP_{770}−Cu(I).

Protein Electroelution from Micropreparative Gels. A strip of the resolving Tris−Tricine gel was cut to detect proteins by immunoblot analysis. Corresponding bands of stained proteins were sliced without dye or silver staining and submitted to electroelution for 4 h at a constant current of 50 mA in 100 mM Tris−CHCOOH, 0.05% SDS, pH 7.4 [molecular mass cutoff of the dialysis membranes was 2 kDa (Sigma, D-7884)].

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI). Analysis was performed on a Vision 2000 (Finnigan) mass spectrometer with a nitrogen laser and operating at an accelerating potential of 85 kV in the reflectron mode. External mass assignments were made with bovine serum albumine (BSA) [mass-to-charge ratio (*m*/*z*) 66 431; Sigma/Finnigan]. In a typical experiment, 0.5 μL of a protein sample was mixed with 0.5 μL of matrix solution consisting of 1% 2,4-dihydroxybenzoic acid (20% acetonitrile, 0.08% TFA). A 1 μL aliquot was placed on the probe tip and allowed to dry.

Electrospray Mass Spectrometry (ESI-MS). On-line analysis was performed with a tandem quadrupole mass spectrometer (TSQ7000; Finnigan-MAT, Bremen) equipped with an electrospray ion source. Each scan was acquired over the *m*/*z* range 400−1500 in 2 s. The peptides were dissolved in 10 μL of methanol/acetic acid, 50/2 (v/v), and 5 μL of the solution was injected. Peptides were identified by their molecular mass calculated from the *m*/*z* peaks of the single- or multiple-charged ions.

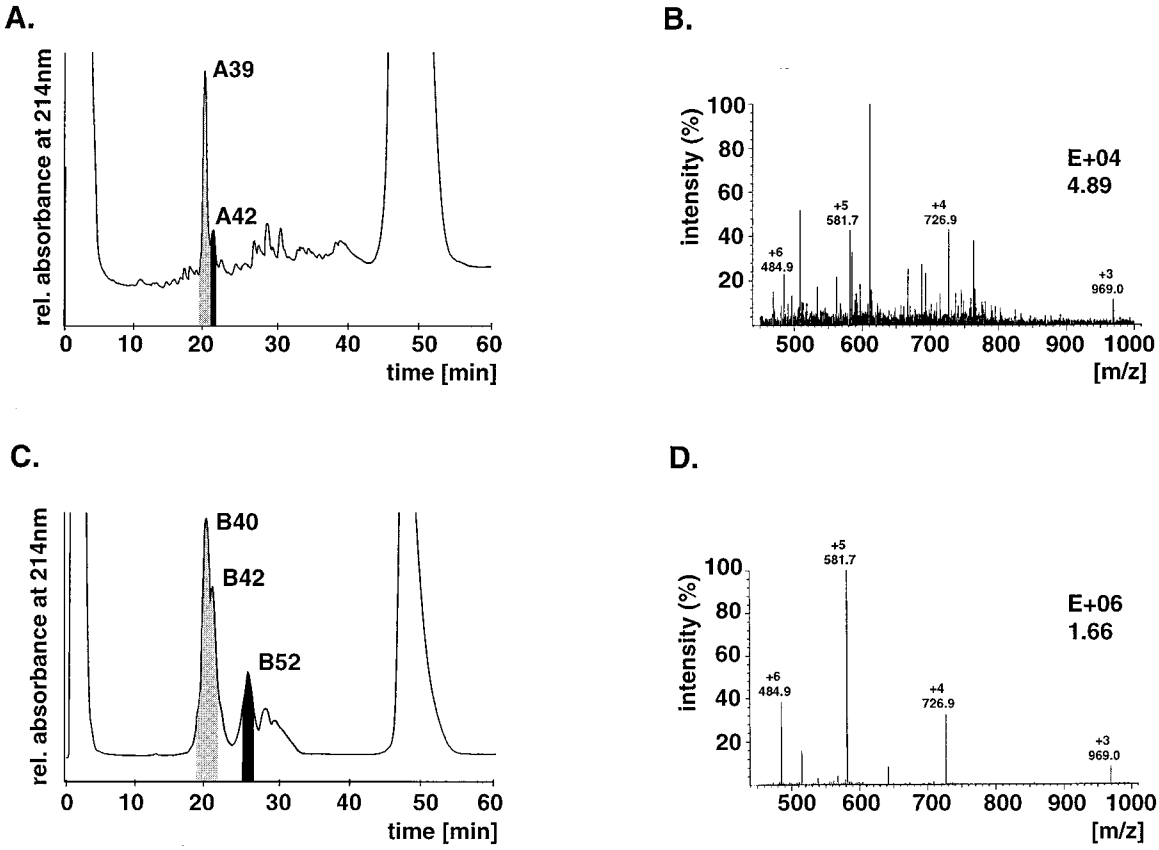


FIGURE 1: HPLC purification of copper-binding peptides obtained by endoproteinase Asp-N digestion of APP_{N262} encoded by exons 1−6. Peptides were those eluted from chelating Sepharose loaded with copper (A). Edman degradation and mass spectrometry revealed the sequence of peak A42 to contain an oxidized peptide with APP residues 142−166 (B) and peak 39 to contain a C-terminally truncated versions of this peptide (B and Table 1). HPLC purification of synthetic peptide APP142−166 eluted from chelating Sepharose loaded with copper in three separate fractions, B40, B42, and B52 (C). Sequence and mass spectrometry analysis identified the majority of oxidized peptides with an intramolecular disulfide bridge in fractions B40 and B42 (D) and the minority to be composed of a mixture of disulfide cross-linked dimers in fraction B52 (C and Table 1).

EPR Spectroscopy. X-band spectra were recorded with a Bruker ESP 300E cw spectrometer, equipped with a helium flow cryostat ESR910 (Oxford Instruments). All buffers were treated with Chelex 100 resin (Bio-Rad) to remove trace metals. Peptide samples were diluted in 23.5 mM $NaHCO_3$/CO_2 buffer (pH 7.5) to 200 μg/mL containing 10 μM $CuCl_2$ and incubated at 37 °C for 15 min. The reaction was stopped after transferring 200 μL of the solution to 4 mm quartz EPR tubes (Spintech, 707SQ) by freezing the samples in liquid nitrogen until EPR analysis. For extended reaction with hydrogen peroxide, individual samples were thawed and solutions adjusted to 2 mM hydrogen peroxide.

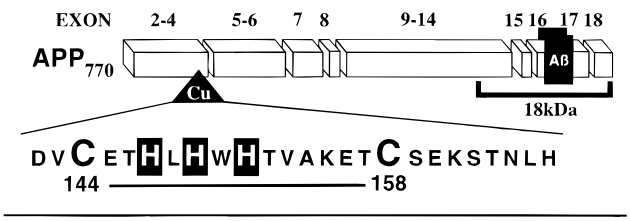
Conditions for EPR measurement were as follows: temperature, 30.0 K; modulation frequency, 100 kHz; scanning field, 310 ± 100 mT; microwave power, 1.0 mW/23 dB; receiver gain, 200 000; modulation frequency, 9.65000 GHz; sweep time, 83.886 s.

RESULTS

To map the cystine formed during the reduction of Cu(II) to Cu(I) in APP, we used proteolytic fragments of the purified fusion protein APP (APP_{N262}) containing the previously identified Cu(II)-binding site of APP residing within APP residues 135−155 (*15*). A digestion of the copper-oxidized and carboxymethylated APP with endoprotease Asp-N yields one major peak (Figure 1A; peak A39) followed by a minor one (Figure 1A; peak A42) after affinity chromatography on copper(II)-charged chelating Sepharose. The control without Cu(II) treatment did not give rise to any peptides eluting at this retention time. This failure was caused by side reactions of iodoacetamide affecting histidines of the copper-binding site whereas in the sample these sites were protected through the copper-induced disulfide bridge and bound Cu(I) itself (data not shown). Edman degradation of corresponding peptides from both peaks A39 and A42 revealed the sequence to commence at residue 142 of APP_{695} (Table 1). To confirm the possible participation of Cys-144 and Cys-158 in the redox reaction and cystine formation (*15*), ESI-MS was employed to obtain a complete analysis of the peptides eluting from the affinity column (Figure 1B). Electrospray mass spectrometry revealed a single series of ions in the minor HPLC peak fraction A42 (Figure 1A) that correspond to a mass of 2903.6 for oxidized APP142−166 (Table 1). These data indicate that the minor HPLC fraction contained a single oxidized peptide (APP residues 142−166) and thus permitted the assignment of a disulfide linkage between Cys-144 and Cys-158, differing in two mass units from the peptide in its reduced form (2905.6). The complete analysis by ESI-MS of the fraction eluting from the affinity column failed to show dimerized or cross-linked APP142−166 peptides. Thus, the lack of detection of any intermolecular disulfide bridges and the tendency to form exclusively an intramolecular disulfide were assumed to be due to an intrinsic activity of the primary

Table 1: Peptides Detected in Endoprotease Asp-N Digests of Oxidized APP and Synthetic Peptide APP142−166 Analyzed by HPLC/ESI-MS

peak	t_R (min)	MW obsd	MW calcd	peptide amino acids
A39	19.5	1745.7	n.d.	n.d.
		2355.9	2352.9	APP142-161
		2471.6	n.d.	n.d.
		2535.9	2541.1	APP142-163
		2658.8	2655.2	APP142-164
A42	21.0	2903.4	2903.6	APP142-166 ox.
B40	20.0	2903.5	2903.6	APP142-166 ox.
B42	21.0	2903.3	2903.6	APP142-166 ox.
B52	26.0	5806.7	5807.2	APP142-166 dimer



sequence. To test this assumption, we synthesized a peptide with APP residues 142−166 (Table 1). This peptide, comprising Cys-144 and Cys-158, was oxidized on a Cu(II)-charged chelating Sepharose column and eluted with EDTA. ESI-MS revealed that HPLC fractions eluting between 20 and 21 min (Figure 1C) contained the peptide with an intramolecular disulfide linkage (Table 1) corresponding to a mass of 2903.6 compared to the nonoxidized peptide with a mass of 2905.6 (Figure 1D). Fraction B52 appeared to be composed of a mixture of disulfide cross-linked dimers (Table 1). Quantification using peak areas gives a 6-fold higher ratio for the formation of an intramolecular disulfide compared to an intermolecular disulfide cross-linked peptide, APP142−166. Thus, this result obtained for the synthetic peptide provides convincing evidence for the intrinsic activity of APP residues 142−166 to form a site-directed intrachain disulfide bond between Cys-144 and Cys-158.

ESI-MS showed that the corresponding HPLC fraction of the major peak A39 in Figure 1A contained ions the molecular masses of which could be assigned to APP142−166 with two, three and five amino acid residues lacking at the ragged C-terminus of APP142−166 (Table 1). Thus, we conclude that there is a significant population of C-terminal microheterogeneity that might be mediated by oxygen radical-induced damage to peptide bonds surrounding the copper-binding site.

To examine this aspect and to confirm our earlier proposal that APP−Cu(I) complexes may be particularly vulnerable to peroxides, we undertook an EPR investigation of APP142−166. The reducing potential of APP142−166 for Cu(II) was identified by the decreased signal of Cu(II) in EPR analysis with a 30-fold molar excess of peptide to $CuCl_2$ (Figure 2A). A peptide, that carried amino acid substitutions at cysteines and the Cu-coordinating histidines, showed an EPR spectrum of a regular type II copper site (*29*), with an axial $g_\perp$ (=2.06) feature and with a hyperfine coupling $A_{||}$ of 142×10^{-4} cm^{-1} and a $g_{||} = 2.28$ (data not shown). These results are consistent with EPR-nondetectable Cu(I) as an EPR-silent complex and corroborate our previous findings of Cu(I) formation by APP using bathocuproine disulfonate as an indicator molecule for Cu(I) (*15*). Most interestingly, the

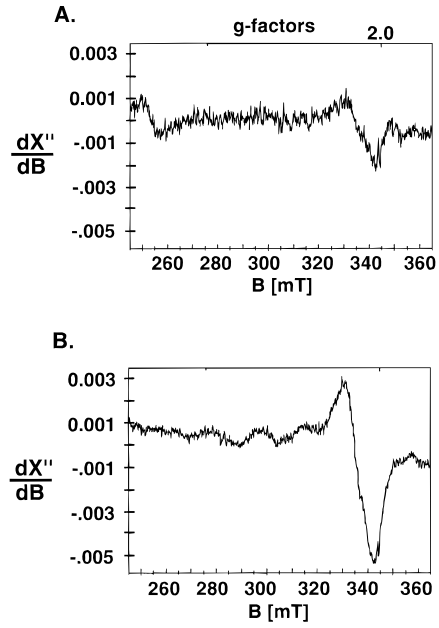


FIGURE 2: EPR spectra of H_2O_2-untreated (A) or treated APP142−166−Cu(I) (B). The EPR spectrum of (A) represents traces of nonreduced Cu(II) present in the initial copper−peptide complex. After addition of H_2O_2, the signal accounted for 10 μM EPR-detectable copper, as measured versus a Cu(II) standard.

EPR signals of paramagnetic copper that disappeared upon reduction of Cu(II) by APP142−166 were reconstituted in the presence of H_2O_2 (Figure 2B). A quantification of this signal indicates that it corresponds to 10 μM Cu(II) and equals the initially added amount of Cu(II). This observation indicates that Cu(I) bound to APP142−166 was rapidly reoxidized to Cu(II) with H_2O_2 according to a reaction process that may be proposed for the Cu(II)/(I)-catalyzed Fenton-like reaction [Cu(I) + H_2O_2 → Cu(II) + ·OH + OH^-].

SDS−PAGE of the H_2O_2-treated APP_{770}−Cu(I) showed fragmentation of the protein, with the appearance of abundant peptide molecules in the molecular mass range from 4 to 16 kDa (Figure 3A) by silver staining and Aβ-containing fragments of 12−20 kDa by Western blot analysis (Figure 3B) using antibody WO-2 raised against Aβ (*28*). An extensive fragmentation of intact full-length APP charged with copper and in the presence of H_2O_2 compared to uncharged APP or in the absence of H_2O_2 became apparent by silver staining and Western blot after a 24 h incubation period (Figure 3A,B). The degradation of APP−Cu(I) complexes was strongly inhibited when the protein was preincubated with Zn(II) prior to charging with Cu(II) (Figure 3A,B). The selective binding of Zn(II), but not of Ca(II) (Figure 3A,B) or other divalent ions to APP (*10*, *12*, *13*), might antagonize a partial denaturation or unfolding in the copper-binding site of APP caused by H_2O_2.

To unravel in more detail the time course and the fragmentation profile of APP, we investigated APP−Cu(I) complexes during treatment with H_2O_2. The amount of intact APP decreased to about 50% in 4 h after treatment with H_2O_2 and disappeared totally between 24 and 48 h incubation time (Figure 4A, left panel). The degradation of H_2O_2-modified APP−Cu(I) complexes gave distinct peptide molecules with an apparent molecular mass of 25 and 18 kDa which appeared 2−4 h after the incubation of APP−Cu(I) with 200

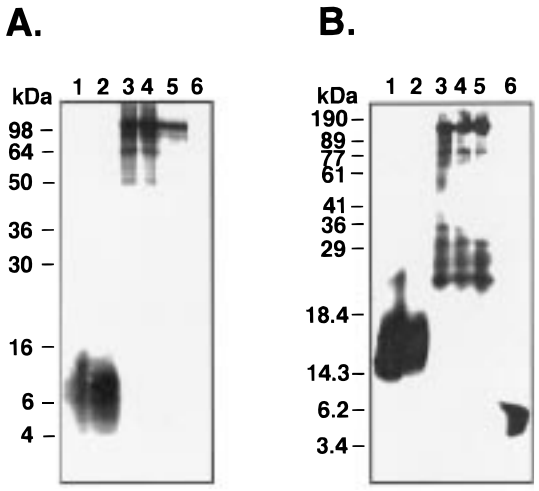


FIGURE 3: SDS−PAGE of APP−Cu(I) after reaction with H_2O_2. APP−Cu(I) (1.5 μg/mL) was incubated with H_2O_2 (100 μM) for 24 h at 37 °C. Proteins were subjected to SDS−PAGE followed by silver staining (lanes 1−5, 400 ng of APP/lane, 180 ng of synthetic Aβ/lane 6) (A) or Western blot analysis (lanes 1−5, 200 ng of APP/lane, 90 ng of Aβ/lane 6) (B). Lanes 1−3, APP−Cu(I) treated with H_2O_2; lane 2, APP pretreated with 10 μM Ca(II); lane 3, APP pretreated with 10 μM Zn(II); lane 4, APP with no Cu (nonoxidized) treated with H_2O_2; lane 5, incubation of APP−Cu(I) without H_2O_2.

μM H_2O_2. The latter peptide was the most stable since it could readily be seen after 4 h and remained resistant to chemical change as the only fragment within the following 44 h incubation period (Figure 4A). The discrete bands seen with oxidative exposure suggest that the reaction of H_2O_2 with APP−Cu(I) cannot be random, but rather may involve site-specific modifications, perhaps dictated by the initial reaction at the active copper-binding site.

The protein with an apparent molecular mass of 18 kDa was electroeluted and reanalyzed by Western blotting using the Aβ-specific W0-2 as primary antibody (Figure 4B, middle panel). The peptide fragments were subjected to matrix-assisted laser desorption/ionization mass spectrometry and Edman degradation. Whereas the N-terminus was apparently blocked, most likely by an isocyanate structure (*24*), two monoisotopic masses of 18 806.3 and 19 874.1 were detected in the eluate. The mass of 19 874.1 matches that of the sequence of amino acids 516−695 of APP_{695} (theoretical mass 19 868.1); the mass of 18 806.3 of the more prominent peptide (Figure 4) matches that of amino acids 526−695 of APP695 (theoretical mass 18 808.8) and thus denote, together with the Western blot result, these polypeptides as 180 and 170 amino acid C-terminal fragments of APP (Figure 4C, right panel, and Table 1), both containing the Aβ and cytoplasmic domains. This result is in agreement with suggested mechanisms for oxygen radical-mediated cleavage reactions of polypeptide chains and a site-specific generation of ·OH, which is likely the most important mechanism of protein damage (*30*, *31*).

Accordingly, the mechanism for fragmentation of APP−Cu(I) complexes in the presence of H_2O_2 was persued by studying the effects of dimethyl sulfoxide (DMSO) as a scavenger for ·OH, diethylenetriaminepentaacetic acid (DTPA) as a chelating agent for Cu(II), and bathocuproine disulfonate (BC) for chelating Cu(I). The data showed that neither DMSO protected APP−Cu(I) from degradation in a reaction with H_2O_2, nor did BC (Figure 5). In contrast, in the presence of DTPA, the intensities of the remaining APP bands were increased and almost equal in the reaction, independent of the agent which was added first, H_2O_2 or DTPA (Figure 5). Most interestingly, BC blocked completely the fragmentation process when BC was added prior to the addition of H_2O_2 (Figure 5). Here, BC chelated and removed Cu(I) from the copper-binding site and thus interfered with the ability of the protein to catalyze the peroxidation reaction of H_2O_2, implying that Cu(I) in APP participates in the catalytic mechanism. From these results, we conclude that Cu(I) in APP−Cu(I) complexes is rapidly reoxidized to Cu(II) with H_2O_2, as also identified by EPR spectroscopy (Figure 2B). Hydroxyl radicals, if generated in this process, are most likely responsible for the fragmentation, and would react with whatever is present at their site of formation (i.e., the copper-binding site of APP).

DISCUSSION

The present study clarified by ESI mass spectrometry and sequencing that an intrachain disulfide bridge is formed between Cys-144 and Cys-158 according to an intrinsic activity of the primary sequence during the reduction of Cu(II) to Cu(I) bound to APP. Fragmentations of APP−Cu(I) complexes were seen upon exposure of the protein to hydrogen peroxide. Electron paramagnetic resonance (EPR) studies revealed that the copper-binding site of oxidized APP contained EPR-silent Cu(I) which is, however, nullified in the presence H_2O_2. Thus, APP reduced H_2O_2 in a "peroxidative" reaction. This indicates that Cu(I) in oxidized APP rapidly reoxidized to Cu(II) with H_2O_2. Therefore, the following reaction process may be proposed for the Cu(I)-catalyzed Fenton-like reaction of APP as the first step:

$$\text{APP}-\text{Cu(I)} + \text{H}_2\text{O}_2 \rightarrow \text{APP}-\text{Cu(II)}\,(\cdot\text{OH}) + \text{OH}^-$$

The reaction scheme implies that a bound hydroxyl radical with Cu(II) (i.e., Cu(II) − ·OH) but not free ·OH was generated upon interaction of APP−Cu(I) complexes with H_2O_2. The selective and time-dependent degradation of H_2O_2-modified APP−Cu(I) complexes suggests that H_2O_2 may cause a partial denaturation or unfolding in the copper-binding site of APP that was observed to be antagonized in the presence of Zn(II). This observation is strengthened by our earlier observation that Zn(II) binding is known not to interfere with Cu(II) binding (*15*).

A denaturation of APP−Cu(I) by H_2O_2 may reveal previously shielded hydrophobic amino acid residues as newly preferred proteolytic substrates. Such fragments, such as the 18 kDa fragment with 170 residues that was liberated by the reaction of APP−Cu(I) and H_2O_2, may be further degraded by intracellular peptidases to the amyloidogenic 100 amino acid C-terminal fragment of APP (*32*). Recently, it has also been reported that the intact Aβ domain can be generated by nonspecific proteases from such molecules (*33*). These findings are in agreement with other suggestions that at least the β-secretase activity necessary to produce the N-terminus of Aβ might not be due to sequence-specific proteases (*34*).

Our results suggest that copper binding to APP and reduction are prerequisites for increased protein degradation as also has been observed for Cu/Zn−SOD (*35*, *36*). The

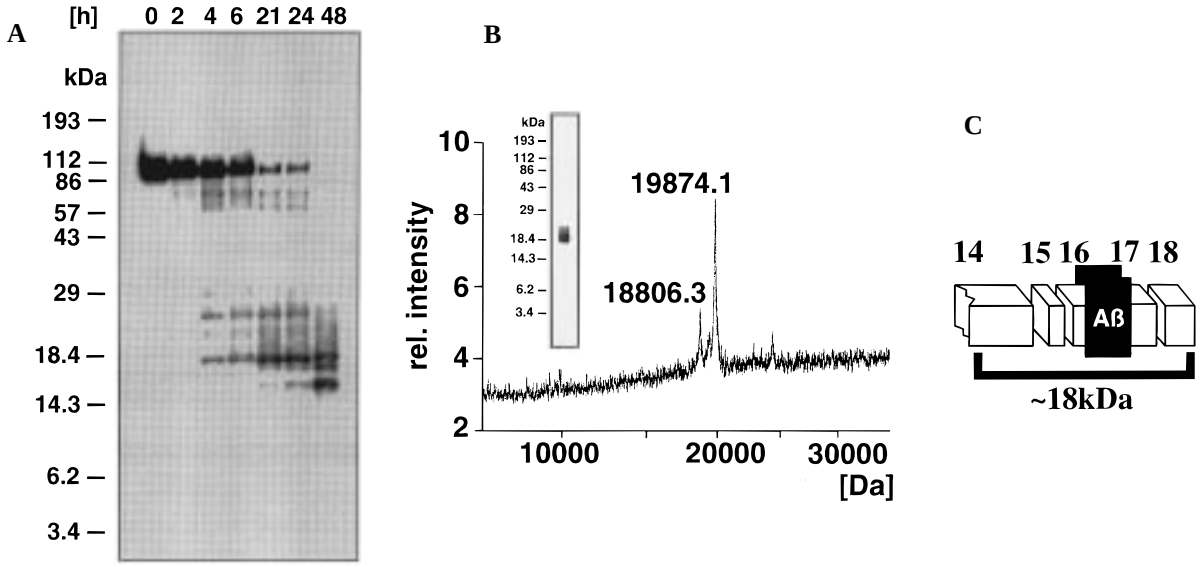


FIGURE 4: Time-dependent changes of the original band of APP−Cu(I) on SDS−PAGE after treatment with H_2O_2 (A, left panel). APP−Cu(I) was treated with H_2O_2 for various time periods as indicated and subjected to SDS−PAGE (16.5%, 3% Tris−Tricine) followed by Western blot analysis. Note that a homogeneous band at 18 kDa containing the Aβ sequence was generated by treatment in the APP−Cu(I)/H_2O_2 system. Molecular mass determination by MALDI of the 18 kDa band (Figure 4B, middle panel) after electroelution. The inset shows the Western blot analysis (WO-2) of the eluted protein (2.5% loaded) (B). Domain structure of the sequence of amino acids 516/526−695 of APP_{695} (C, right panel).

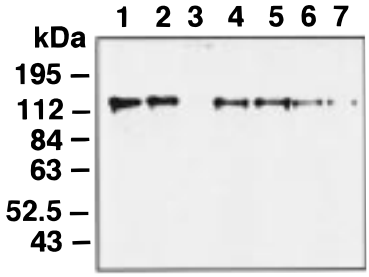


FIGURE 5: SDS−PAGE of APP−Cu(I) after reaction with H_2O_2. APP−Cu(I) (4 μg/mL) was incubated with H_2O_2 (100 μM) for 24 h at 37 °C. Proteins were subjected to SDS−PAGE (12.5%) and stained with Coomassie Briliant Blue. Lane 1, untreated APP; lanes 2−7, APP−Cu(I) treated with H_2O_2; lane 2, in the presence of BC (1 mM), added prior to H_2O_2; lane 3, with BC (1 mM) added after H_2O_2; lane 4, DTPA (1 mM) added prior to H_2O_2; lane 5, DTPA (1 mM) added after H_2O_2; lane 6, DMSO (10%, v/v) added after H_2O_2; lane 7, DMSO (10%, v/v) added prior to H_2O_2.

fragmentation process for Cu-modified APP was strongly inhibited by BC when added prior to H_2O_2, and the chelation of Cu(I) by BC blocked its initiation. This indicates the importance of Cu(I) in the critical step, the reduction of H_2O_2, where H_2O_2 accepts an electron from Cu(I). A chelator for divalent metal ions, DTPA, most likely interrupted the second step of the Fenton-type reaction with the Cu(II) ion cyclically reduced and oxidized during successive encounters with an excess of H_2O_2:

$$\mathrm{Cu(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Cu(I)} + \mathrm{O_2^-} + 2\mathrm{H^+}$$

This implies a continuous production of APP−Cu(II)-(·OH) that was obviously not accessible to the potent hydroxyl radical scavanger DMSO showing no inhibitory action of the fragmentation (Figure 5). Thus, the oxidation of APP−Cu(I) complexes by H_2O_2 catalyzed by bound Cu(I) suggests that the reaction strictly occurs at the copper-binding site of APP. This also implies a three-dimensional structural proximity between the site of APP−Cu(I)/H_2O_2-derived radical formation and their site of reaction (the cleavage site at the N-terminus of the 18 kDa fragment that is encoded by exon 14; Figure 4C and Table 1) since radicals will react with whatever is present at its site of production. The postulated reaction occurs at physiological concentrations of H_2O_2 and perhaps may also occur at as low as nanomolar amounts.

If both H_2O_2 and Cu(I) are available in vivo, then radicals will form. H_2O_2 is produced in vivo (*37*, *38*), either accidentally from electron transport chains, or through specific pathways (*39*). The lens of the human eye contains micromolar concentrations (*40*), and 82 nmol of H_2O_2 is produced per gram of tissue per minute in perfused livers isolated from normally fed rats (*37*). Most importantly, Aβ itself is known to cause the overproduction of H_2O_2 or related peroxides (*41*) and thus may act on APP in a feedback reaction, thereby increasing oxidative stress. For instance, a copper-induced toxicity for neuronal cells is known to result from an increased production of hydrogen peroxide, radical production, and concomitant lipid oxidation that leads to neuronal cell death. Also, oxidative reactions of H_2O_2 may be highly vulnerable for forebrain neurons since there is a variant level of catalase activity reported in those cells (*42*).

Thus, sporadic Alzheimer's disease could arise from a perturbation of free radical homeostasis and resulting neuronal toxicity by reactive oxygen species. This model is consistent with the slow onset of AD: younger persons may have greater antioxidant capacity and can withstand free radical stress. Aging coupled to environmental insults or genetic defects could exacerbate the consequences of APP fragmentation.

Even the most prevalent risk factor associated with late-onset AD has been shown to be linked to cytotoxicity modulated by the varying antioxidant activity of the apoE isoforms (*43*). The E4 allele has a higher frequency in AD patients than in age-matched controls and was found to possess the lowest activity in protecting cells from hydrogen

peroxide cytotoxicity (*43*). Although the link between APP and Aβ toxicity remains obscure, our recent finding that the Aβ domain may serve a functional axonal sorting signal for APP could provide the missing link in explaining the early accumulation of APP (*44*). This accumulation could simply be due to a competition between Aβ and APP for the sorting receptor.

Thus, the hypothesis of radical-based APP fragmentation and neurotoxicity of degradation products (*45*) would present several novel therapeutic strategies for all forms of AD.

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