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Regulation of serine protease activity by aluminum: Implications for Alzheimer disease

(β-amyloid precursor protein/proteolytic processing/inhibitors/brain)

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ABSTRACT **The brain of Alzheimer disease patients contains plaques that are diagnostic for the disease. The plaques also contain β-amyloid peptide, α_1-antichymotrypsin, and the element aluminum. We present indirect evidence that can relate all three components of plaques to each other in such a way as to suggest their involvement in the etiology of the disease. The β-amyloid peptide is derived by proteolytic processing from β-amyloid precursor proteins and some of these proteins contain a domain that is highly homologous to bovine pancreatic trypsin inhibitor. Bovine pancreatic trypsin inhibitor also inhibits α-chymotrypsin and we show that aluminum affects both the activity and the inhibition of this enzyme. At pH 6.5, in the presence of aluminum, the enzyme activity is doubled, and the inhibitor is only 1% as effective as in the absence of the metal ion. The inhibition by BX-9, a protease inhibitor prepared from protein components of amyloid plaques, is also reduced by aluminum; so too is that by α_1-antichymotrypsin but to a lesser degree. In the Alzheimer brain, we propose that aluminum may accelerate proteolytic processing of the β-amyloid precursor protein by suppression of the inhibitor domain. Thus, the β-amyloid peptide may accumulate and initiate plaque formation.**

Increased levels of aluminum have been observed in neuritic deposits, the plaques and the neurofibrillary tangles, of Alzheimer disease (AD) (1) and amyotrophic lateral sclerosis (2). Several *in vitro* studies have demonstrated the neurotoxicity of aluminum (3). Recent epidemiological evidence associates increased bioavailability of aluminum with incidence of AD (4). Because the solution chemistries of aluminum and iron are very similar (5), the observed slow accumulation of aluminum in brain and bone tissue is suggested to occur via the iron transport and storage systems (3). Despite the *in vivo* and *in vitro* evidence, no initial event(s) for the involvement of aluminum in AD or amyotrophic lateral sclerosis has been established. Therefore, some researchers have questioned (35) the importance or the role of aluminum in the early pathogenesis of these neurological disorders.

By contrast, the β-amyloid peptide found at the "heart" of AD plaques remains a consistent feature of AD (6, 7). Recent discoveries of β-amyloid mutations in some cases of early-onset familial AD further underscore the importance of β-amyloid peptide in the etiology of AD (8). The β-amyloid peptide(s) has 39–43 amino acids and is part of the family of β-amyloid precursor proteins that contain 695, 751, and 770 amino acids that upon proteolysis generate the β-amyloid peptide(s). The two larger precursors (751 and 770 amino acids long) also contain a 56-amino acid segment whose sequence is >60% homologous to the bovine pancreatic trypsin inhibitor (bPTI). This discovery suggested the possible role of the inhibitor segment in regulating the proteolytic processing of the precursor proteins to generate β-amyloid peptide(s). However, the pool of β-amyloid protein precursors is neither expressed solely in AD nor only in affected brain areas (9). Distribution of the β-amyloid precursor proteins and their transcripts is still a matter of controversy, but the differences appear to be only quantitative rather than qualitative (10, 11). Therefore, "abnormal" proteolysis of "normal" β-amyloid precursor protein still presents the most likely source of overproduction and accumulation of β-amyloid peptide (6).

Both the identification of a 56-amino acid domain in the 751- and 770-amino acid β-amyloid precursor proteins with high homology to bPTI and the recent isolation of a chymotrypsin-like protease capable of cleaving amyloid proteins in rats (12) led us to investigate *in vitro* the possible effect of aluminum on serine proteases and their regulation by inhibitors. Although the significance of the inhibitory domain for the etiology of AD is somewhat unclear (36), other researchers believe the regulatory function of protease inhibitory domain to be critical (10). Preliminary reports of this work have been presented (13, 14).

MATERIALS AND METHODS

All metal salts were of the highest available purity. All other reagents were purchased from Sigma. BX-9 protein was a generous gift from S. Sinha (Athena Neurosciences, San Francisco). Protease activity was determined spectrophotometrically according to Hummel (15). Proteolysis of transferrin was monitored by HPLC as described in Fig. 4.

RESULTS

The results show that, with benzoyl-tyrosine ethyl ester (BTEE) as a substrate at pH 6.5, aluminum trichloride activated α-chymotrypsin activity to ≈200% with an apparent K_m ($K_{m(app)}$) of 2×10^{-4} M, calculated from the total medium concentration of aluminum (Fig. 1). A Hill plot of the data yielded a one-to-one binding of aluminum trichloride to α-chymotrypsin, without affecting the K_m for the substrate (data not shown). α-Chymotrypsin activity was not affected by Cl^-, Fe(III), or Na^+. Ca(II), a frequently employed stabilizer of serine proteases (15), did activate to a similar extent, yet the $K_{m(app)}$ was 10-fold higher. To identify which of the species of the complex mixture of solution species for aluminum was responsible for activation, the metal buffering systems containing nitrilotriacetate or EDTA were used (16) to regulate the available concentrations of the $Al(H_2O)_6^{3+}$ species. No activation was seen in the presence of these metal chelators. This suggests, although does not prove, that a hydroxylated mononuclear or, more likely, a hydroxylated polynuclear species could be responsible for activation.

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Abbreviations: bPTI, bovine pancreatic trypsin inhibitor; AD, Alzheimer disease; BTEE, benzoyl-tyrosine ethyl ester.

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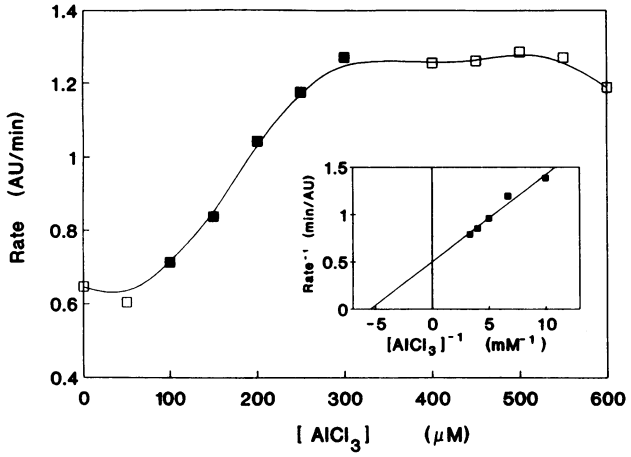

FIG. 1. Aluminum trichloride activates bovine pancreas α-chymotrypsin 2-fold at pH 6.5. (*Inset*) Lineweaver–Burk plot with a $K_{m(app)}$ for aluminum trichloride of 2×10^{-4} M. The solid symbols are those used for the $K_{m(app)}$ determination. Standard assay conditions (12) were used, except 38 mM Pipes (pH 6.5) was employed, the methanol concentration was reduced to 3%, and calcium chloride was absent from the control assays. Fresh solutions of Sigma enzyme were made daily, calibrated for activity, and kept in dilute HCl on ice to prevent autolysis. One-minute preincubations of metal and enzyme were shown to be sufficient for full activation. The synthetic substrate BTEE was not precipitated or hydrolyzed by the addition of aluminum trichloride alone. Different orders of addition did not affect activation. AU, arbitrary unit(s).

Thus, high localized concentrations of polynuclear aluminum deposits discovered in the brains of patients with AD or amyotrophic lateral sclerosis may be biologically relevant (17, 18). Clearly, additional experimentation is needed to establish the biologically "active/activating" species of aluminum (19).

Since the β-amyloid precursor proteins contain a 56-amino acid domain of high homology to the bPTI, we studied the effect of aluminum on the well-characterized interaction of bPTI and α-chymotrypsin (20). Fig. 2 shows that aluminum

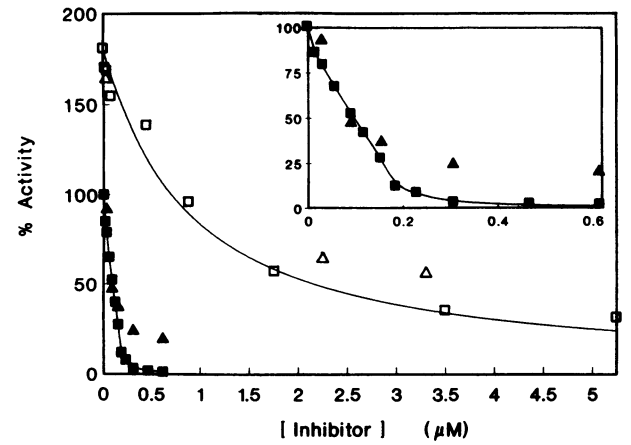

FIG. 2. Aluminum protects α-chymotrypsin from inhibition by bPTI and BX-9 (see text) to a similar extent. The abscissa gives the concentration of either bPTI or BX-9 inhibitor. (*Inset*) Enlargement of the inhibition curve for control enzyme (1.75×10^{-7} M) with bPTI (■) or BX-9 (▲). In the presence of 5×10^{-4} M aluminum, the inhibition constants (K_i) for bPTI (□) and for BX-9 (△) were ≈100-fold higher. The K_i values were calculated as published by Sinha *et al.* (20). The profiles of the control curves were similar to those previously reported, but not all of the BX-9 reacted to form inhibitor–protease complex. This was also seen in part in the original work on BX-9 (20). This may simply relate to the presence of the β-galactosidase portions of the fusion protein. However, an altered binding mechanism at the acidic pH cannot be excluded.

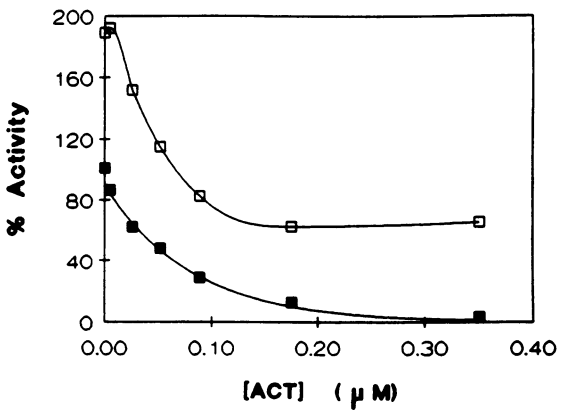

FIG. 3. Aluminum protects α-chymotrypsin from inhibition by α_1-antichymotrypsin (ACT). The K_i was increased ≈10-fold in the presence of 5×10^{-4} M aluminum (□) over control (■). Determination of K_i by using the previously determined quadratic equation (20) was very reproducible but showed a less than good curve fit for the employed conditions. The data fit more closely a single exponential decay curve. Furthermore, it appears that the inhibition in the presence of aluminum is biphasic, with an initial rate of decrease in activity comparable to that of the control curve, yet the final activity does not reach zero. Whether this is reflective of a different binding mechanism for α_1-antichymotrypsin at acidic pH values remains to be established.

dramatically protects α-chymotrypsin from bPTI inhibition (K_i without Al = 3.7×10^{-10} M; $K_{i(Al)} = 4.6 \times 10^{-8}$ M). The BX-9 fusion protein (21) contains the protease inhibitory domain of β-amyloid precursor protein inserted into β-galactosidase. Sinha *et al.* (21) showed that the inhibitory properties of this fusion protein are identical to the secreted 751-amino acid precursor species and similar to the bPTI. We observed that aluminum protected α-chymotrypsin against inhibition by BX-9 (Fig. 2).

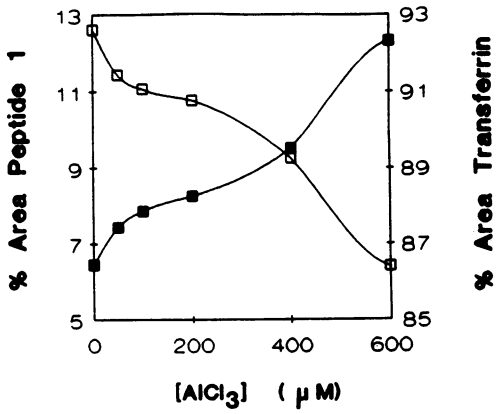

FIG. 4. Human serum transferrin proteolysis is accelerated by aluminum. Early-event limited α-chymotrypsin digestion of transferrin resulted in the formation of peptide 1. The appearance of peptide 1 (■) and disappearance of native transferrin (□) is plotted against aluminum concentration (abscissa). Reverse-phase HPLC separation was employed and the areas under the peaks were calculated. The reverse-phase HPLC was performed on a Vydac 218TP54 5m column with a Waters HPLC system. A linear gradient of 0–60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid over 60 min was employed. Data were collected and peak areas were calculated with a Waters Baseline 810 program. Various times of proteolysis and molar ratios of substrate to enzyme were tested and best results were obtained at a molar ratio of 36 and a 360-min incubation at 37°C. Proteolysis was stopped by addition of a 100 molar excess of phenylmethylsulfonyl fluoride and immediate freezing in an ethanol/dry-ice slurry. Digests were kept at −80°C until immediately before HPLC analysis.

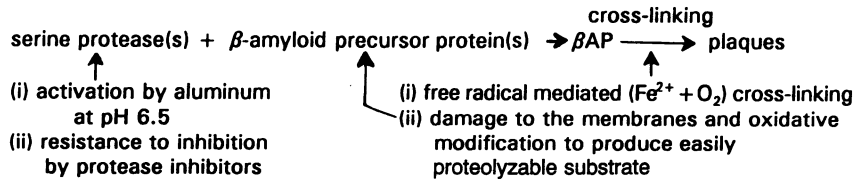

FIG. 5. Schematic representation of the role of aluminum, iron, proteases, and their inhibitors in plaque formation. βAP, β-amyloid protein.

With benzoyl-arginine ethyl ester, aluminum also activated trypsin by 140% with a similar $K_{m(app)}$ and produced only a 15-fold decrease in the binding of bPTI (data not shown). Besides β-amyloid peptide(s), another major component of AD plaques is the protease inhibitor α_1-antichymotrypsin (11). Fig. 3 shows that aluminum protected α-chymotrypsin from inhibition by α_1-antichymotrypsin, although only by ≈10-fold.

The above results were obtained with synthetic substrates. To verify whether the same is true for natural substrates, we studied limited proteolysis of human serum transferrin by α-chymotrypsin with or without added $AlCl_3 \cdot 6H_2O$. Fig. 4 shows the 2-fold activation of proteolysis of transferrin in the presence of aluminum [$K_{m(app)} = 2.1 \times 10^{-4}$ M].

Finally, supernatants of brain homogenates at pH 6.5 obtained after centrifugation at 100,000 × g also demonstrated a maximum of 2.8-fold activation of hydrolysis of BTEE in the presence of 5×10^{-4} M aluminum chloride (data not shown). This suggests that *in vitro* a subset of soluble protease(s) of cytosolic or extracellular origin, which are capable of cleaving BTEE, can be activated by aluminum.

DISCUSSION

The results show that *in vitro* aluminum-activated serine proteases, such as trypsin and α-chymotrypsin, are resistant to inhibition by protease inhibitors. Activation by aluminum is optimal at an acidic pH of 6.5, a pH value that is achieved regularly in various brain regions during ischemia and hypoxia (22), with the hippocampus affected the most (23). Acidic brain pH values have also been reported in cases of AD (24). Significantly, self-aggregation of β-amyloid protein is also suggested to be most rapid under acidic conditions (25).

To date, no single cause for AD has been proven, and it is probable that a combination of factors is responsible, at least in the sporadic or late-onset type of AD. Potential genetic factors involving β-amyloid precursor proteins are now being recognized for early-onset AD. Perhaps not coincidentally, one such mutation (Val → Phe) would generate an additional chymotryptic cleavage site (8).

In contrast, cases of identical twins acquiring AD at very different ages clearly implicate environmental factors such as aluminum (6). Perhaps direct effect of aluminum on several key homeostatic pathways must also be considered. Aluminum enhances iron-catalyzed lipid peroxidation and is thought to cause partial destruction or dismantling of the membrane. These effects are even more pronounced at acidic pH (26, 27). This could explain how protease(s) gain access to the transmembrane domain of β-amyloid precursor protein(s).

Increased aluminum (1) and ferritin (28) colocalize to the plaques in AD brains. *In vitro*, aluminum reduces the rate of iron uptake by ferritin (29), yielding unsequestered iron available for increased ferritin synthesis and production of iron-catalyzed oxygenated free radical (30). Indeed, translatibility of mRNA for ferritin, the major nonheme iron binding protein, is increased in the brains of animals injected intracranially with aluminum salts (31).

Lowered pH values observed during acidosis can increase the release of iron from ferritin and transferrin. Thus these events may lead to increased oxidative free radical damage of β-amyloid precursor proteins yielding a more readily proteolyzable substrate (32). In addition, oxygenated free radicals are also shown to enhance the plaque formation by cross-linking the β-amyloid peptides (33).

Each of these factors individually may not be significant in every case of AD. Rather, colocalization of a "critical mass" of metabolic errors is required for the onset and development of AD, especially for nonfamilial or sporadic AD. Admittedly, some of the connections of our *in vitro* data to AD and amyotrophic lateral sclerosis are as yet hypothetical. However, no definitive cause for AD has yet been demonstrated and all working models are, thus, still hypothetical. The *in vitro* data in this report offer a potential linkage between loss of regulation for processing of β-amyloid precursor proteins and the seemingly diverse changes such as accumulation of iron, aluminum, ferritin, and acidosis observed in AD brains. These relationships are shown in Fig. 5. Although the applicability of these data *in vivo* remains to be established, recent epidemiological studies seem to support this postulate. Accordingly, McLachlan *et al.* (34) reported that desferroxamine, initially shown to overcome dialysis dementia, also aided in slowing the dementia associated with AD. Desferroxamine is an effective chelator for aluminum as well as iron. Therefore, the observed relief in AD patients may be due to chelation of both of these neurotoxic metal ions.

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