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Research Article

4-Hydroxynonenal-modified amyloid-β peptide inhibits the proteasome: possible importance in Alzheimer's disease

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Abstract. The amyloid β-peptide (Aβ) is a 4-kDa species derived from the amyloid precursor protein, which accumulates in the brains of patients with Alzheimer's disease. Although we lack full understanding of the etiology and pathogenesis of selective neuron death, considerable data do imply roles for both the toxic Aβ and increased oxidative stress. Another significant observation is the accumulation of abnormal, ubiquitin-conjugated proteins in affected neurons, suggesting dysfunction of the proteasome proteolytic system in these cells. Recent reports have indicated that Aβ can bind and inhibit the proteasome, the major cytoslic protease for degrading damaged and ubiquitin-conjugated proteins. Earlier results from our laboratory showed that moderately oxidized proteins are preferentially recognized and degraded by the proteasome; however, severely oxidized proteins cannot be easily degraded and, instead, inhibit the proteasome. We hypothesized that oxidatively modified Aβ might have a stronger (or weaker) inhibitory effect on the proteasome than does native Aβ. We therefore also investigated the proteasome inhibitory action of $A\beta_{1-40}$ (a peptide comprising the first 40 residues of Aβ) modified by the intracellular oxidant hydrogen peroxide, and by the lipid peroxidation product 4-hydroxynonenal (HNE). H_2O_2 modification of $A\beta_{1-40}$ generates a progressively poorer inhibitor of the purified human 20S proteasome. In contrast, HNE modification of $A\beta_{1-40}$ generates a progressively more selective and efficient inhibitor of the degradation of fluorogenic peptides and oxidized protein substrates by human 20S proteasome. This interaction may contribute to certain pathological manifestations of Alzheimer's disease.

Key words. Amyloid-beta peptide; Alzheimer's disease; oxidative stress; proteasome; proteolysis; protein degradation; protein oxidation; 4-hydroxynonenal; free radical.

The amyloid-β peptide (Aβ) is a major constituent of senile plaques, the pathological hallmark of Alzheimer's disease. Several lines of evidence have causally implicated Aβ in the development of Alzheimer's disease pathology [1–3], however, its role in the etiology of the disease remains uncertain. Aβ-mediated cell toxicity has been proposed to occur through oxidative stress [4, 5]. Protein modifications such as carbonyl formation and nitration, and lipid peroxidation products such as 4-hydroxynonenal (HNE) and malondialdehyde [4, 6] have been reported in association with Alzheimer's disease.

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Besides providing evidence for oxidative stress, these changes render a possible biochemical explanation for the insolubility of pathologic lesions through protein cross-linking.

Biochemical and immunohistochemical studies have also identified ubiquitin and ubiquitin-conjugated proteins in the intraneuronal neurofibrillary tangles [7, 8]. Ubiquitin is a highly conserved, 76-amino acid protein, which targets several abnormal and short-lived proteins by covalent ligation, for selective degradation by the 26S proteasome [9]. The proteasome is believed to exist in two major forms: the smaller 20S proteasome which comprises the catalytic core, and the larger, 26S proteasome which includes additional subunits for polyubiquitin recognition and ATP hydrolysis [9–13]. While the 20S core proteasome can degrade oxidized proteins in an ATP- and ubiquitin-independent manner, the larger 26S proteasome is stimulated by ATP and degrades only ubiquitinylated substrates [10–12, 14, 15]. Earlier studies identified the proteasome as an alkaline protease which preferentially degraded the oxidatively modified form (over the native form) of glutamine synthase [16, 17]. Our laboratory has also shown that moderately oxidized proteins are selectively degraded by the 20S proteasome [14, 15, 18–21]. Failure to degrade moderately oxidized proteins may result in extensive protein cross-linking and the formation of insoluble aggregates [15, 18–24]. Although moderately oxidized proteins are good substrates, extensively oxidized proteins are actually poor substrates for the proteasome and their proteolytic susceptibility decreases with further oxidation [14, 15, 18–21]. Good evidence is now available that shows that heavily oxidized proteins may actually inhibit the proteasome [24–26]. The accumulation of oxidized and ubiquitinylated proteins in neurons of patients with Alzheimer's disease [27] suggests a possible role of proteolytic dysfunction in the pathogenesis of the disease.

Recent in vitro studies have shown that Aβ binds the catalytic core of the bovine proteasome [28] and inhibits its chymotrypsin-like activity [29]. If such an interaction truly occurs in vivo, it could be responsible for the accumulation of damaged proteins in neurons. Taking into account that several reactive oxygen species and their derivatives found in the Alzheimer's disease brain can interact with the Aβ peptides [4, 6], and that the 20S proteasome preferentially recognizes and degrades oxidized proteins [14, 15, 18–21], we wondered if oxidized Aβ would exhibit a different proteasome inhibition profile than native Aβ. We therefore undertook the current investigation to analyze the potential inhibitory effects of oxidized Aβ_{1-40} (a peptide comprising the first 40 residues of Aβ) on the catalytic activity of the human 20S proteasome. Oxidized Aβ_{1-40} might conceivably be recognized by the proteasome as a potential substrate, but cannot be degraded, perhaps, because it is extensively aggregated or cross-linked [24–27, 30]. Since Aβ can react with other potentially toxic products of oxidation, we also tested the effect of the major lipid peroxidation product HNE-modified Aβ_{1-40} on the activity of the proteasome.

Materials and methods

Purification and analysis of the 20S proteasome. The 20S proteasome was purified from human erythrocytes as described by Hough et al. [31]. Cells were lysed in 1 mM dithiothreitol (DTT) and, after removal of membranes, the lysates were subjected to DEAE-chromatography, followed by sucrose density gradient ultracentrifugation and separation on a monoQ column using an FPLC system (Pharmacia). Active fractions were assayed by measuring degradation of the fluorogenic peptide, suc-LLVY-MCA (a substrate for the chymotrypsin-like activity of the proteasome: succinyl-leucine-leucine-valine-tyrosyl-methylcoumaric acid). The product was analyzed by 15% SDS-polyacrylamide gel electrophoresis, as well as a 5% nondenaturing polyacrylamide gel.

Labeling and oxidation of hemoglobin. Purified hemoglobin (Sigma) was labeled by reductive methylation [32, 33] to a specific activity of 6×10^5 cpm/mg of protein. [^{3}H]-hemoglobin was oxidatively modified with various concentrations of H_2O_2 as described previously [14, 18, 19]. Any remaining H_2O_2 and protein fragments were removed by microconcentration using a membrane with a molecular weight cut-off of 10 kDa (YM-10, Amicon).

Oxidation of Aβ_{1-40}. To test the effect of oxidized Aβ_{1-40}, we used Aβ_{1-40} modified by various concentrations of hydrogen peroxide. In separate experiments, we also tested various concentrations of oxidized Aβ_{1-40}. Oxidation of Aβ_{1-40} was conducted exactly as described for hemoglobin, except that elimination of residual H_2O_2 and possible protein fragments was carried out using a microconcentrator with a membrane molecular weight cut-off of 3 kDa.

Determination of carbonyl content. Carbonyl production in the Aβ_{1-40} peptide was measured by reaction with tritiated borohydride [34], as an index of the degree of Aβ_{1-40} oxidation. Briefly, 6 µl of 1 M Tris-HCl, 10 mM EDTA, and 14 µl of 100 mM [^{3}H]-$NaBH_4$ (specific activity $\sim$100 mCi/mmol, New England Nuclear) were incubated with 50 µl oxidzed Aβ_{1-40} (10 µg) for 30 min at 37 °C. The protein was precipitated with 1 ml of 10% trichloroacetic acid by centrifugation at 11,000 *g* for 5 min. After two washes, the protein was redissolved in 100 mM sodium hydroxide and an aliquot was used for liquid scintillation counting. As suggested by Levine et al. [34], the 100 mCi/mmol

borotritide was estimated to provide about 10,000 cpm for 1 nmol carbonyl.

HNE modification of Aβ. HNE was obtained in the form of 4-hydroxynonenal-diethylacetal, converted to HNE following the manufacturer's instructions, and quantified spectrophotometrically as described by Esterbauer et. al. [35]. HNE modification of $A\beta_{1-40}$ was performed in a buffer containing 50 mM Tris-HCl (pH 7.8), 20 mM KCl, and 5 mM MgOAc for 2 h at 37 °C. The cross-linking action of HNE was stopped by addition of DTT to a final concentration of 1 mM, and HNE-modified $A\beta_{1-40}$ was used immediately.

Chymotrypsn-like activity of the proteaosme. The chymotrypsin-like activity of the proteasome was measured using the fluorogenic peptide suc-LLVY-MCA as a substrate. Purified proteasome (2 μg) was preincubated with various concentrations of $A\beta_{1-40}$ or oxidized $A\beta_{1-40}$ for 1 h at room temperature, in a buffer containing 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 5 mM MgOAc, and 0.5 mM DTT. The fluorogenic substrate suc-LLVY-MCA was then added (at 100 μM) to the reaction mix and proteolysis was allowed to continue for 1 h at 37 °C. Proteolysis was stopped by addition of an equal volume of ice-cold ethanol and 10 vol of 0.125 M sodium borate (pH 9.0). Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

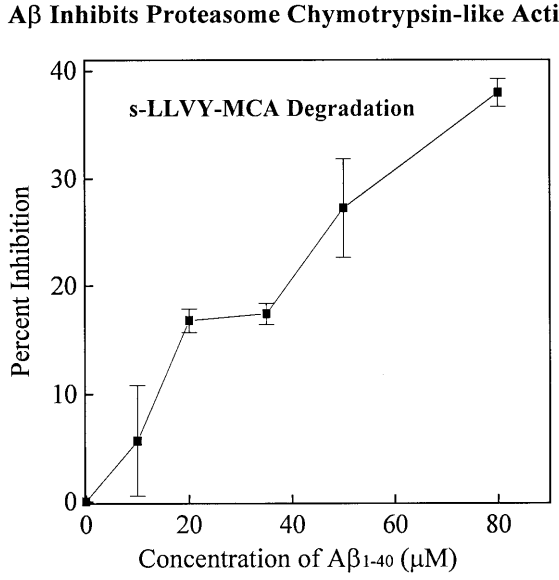

Figure 1. $A\beta_{1-40}$ inhibits proteasome chymotrypsin-like activity. The chymotrypsin-like activity of the proteasome was monitored by measuring degradation of the fluorogenic peptide substrate, suc-LLVY-MCA in the presence of increasing concentrations of $A\beta_{1-40}$. Data are represented as percent inhibition (means ± SEs of three independent experiments).

To test the effect of HNE-modified $A\beta_{1-40}$, identical assay conditions were used, except that after HNE modification, proteasome and proteolytic substrates were added directly to HNE-modified $A\beta_{1-40}$ without a preincubation period with proteasome alone. The fluorogenic peptides, suc-LLVY-MCA (100 μM) and z-LLL-MCA (carbobenzoxy-leucine-leucine-leucine-methylcoumaric acid; 100 μM) were used as substrates. Controls used were vehicle alone (water), 50 μM $A\beta_{1-40}$ alone, and 50 μM and 100 μM HNE alone.

Degradation of oxidized protein substrate. Since fluorogenic peptides are only synthetic substrates, we also tested the ability of the proteasome to degrade oxidized proteins. We have previously shown that the 20S proteasome preferentially degrades oxidized proteins, in an ATP-independent manner. Purified proteasome (5 μg) was preincubated with various concentrations of $A\beta_{1-40}$ for 1 h at room temperature and 10 μg of oxidized (or untreated) [^{3}H]-hemoglobin was then added as a proteolytic substrate, in a total reaction volume of 120 μl. Proteolysis was allowed to continue for 1.5 h at 37 °C. The reaction was stopped by addition of equal volumes of 20% trichloracetic acid (TCA) with 3% bovine serum albumin as a carrier and incubated on ice for 30 min. The TCA-soluble fraction was separated by centrifugation at 14,000 *g* for 15 min. Acid-soluble counts were measured in a scintillation counter and percent degradation was calculated as follows:

% degradation = (acid-soluble counts − background counts)/(total counts − background counts)

Results and discussion

Recent studies by Gregori et al. [28, 29] showed that $A\beta_{1-40}$ binds the catalytic core of the bovine proteasome and can inhibit its chymotrypsin-like activity. Given that several reactive oxygen species have been implicated in the pathology of Alzheimer's disease, and knowing that the proteasome preferentially recognizes and degrades oxidized proteins, we compared the effects of both oxidized $A\beta_{1-40}$ and untreated $A\beta_{1-40}$ on the activity of purified proteasome.

$A\beta_{1-40}$ did have an inhibitory effect on the proteasome, but a concentration of 80 μM Aβ was required to inhibit the degradation of suc-LLVY-MCA even by 40% (fig. 1). A concentration of 50 μM $A\beta_{1-40}$ produced only a 10% inhibition of z-LLL-MCA degradation (not shown in fig. 1, but seen later in fig. 6). These results are not consistent with the previously published inhibitory concentrations of $A\beta_{1-40}$ for the chymotrypsin-like activity of the proteasome, with an IC_{50} of 3–6 μM

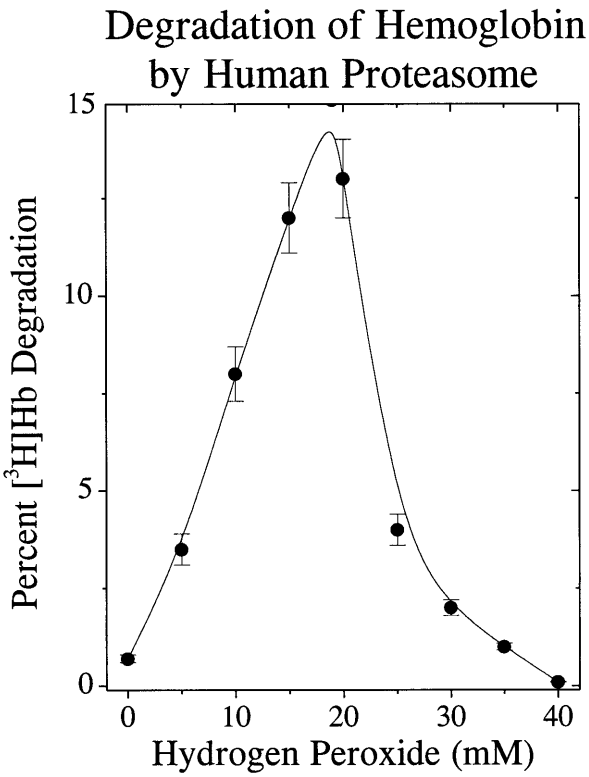

Figure 2. Effects of oxidative modification on the proteolytic susceptibility of hemoglobin. [^{3}H]-hemoglobin was exposed to various concentrations of hydrogen peroxide, microconcentrated, and then incubated for 1 h at 37 °C with purified human proteasome, to measure proteolytic susceptibility. All data are the means and SEs of three experiments.

measured by z-LLL-MCA degradation [29]. The fact that the source of the proteasome was different in these two investigations could be a contributing factor. Since only humans are known to be affected by Alzheimer's disease, we used purified human proteasome, while Gregori et al. [28, 29] used purified bovine proteasome. Although little is known about the significance of tissue- and species-specific differences in subunit composition of the proteasome, recent studies by Orlowski and colleagues [36, 37] suggest that tissue-specific subunit composition can contribute to great differences in kinetic properties and substrate specificity.

Since fluorogenic peptides are artificial substrates, we also wanted to measure the effect of $A\beta_{1-40}$ on the degradation of 'real' substrates, such as oxidized proteins. We have previously reported that moderately oxidized proteins are selectively degraded by the 20S proteasome in an ATP- and ubiquitin-independent manner [14, 15, 18–21]. Hemoglobin was labeled by reductive methylation with [^{3}H]-formaldehyde and oxidatively modified with H_2O_2. As expected, exposure to H_2O_2 progressively increased the proteolytic susceptibility of [^{3}H]-hemoglobin, up to a concentration of 20 mM H_2O_2 (fig. 2) (it should be noted that most of the H_2O_2 added to the incubation mixture is actually dissipated harmlessly without reacting with the hemoglobin). Higher concentrations of (added) H_2O_2 caused an expected decrease in proteolytic susceptibility (fig. 2), in agreement with previous studies [14, 15, 18–21].

After a 1-h pre-incubation of proteasome with various concentrations of $A\beta_{1-40}$, [^{3}H]-hemoglobin was added to the reaction mixture. $A\beta_{1-40}$ inhibited the degradation of both untreated and oxidized hemoglobin in a dose-dependent manner (fig. 3A). Although the pathophysiological concentrations of $A\beta$ are not very well-established, it should be noted that the concentration ratio of $A\beta_{1-40}$ to [^{3}H]-hemoglobin in figure 3A varied from 4:1 to 20:1. These results suggest that $A\beta_{1-40}$ is a rather poor inhibitor of the human proteasome.

As proposed in our introduction, we expected to find altered inhibition of the proteasome with oxidized $A\beta_{1-40}$. To test this hypothesis, we used increasing concentrations of hydrogen peroxide to modify $A\beta_{1-40}$, and tested for oxidative modification of the peptide using sodium borohydride reduction to quantify the carbonyl content. As shown in figure 4A, the $A\beta_{1-40}$ peptide was progressively oxidized with increasing H_2O_2 concentrations. When we tested oxidized $A\beta_{1-40}$ as a potential inhibitor of the proteasome, however, it appeared to

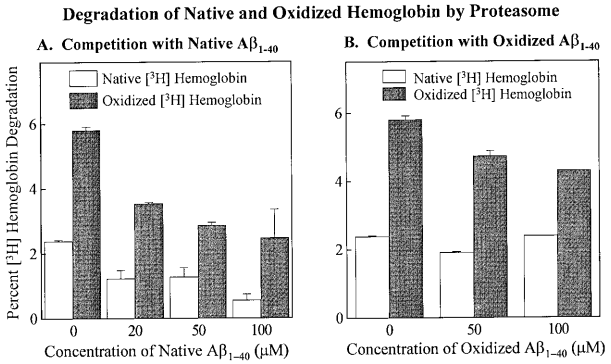

Figure 3. Effects of $A\beta_{1-40}$ on the degradation of native and oxidized hemoglobin by the proteasome. $A\beta_{1-40}$ was oxidized as described in Materials and methods. Purified proteasome was incubated with various concentrations of oxidized $A\beta_{1-40}$ for 1 h at room temperature. Next, native [^{3}H]-Hb or [^{3}H]-Hb oxidized with H_2O_2 was added, and proteolysis was allowed to proceed for 1 h at 37 °C. Percent hemoglobin degradation was calculated as described in Materials and methods. Data are represented as means ± SEs of triplicate samples from a total of four experiments. (*A*) Percent degradation of both native and oxidized (20 mM H_2O_2) [^{3}H]-hemoglobin by the purified human 20S proteasome, in the presence of native $A\beta_{1-40}$. (*B*) Effect of oxidized $A\beta_{1-40}$ (30 mM H_2O_2) on the degradation of both native and oxidized (20 mM H_2O_2) hemoglobin by the proteasome.

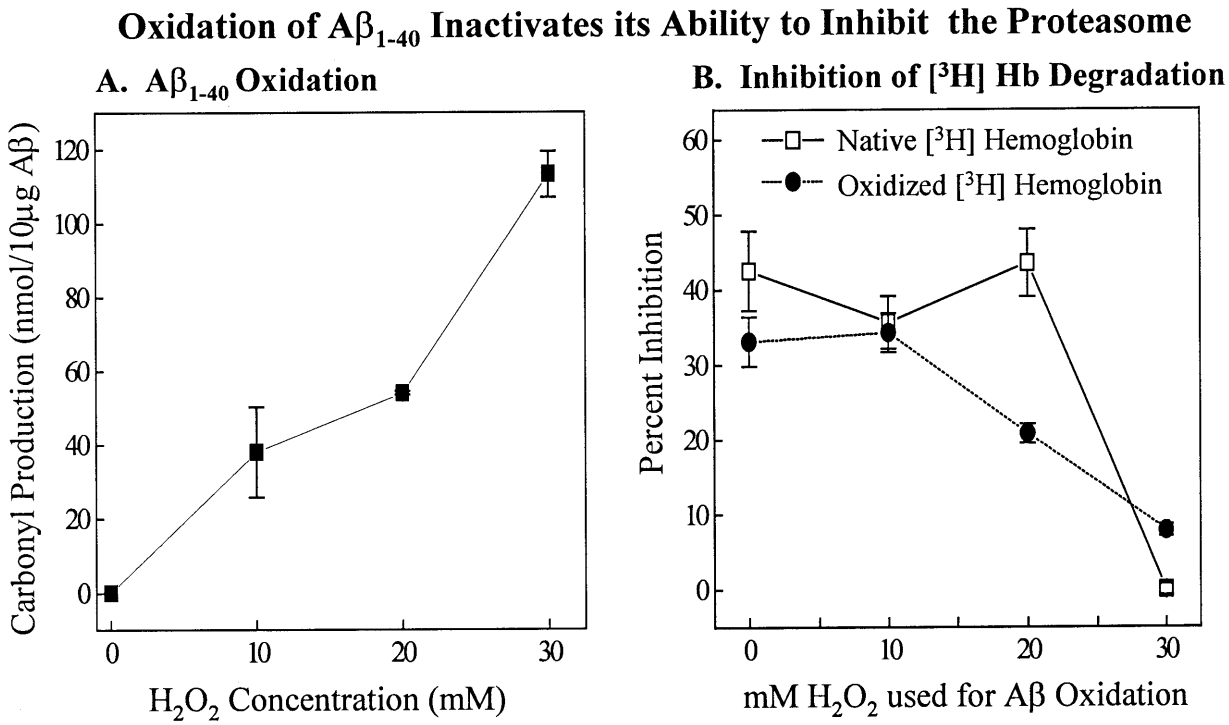

Figure 4. Oxidation of $A\beta_{1-40}$ inactivates its ability to inhibit the proteasome. Shown is the progressive oxidation of $A\beta_{1-40}$ as measured by carbonyl production with exposure to increasing concentrations of H_2O_2 (*A*) and that increasing oxidation progressively inactivates the ability of $A\beta_{1-40}$ to inhibit degradation of both native and oxidized hemoglobin by the proteasome (*B*). Carbonyl measurements, exposure of $A\beta_{1-40}$ and hemoglobin to H_2O_2, and calculation of percent hemoglobin degradation were performed as described in Materials and methods. Data are represented as means and SEs from four experiments. (*A*, *B*) The extent of $A\beta$ oxidation was varied by pre-exposure to 0–30 mM hydrogen peroxide. (*B*) Purified human 20S proteasome was incubated with progressively oxidized $A\beta$ for 1 h at room temperature. Next, native [^{3}H]-Hb or [^{3}H]-Hb oxidized with 20 mM H_2O_2 was added and proteolysis was allowed to proceed for 1 h at 37 °C.

actually lose its inhibitory potential (fig. 4B). In fact, increasing oxidation of $A\beta_{1-40}$ progressively abolished its inhibitory action on hemoglobin degradation by the proteasome; the degradation of native and oxidized hemoglobin were similarly affected (fig. 4B). We also tested increasing concentrations of oxidized $A\beta_{1-40}$ but found that even 100 μM oxidized $A\beta_{1-40}$ did not selectively inhibit the proteasome (fig. 3B).

Oxidative inactivation of the inhibitory action of $A\beta_{1-40}$ could be due to a direct modification of its proteasome binding site by hydrogen peroxide. Alternatively, due to the ability of $A\beta$ to aggregate randomly, oxidation possibly leads to formation of aggregates which are too large to enter the catalytic core of the proteasome. Finally, $A\beta$ could be an alternate substrate (to hemoglobin) for degradation by the proteasome, but Gregori et al. [28] have already demonstrated that the proteasome does not degrade $A\beta$.

Senile plaques containing amyloid deposits have also been reported to contain several oxidative stress markers [4, 6], and increased HNE immunoreactivity of amyloid deposits in brains of patients with Alzheimer's disease has been described [38]. Stadtman and colleagues [39–41], and we [19] have demonstrated that products of lipid peroxidation such as malondialdehyde or HNE can cross-link proteins which in turn inhibit the proteasome. Since increased levels of HNE as well as $A\beta$ are observed in the brains of patients with Alzheimer's disease, and in light of the potent toxic effects of HNE, further modification of $A\beta$ by HNE seems likely. We therefore tested the effect of HNE-modified $A\beta_{1-40}$ on the chymotrypsin-like activity of the proteasome.

Exposure of $A\beta_{1-40}$ to HNE clearly caused progressive covalent cross-linking of the peptide in a dose-dependent manner, as shown in figure 5. Although 50 μM $A\beta_{1-40}$ by itself did not affect proteasome activity to a considerable extent, HNE-modified $A\beta_{1-40}$ significantly inhibited the chymotrypsin-like activity of the proteasome, as measured by degradation of the fluorogenic peptides, suc-LLVY-MCA (fig. 6A) and z-LLL-AMC (fig. 6B). This inhibition could be further augmented by increasing the severity of cross-linking of $A\beta_{1-40}$ using a higher concentration of HNE. Importantly, HNE by

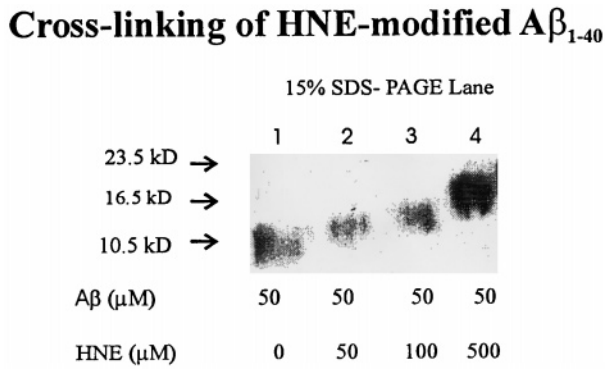

Figure 5. Cross-linking of Aβ_{1-40} with exposure to HNE. Aβ_{1-40} was modified with various concentrations of HNE and separated by 15% SDS-PAGE. The bands were visualized by staining with Coomassie blue.

itself did not affect the activity of the proteasome. The apparent discrepancy in the inhibitory effect of 50 μM Aβ_{1-40} in figures 1 and 6 was caused by a 1-h pre-incubation of Aβ_{1-40} with purified proteasome before addition of the substrate in figure 1, whereas the experiments of figure 6 were conducted without Aβ_{1-40} pre-incubation.

Although Aβ is found predominantly in extracellular plaques, its occurrence inside the cell has been reported for different cell types by many laboratories [42–45]. There are also data documenting distinct processing pathways of the Swedish mutant amyloid precursor protein for intracellular and secreted forms of Aβ [44]. Thus, the possibility that Aβ is available to interact with cytoplasmic components cannot be ruled out.

Although our results on unmodified Aβ_{1-40} do not completely agree with the findings of Gregori et al., these differences possibly arise due to tissue- and species-specific properties of the proteasome. In conclusion, our results show that HNE-cross-linked Aβ_{1-40} significantly inhibits the chymotrypsin-like activity of the proteasome. In light of the increased HNE immunoreactivity and accumulated Aβ in affected areas of the brain with Alzheimer's disease, this interaction possibly contributes to certain pathological manifestations of Alzheimer's disease and may partially explain the accumulation of oxidized and ubiquitinylated proteins.

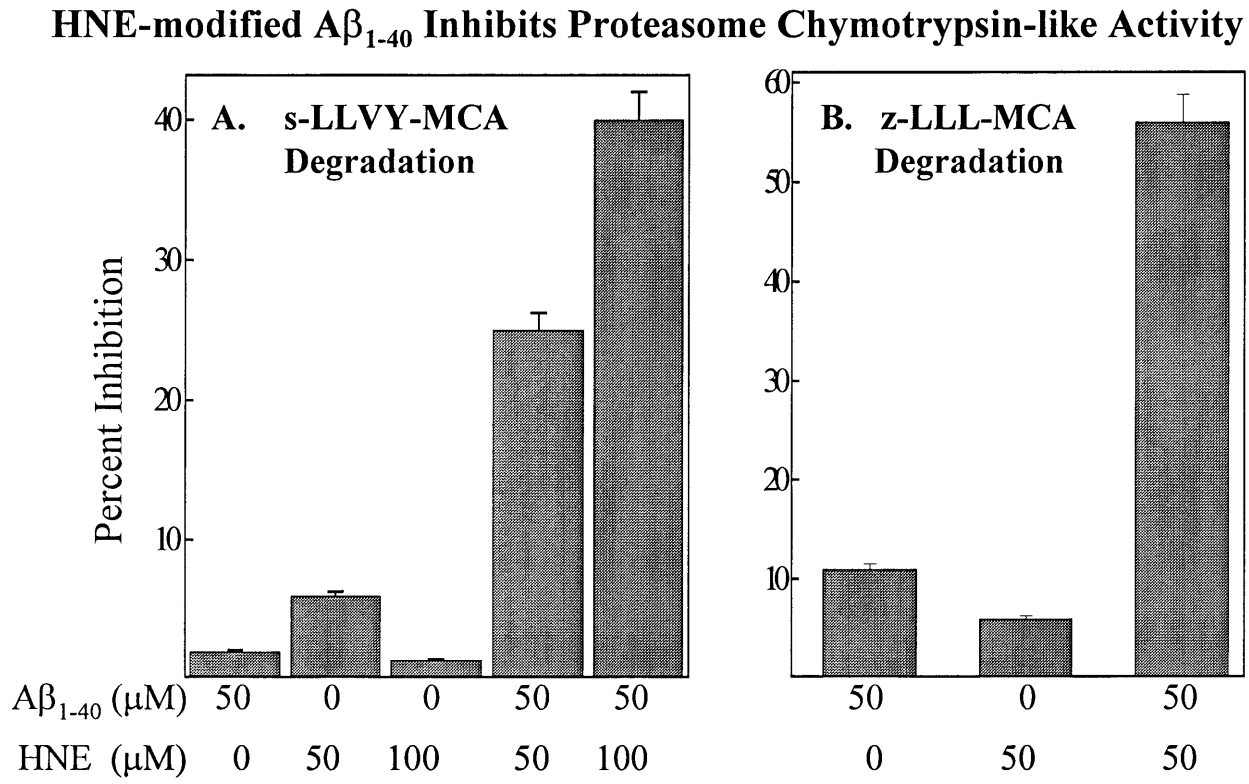

Figure 6. HNE-modified Aβ_{1-40} inhibits the chymotrypsin-like activity of the proteasome. Aβ_{1-40} was modified with 50 and 100 μM HNE as described in Materials and methods. Degradation of the fluorogenic substrates suc-LLVY-MCA (*A*) and z-LLL-MCA (*B*) was measured in the presence of HNE-modified Aβ_{1-40}. Where used, Aβ_{1-40} or HNE were added to purified human 20S proteasome at the same time as suc-LLVY-MCA or z-LLL-MCA, without any pre-incubation. This precaution was taken to prevent possible reactions of HNE with the proteasome. Data are represented as means ± SEs of three independent experiments.

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