Endogenous Proteins Controlling Amyloid β-Peptide Polymerization

POSSIBLE IMPLICATIONS FOR β-AMYLOID FORMATION IN THE CENTRAL NERVOUS SYSTEM AND IN PERIPHERAL TISSUES*

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We report that certain plasma proteins, at physiological concentrations, are potent inhibitors of amyloid β-peptide (Aβ) polymerization. These proteins are also present in cerebrospinal fluid, but at low concentrations having little or no effect on Aβ. Thirty-three proteins representing more than 90% of the protein content in plasma and cerebrospinal fluid were studied. Quantitatively, albumin was the most important protein, representing 60% of the total amyloid inhibitory activity, followed by α1-antitrypsin and immunoglobulins A and G. Albumin suppressed amyloid formation by binding to the oligomeric or polymeric Aβ, blocking a further addition of peptide. This effect was also observed when the incorporation of labeled Aβ into genuine β-amyloid in tissue section was studied. The Aβ and the anti-diabetic drug tolbutamide apparently bind to the same site on albumin. Tolbutamide displaces Aβ from albumin, increasing its free concentration and enhancing amyloid formation. The present results suggest that several endogenous proteins are negative regulators of amyloid formation. Plasma contains at least 300 times more amyloid inhibitory activity than cerebrospinal fluid. These findings may provide one explanation as to why β-amyloid deposits are not found in peripheral tissues but are only found in the central nervous system. Moreover, the data suggest that some drugs that display an affinity for albumin may enhance β-amyloid formation and promote the development of Alzheimer's disease.

Alzheimer’s disease (AD)† is associated with the accumulation of a specific form of amyloid in the brain parenchyma and in meningocerebral blood vessels (1–3). The primary components of the amyloid are polymers of a short peptide derived from proteolytic processing of a ubiquitous transmembrane protein (4, 5) termed the amyloid β-precursor protein. The amyloid β-peptide is usually referred to as the Aβ. It is present in two principal variants (2, 6), one that contains 40 amino acid residues (Aβ1–40), and one C-terminally extended variant that contains 42 amino acid residues (Aβ1–42). The longer variant has been suggested to be of major importance in the pathogenesis of AD because it has a greater tendency to form amyloid fibrils in vitro and possibly also in vivo (7–9). Certain mutations associated with familial AD lead to an increased secretion of the 42-amino acid form (10) and an enhanced accumulation of amyloid.

β-Amyloid displays several important features that distinguish it from other types of amyloid. (i) The peptide forming the amyloid deposits is present at very low concentrations in the circulation. This is in contrast to peripheral amyloid disorders in which the amyloid proteins are present at high concentrations. Examples of such non-central nervous system amyloid proteins include serum amyloid A, myeloma protein, and transthyretin (11). (ii) The levels of Aβ are not higher and the peptide is not structurally different (except in extremely rare cases of familial AD) in individuals with the disease than in healthy controls (for a review, see Ref. 3). (iii) It is well known that most and possibly all nucleated cells in the body produce the Aβ (12, 13); however, for unknown reasons, β-amyloid is only deposited in the central nervous system.

In the present study, we aimed at investigating why β-amyloid exclusively is formed in the central nervous system. Previous work has demonstrated that some plasma proteins and lipoproteins bind Aβ and serve as carrier proteins (14, 15). Protein binding is a general mechanism for the transport of endogenous substances such as hormones and lipids as well as clinically used drugs (16). Generally, it is only the non-protein-bound fraction of the substances that is biologically active. We therefore hypothesized that only the free fraction of Aβ can take part in the polymerization process generating amyloid fibrils. Hence, Aβ-carrier proteins may have an important role in preventing amyloid formation by increasing the bound fraction.

The bulk of large proteins do not penetrate the blood-brain barrier efficiently. Thus, the levels of soluble proteins in the central nervous system are much lower than those in peripheral tissues. It has been estimated that the ratio between protein content in the CSF and plasma is approximately 0.004 (17). However, in contrast to large proteins, the Aβ levels are higher in CSF than in plasma (18, 19), which probably reflects a higher rate of secretion from neuronal cells than from other cell types. Overall, this suggests that a smaller fraction of the Aβ is protein-bound in the central nervous system than in the periphery.

With this background, we decided to investigate whether plasma and CSF proteins can indeed inhibit β-amyloidogenesis. We established in vitro assays allowing quantitative and qualitative studies of amyloid formation in the presence of several different proteins. The proteins studied here represent...
more than 90% of the protein content in plasma and CSF (20–25). Many drugs bind to plasma proteins, which can lead to interactions with severe consequences (16). If some drugs bind to the same site on plasma protein molecules as Aβ, it may lead to increased levels of free Aβ and enhanced amyloid formation. Therefore, we decided to also address this possibility experimentally.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic Aβ1–40, Aβ1–40, and PrP106–126 biotinylated at the N-terminus were obtained from ANAWA (Wangen, Switzerland). Nonlabeled Aβ1–40, Aβ1–40, and PrP106–126 were obtained from Merck (Bubendorff, Switzerland). The peptides were stored in Me2SO at −20 °C. Human serum albumin, (fatty acid-free; 99% purity) was from Sigma. All other proteins were from Calbiochem. Streptavidin-peroxidase was bought from Roche Molecular Biochemicals. All other reagents were from Sigma. Iodinated Aβ1–42 was obtained from Amersham Pharmacia Biotech.

**Analysis of Aβ Polymerization**—96-well plates (Maxisorp; Nunc) were coated with peptide by incubating them with a solution of Aβ1–42 or Aβ1–40 (2.5 μM) in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and Na2SO4). Solution (100 μL) was added to each well, and the plates were incubated at 37 °C with shaking for 48 h. The peptide solution was then flicked off. Staining with a solution of Coomassie Blue (20 μM) in Tris-buffered saline showed that the polymeric forms had bound to the wells (data not shown). After removal of the peptide solution, the plates were placed upside down on absorbing paper and allowed to dry. Coated plates were stored at −20 °C in a desiccator. On the day of experiment, the plates were blocked by the addition of 300 μL of PBS containing 0.05% Tween 20 (PBS-T) and 1% bovine serum albumin/well for 2 h at room temperature. The plates were then washed with PBS-Tween (0.05% Tween 20), and the fluid was flicked off. Biotin-Aβ1–40 or biotin-Aβ1–42 was dissolved in Me2SO and diluted with Tris-buffered saline with Na2SO4 (0.05%). Unless stated otherwise, the final concentration of the labeled peptide was 20 nM. The plates were incubated overnight at 37 °C with agitation. Nonbound peptide was removed by washing the plates three times with PBS-T (300 μL/well). Streptavidin-peroxidase was diluted with PBS-T and 1% BSA and added to the plates (150 μL/well). After incubation (2 h at room temperature), the solution was flicked off, and the plates were washed four times with PBS-T. Tetramethyl-benzidin was used as chromogenic substrate for the peroxidase. After termination of the reaction with sulfuric acid (0.33 M, final concentration), absorbance was measured at 455 nm with a SpectraMax 250 96-well plate reader. Nonspecific binding was defined as the binding of biotin-Aβ to wells that had not been coated with Aβ. There was a linear relationship between peroxidase activity and the amount of peptide bound (data not shown). Nonspecific binding was, on average, approximately 15% of total binding (data not shown). We also studied the incorporation of 125I-Aβ into tissue sections of human AD brain using the method of Maggio et al. (129). In experiments with a SpectraMax 250 96-well plate reader, nonspecific binding was defined as the binding of biotin-Aβ to wells that had not been coated with Aβ. There was a linear relationship between Aβ binding and activity of the peptide. The effect of peptide on the binding of Aβ to tissue sections was measured using a BiaCore 2000. The effects of HSA on the polymerization of Aβ1–42 in the presence of human serum albumin (HSA) or BSA were also studied. The highest concentrations of albumin used corresponded approximately to the plasma levels of a healthy human adult (21). Both HSA and BSA had the capacity to completely inhibit the incorporation of biotin-Aβ1–40 into immunolabeled fibrils. The test system used is based on the finding that Aβ monomers bind with high affinity to preformed polymers of Aβ (29). We immobilized Aβ1–42 polymers as seeds (7) in 96-well plates and measured the incorporation of soluble biotin-Aβ1–42 in the presence of human serum albumin (HSA) or BSA. In Fig. 1A, the effects of different concentrations of HSA and BSA on biotin-Aβ1–42 incorporation are shown. The lowest concentrations of albumin used corresponded approximately to the plasma levels of a healthy human adult (21). Both HSA and BSA had the capacity to completely inhibit the incorporation of biotin-Aβ1–40 into immobilized Aβ polymers with apparent IC50 values of 10 and 12 μM, respectively. The effects of HSA on the polymerization of Aβ1–42 in the presence of various concentrations of HSA were also studied. Here, nonlabeled Aβ1–40 or Aβ1–42 was immobilized in the Maxisorp plates as described under “Experimental Procedures.” Biotinylated Aβ1–40 or Aβ1–42 was then allowed to bind the immobilized peptide in the presence of various concentrations of HSA. The IC50 values of HSA on the inhibition of Aβ1–40 or Aβ1–42 binding were essentially identical (data not shown), suggesting that HSA is indeed capable of inhibiting polymerization of the two major forms of the Aβ.

These findings were confirmed in a second set of experiments in which the incorporation of 125I-Aβ1–42 into brain tissue sections from an individual with AD was measured. As seen in Fig. 1B, the radiolabeled peptide bound to the amyloid deposits in the tissue, as demonstrated previously (29). In the presence of 227 μM HSA, binding was heavily reduced (Fig. 1C). Measurement of the incorporated radioactivity using a phosphor imager showed that overall binding (binding to amyloid deposits in the tissue and background together) had been reduced with 55% by the addition of HSA.

Aβ incubated at high concentrations also rapidly polymerizes in the absence of preformed polymers, but through primary nucleation (30, 31). Therefore, in other experiments, we studied the effects of HSA on soluble Aβ1–40 and Aβ1–42 fibrils in the absence of seeds. As seen in Fig. 2, A and C, both peptide formed fibrils when incubated for 24 h at 37 °C at a concentration of 20 μM. This concentration is approximately 6,000 times higher than that in CSF (17, 29, 39). When incubated in the presence of 227 μM HSA, the polymerization of Aβ1–40 into amyloid fibrils was...
completely inhibited (Fig. 2B). Under the same conditions, Aβ1–42 only formed occasional fibrils (Fig. 2D). Moreover, spherical structures of 10–30 nm in diameter were also detected frequently.

**HSA Inhibits the Polymerization of a Prion-derived Peptide—**

PrP106–126 represents the central core of the prion protein (for a recent review, see Ref. 26) and spontaneously forms amyloid-like fibrils. Similar to the Aβ, prion protein can form amyloid deposits in the CNS and cause neurodegeneration. We therefore decided to study whether albumin can also prevent polymerization of this peptide. As seen in Fig. 3, HSA dose-dependently inhibited the binding of biotinylated PrP106–126 to immobilized homologous peptide. The IC50 value for HSA in this system was approximately 100 μM, 10 times higher than that for Aβ. This concentration represents less than one-sixth of the albumin concentration in blood but is more than 30 times higher than the albumin concentration in CSF. Hence, these

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**Fig. 1.** Albumin inhibits the incorporation of biotin-Aβ1–40 into immobilized amyloid polymers. Biotin-Aβ1–40 was incubated at a concentration 20 nM in 96-well plates coated with Aβ1–42 polymers in the presence of the indicated concentrations of HSA (●) or BSA (○) (A). After an overnight incubation at 37 °C with agitation, the reaction was stopped and processed as described under “Experimental Procedures.” The biotin-Aβ1–40 incorporation in the absence of albumin is equal to 100%. The experiments were performed in quadruplicate. The experiment was repeated three times with essentially identical results. Data are indicated as the mean ± S.E. The effect HSA on the incorporation of 125I-Aβ1–42 into genuine amyloid deposits in human AD brain tissue was also studied. In B and C, the effect of buffer alone or buffer containing HSA (227 μM), respectively, is demonstrated.

**Fig. 2.** Effects of HSA on Aβ1–40 and Aβ1–42 polymerization studied with electron microscopy. A and B, Aβ1–40 (20 μM) incubated for 24 h in the absence (A) and presence (B) of 227 μM HSA. C and D, Aβ1–42 (20 μM) incubated in the absence (C) and presence (D) of 227 μM HSA. Scale bars, 500 nm. The area in each panel that is enclosed by a square is shown at a higher magnification in the inset (upper left).
findings demonstrate that HSA displays a certain degree of specificity for β-amyloid.

Characterization of the Mode of Action for Albumin on Aβ Polymerization—Surface plasmon resonance spectroscopy allows protein-protein interaction studies in real time (28), and this methodology was therefore used to study how BSA and HSA interact with monomeric and polymeric Aβ. Preformed Aβ1–42 fibrils were immobilized to the sensor chip as described under “Experimental Procedures.” A solution (25 mM) of BSA (Fig. 4A) or HSA (Fig. 4B) was then allowed to flow through the cell. The protein bound avidly to the polymers, indicating that albumin indeed has an affinity for the polymeric peptide. In parallel flow cells, monomeric biotin-Aβ1–40 was immobilized using streptavidin. In this case, no binding was observed with either BSA (Fig. 4A) or HSA (Fig. 4B). When the experiment was repeated using nonbiotinylated Aβ1–40 that was immobilized with a monoclonal antibody, essentially identical results were obtained (data not shown).

Drug-enhanced Aβ Polymerization—It is well known that several clinically important drugs bind to albumin with various affinities. We speculated that some of these substances may bind to the same site(s) on the albumin molecule as Aβ and may therefore be able to displace the peptide from its binding site(s). This may lead to increased levels of free Aβ and the enhancement of amyloid formation. We therefore screened a number of albumin ligands with regard to their effects on biotin-Aβ1–40 incorporation into preformed amyloid polymers in the presence or absence of 100 μM HSA. It was found that tolbutamide, at concentrations corresponding to therapeutic levels (17), enhanced biotin-Aβ1–40 incorporation in the presence but not in the absence of HSA (Fig. 5). This strongly suggests that tolbutamide is capable of interfering with Aβ-albumin binding and indirectly stimulating amyloid fibril formation.

Endogenous Regulators of Amyloid Formation—In these experiments, we investigated the effects of various plasma/CSF proteins on Aβ polymerization (Table I). The proteins listed in Table I represent more than 90% of the protein content in plasma and CSF. The concentrations tested covered the levels in plasma and CSF for all but two proteins, IgM and α1-antichymotrypsin. The highest concentrations used were 0.55 and 1.0 μM, respectively, which were lower than their plasma concentrations but higher than their CSF concentrations (see Table I). Seven of the 13 tested proteins had very little or no effect (i.e., the IC50 was higher than the plasma concentrations). Of the remaining six proteins, three had IC50 values below 10 μM, and three had IC50 values below 10 μM. Albumin, α1-antitrypsin, IgG, and IgA had IC50 values that were substantially below their plasma concentrations, which strongly suggests that these proteins may be potent inhibitors of β-amyloidogenesis in vivo.

1 unit of inhibitory activity was defined as the number of μmol/liter of protein required to inhibit polymerization by 50%.

![Fig. 3. Effects of HSA on PrP106–126 polymerization.](image)

![Fig. 4. Albumin binding to HSA studied using surface plasmon resonance spectroscopy.](image)

![Fig. 5. Tolbutamide increases biotin-Aβ1–40 incorporation in the presence but not in the absence of HSA.](image)
under the conditions specified under "Experimental Procedures." When taking the plasma concentration of the studied proteins into consideration, it is possible to estimate how much inhibitory activity each protein contributes (Fig. 6). Albumin is probably the most important regulator of β-amyloidogenesis in plasma. Although α₁-antitrypsin has an IC₅₀ value eight times lower than that of albumin (1.25 and 10 μM, respectively), the concentration of the former is substantially lower (25.3 and 644 μM, respectively). Therefore, despite its higher efficacy, it probably plays a less important role in the regulation of Aβ polymerization.

Cerebrospinal fluid contains essentially the same proteins as plasma, but the concentrations are considerably lower (see Table I). None of the tested proteins are present in the CSF in a concentration equal to or higher than its IC₅₀ value, which was obtained in the amyloid formation assay (see Table I). When comparing the total amount of inhibitory activity in plasma and CSF, we found that CSF contains only about 0.3% of that seen in plasma (Fig. 6).

**DISCUSSION**

Plasma and CSF proteins with affinity to Aβ serve as carriers for the peptide (14, 15). This study and previous studies (32, 33) have demonstrated that Aβ-binding proteins in plasma and CSF may also have a function in regulating β-amyloid formation. The most abundant plasma protein, albumin, is present in a concentration more than 60-fold higher than its IC₅₀ in the Aβ polymerization assay used here (see Table I). Albumin is the most abundant protein in CSF, but it is present at a concentration of thioflavin-binding amyloid from synthetic Aβ₁–40.

Pathologically reduced levels of albumin might promote β-amyloidosis and possibly also AD. In clinical studies, it was observed that anti-inflammatory drugs may have beneficial effects on AD (36). Levels of albumin are often reduced in association with inflammation (25) and, hence, the anti-amyloidiogenic activity in plasma and CSF is also reduced. However, even heavily reduced plasma levels of albumin are probably still sufficiently high to prevent amyloid formation in peripheral tissues. It may be different in the central nervous system. Because albumin (and other inhibitory proteins) is present in low concentrations having limited effects on amyloid formation

### Table I

IC₅₀ values for the inhibition of biotin-Aβ₁–40 incorporation into immobilized Aβ₁–42 polymers

| Protein              | IC₅₀ (µM) | n  | Plasma concentration (µM) | CSF concentration (µM) |
|----------------------|-----------|----|---------------------------|------------------------|
| Albumin              | 10        | 10 | 644                       | 3.0                    |
| IgG                  | 10        | 4  | 75                        | 0.3                    |
| IgA                  | 1.3       | 4  | 13                        | 0.007                  |
| IgM                  | >0.55     | 4  | 1.9                       | 0.0002                 |
| α₁-Antitrypsin       | 1.2       | 4  | 25                        | 0.12                   |
| Tranferrin           | 30        | 4  | 38                        | 0.17                   |
| α₂-Macroglobulin     | 2.5       | 4  | 3.4                       | 0.0024                 |
| Insulin              | >100      | 4  | 0.0001¹                   | 0.000008²              |
| α₁-Antichymotrypsin  | >1.0      | 4  | 8.6                       | 0.038                  |
| Antithrombin III     | >7.7      | 4  | 4.0                       | N.I.³                  |
| Serum amyloid P      | >0.2      | 4  | 0.2                       | 0.00033                |
| Transthyretin        | >5.5      | 4  | 5.5                       | 0.27                   |
| Fibrinogen           | >8.8      | 4  | 8.8                       | N.I.                  |

¹ Highly variable.
² N.I., no information available.
³ Fibrinogen

**Fig. 6.** Aβ polymerization inhibitory activity of individual proteins and the total content of inhibitory activity in plasma and CSF of these proteins. A, 1 inhibitory unit is defined as the number of μmol/liter of the indicated protein required for a 50% inhibition of Aβ polymerization under the conditions defined under “Experimental Procedures” and the Fig. 1 legend. B, the total inhibitory activity in plasma and CSF contributed by the studied proteins specified in Table I.
the structural background as to why Aβ binds albumin and other proteins is not known. However, it is reasonable to assume that hydrophobic interactions are involved. It was surprising that monomeric Aβ did not display binding to albumin when studied by surface plasmon resonance spectroscopy, considering the findings of Bieri et al. (14) showing that soluble Aβ binds albumin and lipoproteins. One explanation may be that Aβ molecules rapidly form small, soluble, oligomers with an affinity to albumin (37, 38).

Tolbutamide is a drug used to regulate blood glucose levels in diabetes mellitus. It also displays a high affinity for albumin. As a result, its clinical use is often associated with interactions with other drugs when the compounds compete for the same binding site on the albumin molecule (17). Here, we found that tolbutamide, at concentrations corresponding to therapeutic levels, enhanced amyloid formation in the presence but not in the absence of HSA. A reasonable explanation is that tolbutamide and Aβ bind to the same site on albumin. Tolbutamide may therefore displace Aβ from albumin and generate higher free Aβ fractions that can participate in amyloid formation. Drugs that can penetrate into the central nervous system, bind to the Aβ site(s) on albumin, and increase the free fraction of the peptide may thus be capable of enhancing amyloid formation in vivo.

Mutations affecting proteins capable of binding Aβ may promote the development of AD (39, 40). It is therefore possible that mutations affecting the proteins studied here may also have an impact on the development of AD through a similar mechanism.

In conclusion, the present data suggest a novel and possibly important physiological role for albumin and other plasma/CSF proteins in controlling amyloidogenesis in the central nervous system and possibly also in peripheral tissues. The data also suggest that drugs with certain pharmacokinetic properties may be capable of enhancing amyloidogenesis. Moreover, the reduced levels of albumin seen in association with inflammatory reactions may provide an opportunity for the Aβ to polymerize and thereby more easily form amyloid in the central nervous system.

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