Amphoterin Includes a Sequence Motif Which Is Homologous to the Alzheimer's β-Amyloid Peptide (Aβ), Forms Amyloid Fibrils in Vitro, and Binds Avidly to Aβ†

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ABSTRACT: Many of the proteins associated with amyloidoses have been found to share structural and sequence similarities, which are believed to be responsible for their capability to form amyloid fibrils. Interestingly, some proteins seem to be able to form amyloid-like fibrils although they are not associated with amyloidoses. This indicates that the ability to form amyloid fibrils may be a general property of a greater number of proteins not associated with these diseases. In the present work, we have searched for amyloidogenic consensus sequences in two current protein/peptide databases and show that many proteins share structures which can be predicted to form amyloid. One of these potentially amyloidogenic proteins is amphoterin (also known as HMG-1), involved in neuronal development and a ligand for the receptor for advanced glycation end products (RAGE). It contains an amyloidogenic peptide fragment which is highly homologous to the Alzheimer's amyloid β-peptide. If enzymatically released from the native protein, it forms amyloid-like fibrils which are visible in electron microscopy, exhibit apple green birefringence under polarized light after Congo red staining, and increases thioflavin T fluorescence. This fragment also shows high affinity to Aβ as a free peptide or while part of the native protein. Our results support the hypothesis that the potential to form amyloid is a common characteristic of a number of proteins, independent of their relation to amyloidoses, and that this potential can be predicted based on the physicochemical properties of these proteins.

Insoluble aggregates of normally soluble proteins or fragments thereof are involved in a variety of human disease states including different forms of amyloidoses such as Alzheimer's disease (AD)[1] and prion diseases. Human diseases associated with amyloid fibril formation are caused by a variety of proteins which do not show obvious sequence homology or similarities in their three-dimensional structure. Human transthyretin, forming amyloid fibrils in senile systemic amyloidosis and familial amyloid polyneuropathy type I (*1*), and the immunoglobulin light chain, related to myeloma-associated amyloidosis (*2*), have both an all β fold in the native crystal structure (*3*, *4*), whereas lysozyme, found to form amyloid in hereditary nonneuropathic systemic amyloidosis (*5*), has a mainly helical native fold (*6*).

Interestingly, the amyloidogenic potential does not seem to be limited to only those proteins which lead to amyloidosis. Guijarro et al. recently found that the SH3 domain of phosphatidylinositol 3-kinase can also form amyloid-like structures, although it has not yet been linked to any amyloidogenic disease (*7*). In addition, huntingtin-encoded polyglutamine expansions, associated with neurodegeneration in Huntington's disease, can make amyloid fibrils (*8*), although the disease is not grouped among the amyloidoses. Cribbs et al. (*9*) proposed that Herpes Simplex Virus, suggested to be a risk factor for AD (*10*), is involved in the pathophysiology of sporadic cases of AD through fragments on the glycoprotein B, homologous to the Alzheimer's amyloid β-peptide (Aβ). Finally, Chiti et al. were recently able to convert a small α/β protein, acylphosphatase, to an amyloid-like form (*11*). Thus, the potential to form amyloid may be a common characteristic of a number of proteins, independent of their relation to amyloidoses.

In the present paper, we have searched for potentially amyloidogenic proteins. We show that a simple secondary structure prediction (*12*, *13*) combined with a short but essential sequence homology to one of the known amyloids can be used to identify proteins with potentially amyloidogenic sequences. We used a four amino acid stretch (AFFV or VFFA) from Aβ as the homologue sequence. These amino acids have been defined to be essential for aggregation and fibril formation in AD (*14*, *15*). One of the proteins identified with this method is amphoterin. Amphoterin is a heparin-binding, neurite outgrowth-promoting protein which is highly

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[1] Abbreviations: Aβ=Aβ_{1-40}, Alzheimer's amyloid β-peptide; AGel, Gelsolin-based amyloid of the Finnish type; PrP, Prion protein; IAPP, Islet amyloid polypeptide; Atn$_p$, amphoterin amyloid peptide; AD, Alzheimer's disease; HMG-X, High mobility group protein type X; RAGE, receptor for the advanced glycation end products; ApoE, apolipoprotein E; ESI-IT-MS, electrospray-ion trap mass spectrometry; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; T, percentage of the acrylamide; C, percentage of the cross-linker bisacrylamide; ThT, Thioflavin T; PBS, phosphate-buffered saline; kDa, kilodalton; SPR, surface plasmon resonance spectroscopy.

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expressed in the neurons of the developing central nervous system. It is specifically localized to filopodia of neuronal cells and required for neurite outgrowth. The amino acid sequence of amphoterin is homologous to the high mobility group protein 1 and 2 (HMG-X) type sequences (*16*). Amphoterin is a ligand for the receptor for advanced glycation end products (RAGE) (*17*). Blocking the RAGE-amphoterin signaling supresses tumor growth and metastases (*18*) and RAGE can act as an amyloid receptor molecule (*19*). The motif on amphoterin responsible for the RAGE-amphoterin interaction is currently not known.

EXPERIMENTAL PROCEDURES

Materials. Alzheimer's amyloid β-peptide (Aβ) (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) was synthesized at the Centre for the Analysis and Synthesis of Macromolecules (SUNY, Stony Brook, NY). Amphoterin peptide (Atn_p) (MSSYAFFVQTCREEHK), corresponding to amino acids 12−27 of amphoterin (*20*), was purchased from Bio-Synthesis, Inc. (Lewisville, TX). All peptides were purified by microbore HPLC. The sequence and purity of each peptide was verified by electrospray-iontrap mass spectrometry (ESI-IT-MS, Bruker-Daltonics Esquire-LC, Bremen, Germany) and/or automated Edman degradation. Stock solutions of each peptide (2 mg/mL) were prepared in 50% acetonitrile in water and stored at −70 °C. Human plasma apoE (apoE) was purchased from Calbiochem-Novabiochem (Switzerland). Recombinant rat amphoterin was produced as described (*16*). This protein was a kind gift from Dr. H. Rauvala (Institute of Biotechnology, University of Helsinki, Helsinki, Finland). Amphoterin was more than 95% pure as determined by silver stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE). The antibody against Aβ (4G8) recognizing amino acids 17−24 was purchased from Senetek (St. Louis, MO). The solubility of each peptide was tested after lyophilization by analytical high-resolution tricine-sodium dodecyl sulfate−polyacrylamide gel electrophoresis (TT-SDS−PAGE) and microbore gel filtration (Superdex Peptide PC 3.2/30) liquid chromatography (SMART System, Pharmacia Biotech, Sweden).

Recognition of Amyloidogenic Proteins. Two databases, PIR (protein identification resource) and SWISS−PROT (Swiss national protein database) were searched for proteins and peptide fragments with the sequences AFFV and VFFA. Each hit was further analyzed by the Chou and Fasman secondary structure prediction (*12*, *13*). Only fragments with a predicted high β-sheet, neutral α-helix, and a low β-turn secondary structure score were considered to be potentially amyloidogenic.

Enzymatic Release of the Amyloid Forming Fragment Atn_p. Rat recombinant amphoterin was digested with endoproteinase Lys-C in 1% NH_4HCO_3. Purified amphoterin was incubated with 1.5% (w/w) of the enzyme for 2 h at 37 °C, followed by addition of another 1.5% (w/w) of the same enzyme. The incubation was continued for another 2−4 h. Peptides were separated by reversed-phase high-performance liquid chromatography (HPLC). Each peptide was identified by automated N-terminal sequence analysis using an Applied Biosystems 477A/120A Sequencer.

Congo Red Staining. Amyloid detection by Congo red staining was performed as described (*21*).

Electron Microscopy. For fibril formation, the peptide (0.2 μg/μL) was incubated in phosphate-buffered saline (PBS, pH 7.2) for 3−4 days at 37 °C. Formvar-coated grids (300-mesh) were floated on the peptide solution, air-dried, and negatively stained with 1% uranyl acetate.

Fluorometric Experiments. For fluorometric experiments, ~30 μg (15×10^{-9} M) of Atn_p or 30 μg (7.0×10^{-9} M) of Aβ was incubated alone or with 1/200 (mol of lipoprotein/mol of peptide) of apoE in 50 μL PBS for 0−96 h. Incubated samples were then added to 50 mM glycine, pH 9, with 2 μM thioflavin T (ThT) (Sigma Corporation, St. Louis, MO) in a final volume of 2 mL. Fluorescence was measured at the excitation and emission maxima of 435 and 485 nm, respectively, in a Hitachi F-2000 fluorescent spectrophotometer. A time scan of fluorescence was performed, and triplicate values after the decay reached a plateau were averaged after subtracting the background fluorescence of the ThT control. Three identical samples were measured.

Studying the Complex Formation of Amphoterin, Aβ or Atn_P. Complex formation was studied by incubating 3 μg (0.1×10^{-9} M) of amphoterin with 2 μg (0.46×10^{-9} M) of Aβ or 2 μg (1.0×10^{-9} M) of Atn_p for 6 h at 37 °C in 25 μL PBS. Formed complexes were analyzed by TT-SDS−PAGE.

Competitive Binding Experiments with Amphoterin, Aβ and Atn_p. Competitive binding experiments were performed incubating 3 μg (0.1×10^{-9} M) of amphoterin at 37 °C for 6 h with 0.5 μg (0.25×10^{-9} M) of Atn_p together with 0.1−1 μg ($0.023-0.23 \times 10^{-9}$ M) of Aβ in 25 μL PBS, pH 7.2. Complexes were analyzed by TT−SDS−PAGE.

Surface Plasmon Resonance Spectroscopy. Surface plasmon resonance spectroscopy was performed on a Biacore 2000TM instrument using a CM5 sensor chip and the manufacturer's recommendations for amine coupling. Ligand levels used, gave a clearly positive signal for the analyte binding. Atn_p and Aβ were immobilized on the sensor chip in separate channels. The running buffer (PBS) was applied at a flow rate of 20μL/min with Aβ (8 μg/60 μL) as the analyte, at 25 °C. The binding response of each sample solution was determined by measuring the resonance units. Prior to the numerical analysis, data were adjusted to zero immediately before injection of the analyte.

Tricine-Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. The protein/peptide complexes were analyzed by TT-SDS−PAGE (10−14.5% T, 3% C, with a spacer gel 10% T, 3% C) according to Schaegger and von Jagow (*22*). Protein complex formation (see above) was terminated by adding modified sample buffer (without β-mercaptoethanol and with 50% of the recommended SDS) into each sample vial. The samples were incubated at 37 °C for 5−10 min prior to PAGE. Protein bands were visualized by Coomassie blue or silver staining.

RESULTS

Building up a strategy to search for potentially amyloidogenic proteins. The search for new amyloids was based on the assumption that amyloid fragments must share a sequence motif which is responsible for their amyloidogenicity.

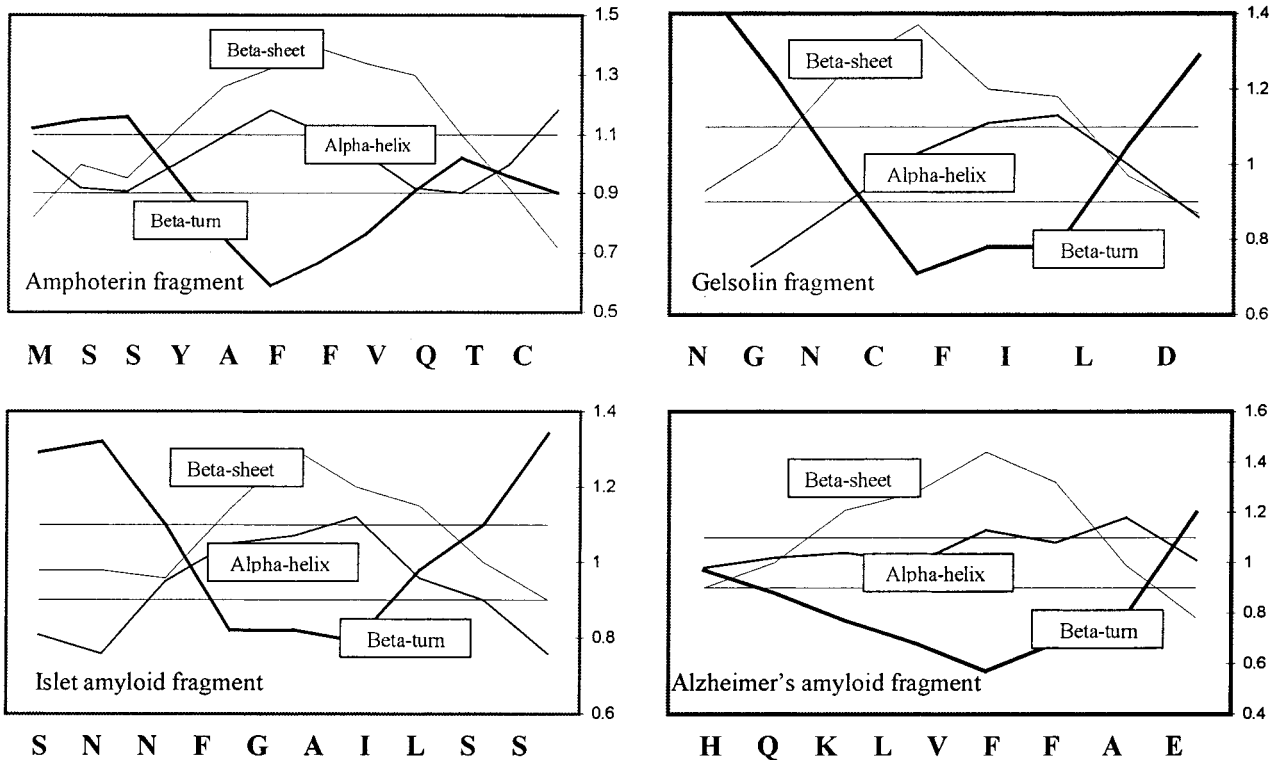

FIGURE 1: This figure shows the amyloidogenic fragments of amphoterin (Atn_p), islet amyloid polypeptide (IAPP), gelsolin amyloid (AGel), and Alzheimer's amyloid (Aβ) with their most amyloidogenic amino acids analyzed by the Chou-Fasman secondary structure prediction. These predictions illustrate the typical amyloidogenic potential for α-helix, β-sheet and β-turn formation (arbitrary units).

Thus, we looked for structural similarities among the three well-characterized amyloid-forming polypeptide fragments; islet amyloid polypeptide (IAPP), gelsolin amyloid of the Finnish type (AGel), and Aβ (Figure 1). Using the Chou and Fasman secondary structure prediction algorithm (*12*, *13*), we were able to identify a structural cluster which is characterized by a high probability of adopting a β-sheet structure, having at the same time a very low propensity for β-turn and an almost neutral value for α-helical conformation (Figure 1).

In addition, each potentially amyloidogenic protein had to contain a sequence of four or more amino acids homologous to one of the known amyloid-forming polypeptides. This sequence homology was necessary for the protein database search. In the present study, we used amino acids AFFV or VFFA, corresponding to amino acids 18−21 of Aβ. The order of these amino acids did not influence the potential to form β-sheet, β-turn, or α-helix. Indeed, it has been shown that $A\beta_{1-40}$ and $A\beta_{40-1}$ are very similar in their aggregation properties (*23*). Searches in the SWISS−PROT and PIR databases showed that several proteins contain the sequence AFFV (478 entries) or VFFA (437 entries); e.g., 5-hydroxytryptamine 2B receptor (Q02152/Mouse), ATP-binding cassette transporter 2 (P41234/Mouse), sodium/potassium-transporting ATPase from several species (P05023/Human) and amphoterin (P27428/Rat). A short stretch of amphoterin near the N-terminus expanding the amino acids 12−27 fulfilled the criteria to be amyloidogenic according to its secondary structure prediction as well (Figure 1). The amino acids VFFA are present in a longer stretch which is released by trypsin or endoproteinase Lys-C. In the present paper, we have used both native and synthetic M^{12}-K^{27} (Atn_P) to determine its amyloidogenic properties.

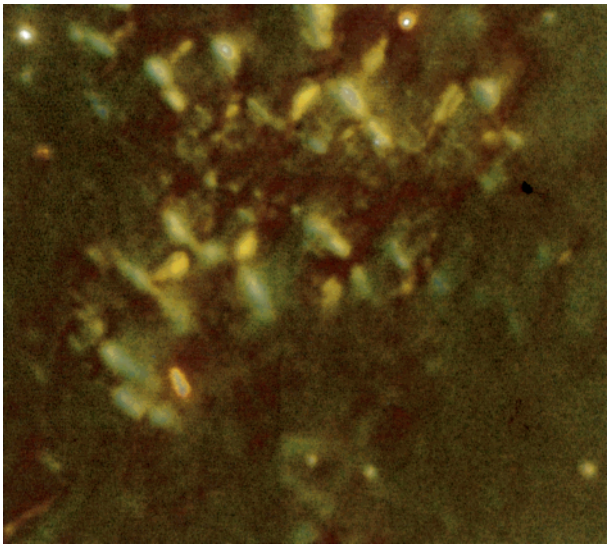

FIGURE 2: Congo red staining of preincubated Atn_p as viewed under polarized light. The apple green birefringence is typical for an amyloid.

Atn_p Forms Amyloid-Like Fibers. The amyloidogenicity of Atn_P was tested by incubating a 0.2 μg/μL solution for 1−2 days at 37 °C in PBS. Under these conditions, Atn_P formed amyloid-like structures which showed apple-green birefringence when viewed under polarized light after Congo red staining (Figure 2). In addition, electron microscopic (EM) examination showed that these fibers had a typical amyloid-like appearance (Figure 3). Amyloid fibril formation by this peptide was also monitored by ThT fluorescence. ThT has been shown to bind amyloid producing a shift in the emission spectrum and a fluorescence signal proportional to the amount of amyloid formed (*24*). The peptide

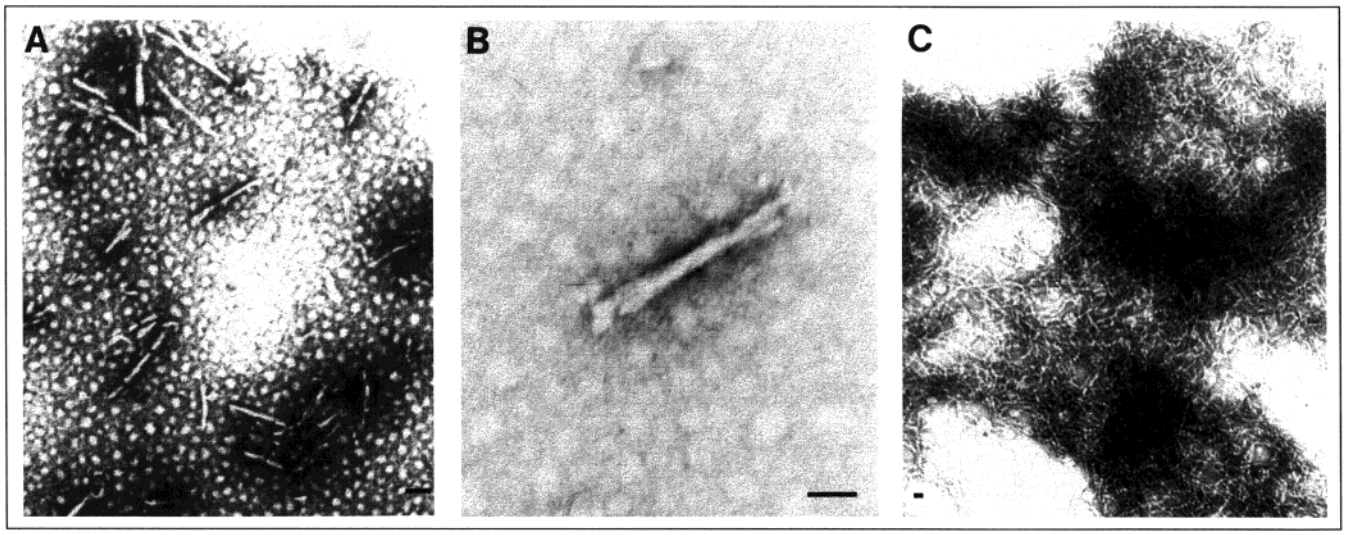

FIGURE 3: Electron micrograph of Atn_p amyloid fibers. (A) Atn_p fibers. (B) One of the fibers enlarged. (C) Enhanced amyloid fibrillization of Atn_p after incubation with human plasma apoE for 24 h (bar = 500 nm).

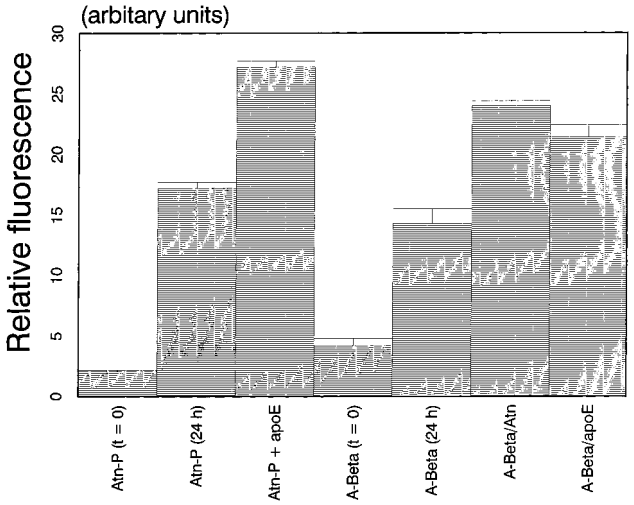

FIGURE 4: ThT fluorescence of Atn_p (Atn-P) at time 0 and after 24 h incubation; Atn_p with human plasma apoE (apoE) after 24 h incubation; Aβ (A-Beta) alone at time 0 and after 24 h incubation; Aβ with amphoterin (Atn) after 24 h incubation, and Aβ with human plasma apoE after 24 h incubation. All incubations were carried out at 37 °C.

showed a clear increase in the fluorescence signal after 24 h incubation, indicating amyloid-like polymerization (Figure 4).

Aβ Is Recognized by Amphoterin and Atn_P. The close homology in the amino acid sequences of Aβ and Atn_P led us to study whether Aβ would bind to native amphoterin and Atn_p. A 6 h incubation of native amphoterin with Aβ resulted in the formation of a complex which could be visualized by TT-SDS−PAGE as a faint band just above the native amphoterin band (Figure 5A, lane A). Not surprisingly, Atn_P formed stable complexes with native amphoterin (Figure 5A, lane B). Both peptides showed monomeric and dimeric complex formation. Interestingly, all complexes were SDS-stable, similar to the complexes formed between Aβ and apolipoprotein E (apoE) (*25*). The interaction between Atn_p and Aβ was measured by plasmon resonance spectroscopy, according to which Atn_p bound to Aβ with an affinity of 38.3% (±5.9%) as compared to that of Aβ to itself.

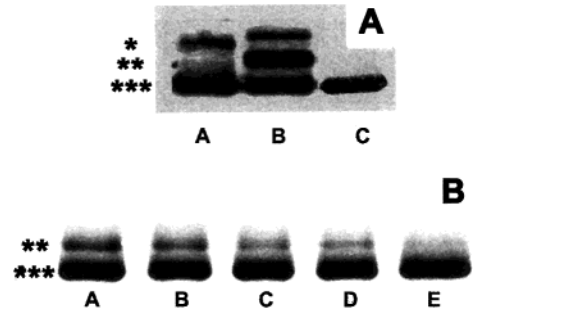

FIGURE 5: Tris-Tricine polyacrylamide gel electrophoresis of amphoterin and the formed complexes. (A) Complex formation. Lane A: 3 μg of amphoterin incubated with 2 μg of Aβ at 37 °C for 6 h. Lane B: 3 μg of amphoterin incubated with 2 μg of Atn_p at 37 °C for 6 h (the highest band illustrates the complex formation between Atn_p -doublet and amphoterin). Lane C: 3 μg of amphoterin alone. [(***) amphoterin, (**) amphoterin complexed with Atn_p, (*) amphoterin complexed with Aβ]. (Coomassie blue staining) (B) Competitive binding. Lane A: 3 μg of amphoterin incubated with 0.5 μg of Atn_p at 37 °C for 6 h. Lane B: 3 μg of amphoterin incubated with 0.5 μg of Atn_p and 0.1 μg Aβ at 37 °C for 6 h. Lane C: 3 μg of amphoterin incubated with 0.5 μg of Atn_p and 0.3 μg Aβ at 37 °C for 6 h. Lane D: 3 μg of amphoterin incubated with 0.5 μg of Atn_p and 0.6 μg Aβ at 37 °C for 6 h. Lane E: 3 μg of amphoterin incubated with 0.5 μg of Atn_p and 1 μg Aβ at 37 °C for 6 h. [(***) amphoterin, (**) amphoterin complexed with Atn_p]. (Coomassie blue staining). Due to the lower binding affinity of Aβ to amphoterin only the amphoterin−Atn_p complex is visible (see also insert A).

Aβ and Atn_p Compete for the Binding to Amphoterin. Incubating various amounts of Aβ and Atn_P as competing agents with native amphoterin resulted in a clear change in the complex formation. Increasing the amount of Aβ while keeping the amount of Atn_P constant, gradually inhibited the binding of Atn_P to native amphoterin as shown in Figure 5B. This result indicates that the interaction of Aβ and amphoterin is mediated through the amyloidogenic (Atn_p) sequence of amphoterin.

Atn_P Amyloidogenicity Is Influenced by apoE. We reported earlier that apoE can accelerate fibril formation of an amyloid peptide fragment in vitro (*26*). In the present work, we studied the effect of human plasma apoE on Atn_P. The influence of apoE on amyloid fibril formation of Atn_p was monitored by ThT fluorescence and EM. Both techniques

revealed that apoE accelerates amyloid fibril formation of Atn_P (Figures 3C and 4).

Amphoterin Accelerates the Polymerization of Aβ. The capability of Aβ to form a complex with amphoterin prompted us to study whether this interaction would increase amyloid fibril formation, i.e., whether amphoterin would also act as an amyloid polymerization-enhancing factor (AEF) for Aβ. The increase in the ThT fluorescence revealed that amphoterin accelerates amyloid fibril polymerization of Aβ nearly 2-fold, closely resembling the enhancing capacity of human plasma apoE (Figure 4).

DISCUSSION

In the present paper, we show that several of the known amyloidogenic polypeptide fragments share a structural pattern which can be identified by a simple secondary structure prediction. Amyloid is formed, according to this prediction, if a polypeptide fragment has a high β-sheet, neutral α-helix and low β-turn probability score.

Our approach was based on the idea that amyloids must share unifying structural determinants which cannot be defined by simple sequence homologies, but that in many amyloidogenic proteins only a short sequence stretch of amino acids is actually responsible for the amyloidogenicity of the corresponding protein. Indeed, AGel and Aβ can form amyloid fibrils with a minimal requirement of 10 amino acids [AGel, ^{183}FNNGDCFILD^{192} (*27*); Aβ, ^{14}HQKLVFFAED^{23} (*15*)] while the most amyloidogenic fragment in the prion protein (PrP) is only 8 amino acids long (^{113}AGAAAAGA^{120}) (*28*). The critical amino acids for fibril formation of IAPP are positioned at 20−29 (*29*) and of amylin at the amino acids 22−28 (*30*, *31*). Some earlier observations indicate that many of the amyloid-forming peptides include a similar positional pattern of hydrophobic, small, charged and polar amino acids, also known as the consensus sequence for amyloid-forming peptides (*32*, *33*). Actually, even a simple pattern of alternating polar and nonpolar residues can cause a protein to fold like an amyloid under certain conditions (*34*).

Public databases do not provide programs which would, based on a secondary structure prediction, directly be able to identify amyloid forming proteins. Thus, we had to imply a second criteria for the existence of an amyloidogenic protein fragment. Following this criteria, we first screened for proteins having sequence homology to amino acids AFFV or VFFA of Aβ. As a result, we were able to identify several proteins with close or exact homology to this peptide. Amphoterin was just one of the candidate proteins including a peptide fragment with sequence homology to Aβ. However, also the secondary structure prediction indicated that this fragment would be amyloidogenic.

Amyloid fibril formation can be visualized by EM and by staining with the amyloid-specific dye Congo red. In addition, amyloids cause a shift in the fluorescence signal of ThT. The amphoterin fragment Atn_p (M^{12}−K^{27}) is clearly amyloidogenic according to these criteria. Moreover, it binds to Aβ as does the native amphoterin. The interaction between these two amyloidogenic peptides, only homologous for the amino acids VFFA (AFFV), and the amyloid enhancing effect of native amphoterin, highlight the importance of these amino acids for the initiation of the polymerization, an observation confirmed by experiments performed on Aβ (*15*). The interaction of Aβ and Atn_p indicates that two closely related amyloidogenic peptides might, under certain conditions, interact with each other. Interestingly, Johan et al. (*35*) have recently shown that the amyloid fibril formation of the protein-A amyloid is enhanced by several unrelated amyloids, further supporting the concept that amyloidogenic sequences enhance the amyloid fibril formation of each other.

The SH3 domain (PI3−SH3) of the p85α subunit of bovine phosphatidylinositol 3-kinase has recently been shown to make amyloid-like fibers in vitro (*7*). According to our prediction, the amyloidogenic sequence in this protein is located between the amino acids 22−33 (LLPGDVLVVS-RAA). We have performed amyloidogenicity tests on a homologous SH3 domain from the tyrosine kinase HcK. The predicted amyloidogenic core sequence in this particular protein-domain is located between the amino acids 20−31 (QKGDQMVVLEES). Our preliminary results show that only the tryptic peptide expanding the amino acids 22−36 (GDQMVVLEESGEWWK) is able to form amyloid-like structures as judged by EM and Congo red staining (Baumann et al., unpublished material).

The amyloidogenic sequence of human muscle acylphosphatase seems to locate to amino acid sequences 8−25 and 30−40, according to the predicted secondary structure, with the former amino acid sequence being a stronger amyloid former. These predictions are in partial agreement with a preliminary mutational study on this protein (*36*). The amyloid-forming protein transthyretin has an amyloidogenic sequence at amino acids 10−20 according to the Chou and Fasman prediction. Indeed, Gustavsson et al. have shown that this particular peptide sequence forms amyloid in vitro (*37*). Another transthyretin peptide expanded to amino acids 105−115, not predicted to induce amyloidogenesis, does however, also produce amyloid-like fibrils in vitro (*37*), but only if incubated with a strong base. A simple secondary structure prediction, such as the Chou and Fasman model, cannot take into account environmental changes, thus probably missing those amyloidogenic polypeptides which can only form amyloid under extreme conditions.

We have shown that many of the known amyloid fragments share structural similarities which are also found in several polypeptides not known to be associated with amyloidoses. Whether a protein forms amyloid seems to depend on the physicochemical properties of that particular protein, and/or on the chemical nature of the environment. Thus, even those proteins which are normally not amyloidogenic may form amyloid under certain conditions. Moreover, proteins which originally do not show an amyloidogenic structure may become highly amyloidogenic due to amino acid substitutions during evolution.

Yan et al. (*19*) have recently shown that AA amyloid and possibly also other amyloids are capable of binding to the monocyte/macrophage RAGE, inducing altered cell function. At this time, no information is available about the RAGE-amphoterin interaction. It would be premature to conclude that the amyloidogenic motif of amphoterin would act as such a recognition site, although such a possibility is nonetheless intriguing. The fact that RAGE is recognized by various amyloidogenic peptides, and that amphoterin, a ligand to RAGE, includes an amyloidogenic motif, suggests

that such a sequence motif may have a signaling activity toward the RAGE receptor.

To test whether Atn_p can cause amyloid fibril formation in vivo, we are currently creating a proteolytically cleavable protein which, after proteolysis, would be able to release Atn_p in a cell or animal model. Since amphoterin is highly expressed in neurons of the developing central nervous system, this would provide an excellent tool to study the cascades leading to amyloid fibril formation in a neural system, and also to study the mechanisms leading to amyloidotic neurodegeneration in an artificial amyloidosis model. Our preliminary observations show that Atn_p is neurotoxic in cell culture experiments.

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