Prevention of Alzheimer’s Disease-associated Aβ Aggregation by Rationally Designed Nonpeptidic β-Sheet Ligands

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A new concept is introduced for the rational design of β-sheet ligands, which prevent protein aggregation. Oligomeric acylated aminopyrazole derivatives with a donor-acceptor-donor (DAD) hydrogen bond pattern complementary to that of a β-sheet efficiently block the solvent-exposed β-sheet portions in Aβ(1–40) and thereby prevent formation of insoluble protein aggregates. Density gradient centrifugation revealed that in the initial phase, the size of Aβ aggregates was efficiently kept between the trimeric and 15-meric state, whereas after 5 days an additional high molecular weight fraction appeared. With fluorescence correlation spectroscopy (FCS) exactly those two, i.e. a dimeric aminopyrazole with an oxalyl spacer and a trimeric head-to-tail connected aminopyrazole, of nine similar aminopyrazole ligands were identified as efficient aggregation retardants whose minimum energy conformations showed a perfect complementarity to a β-sheet. The concentration dependence of the inhibitory effect of a trimeric aminopyrazole derivative allowed an estimation of the dissociation constant in the range of \(10^{-5} \; m\). Finally, electrospray ionization mass spectrometry (ESI-MS) was used to determine the aggregation kinetics of Aβ(1–40) in the absence and in the presence of the ligands. From the comparable decrease in Aβ monomer concentration, we conclude that these β-sheet ligands do not prevent the initial oligomerization of monomeric Aβ but rather block further aggregation of spontaneously formed small oligomers. Together with the results from density gradient centrifugation and fluorescence correlation spectroscopy it is now possible to restrict the approximate size of soluble Aβ aggregates formed in the presence of both inhibitors from 3- to 15-mers.

Several pathological processes are connected with the formation of a β-sheet structure and subsequent protein aggregation. In particular amyloid deposition of the Aβ peptide is discussed as the primary cause for Alzheimer’s disease. Structural conversions of proteins leading to the formation of β-sheet-rich aggregates are also documented for Creutzfeldt-Jakob disease, bovine spongiform encephalopathy (BSE), Parkinson’s disease, and other protein misfolding diseases (1, 2). To date only palliative therapies for those diseases are available. For this reason understanding the mechanism of aggregation and understanding the development of β-sheet binders, which can slow down or even prevent the pathological process, are of great importance both from a mechanistic and a therapeutic view (3–5). Approaches for Alzheimer’s disease therapy, which are studied at present in several laboratories, constitute the activation of brain activity as well as recent experiments concerning a retarded aggregation, e.g. by the inhibition of secretases (6, 7). In the future, the dissolution or clearance of aggregates from the neurocortex will definitely become an important research goal. Studies with active and passive Aβ immunization also appear very promising (8–10).

In recent years experimental evidence has grown that Aβ deposition predates other pathological events in Alzheimer’s disease (11). Based on solid state NMR investigations it is today generally assumed that the fibrils form a parallel β-sheet with residues in exact register and an alternating order of charged and hydrophobic side chains (12, 13), whereas the solution structure displays two short α-helical regions surrounded by random coils dependent on the solvent (14). Several newer investigations indicate that medium size oligomers are the most neurotoxic species involved in Alzheimer’s disease (15). Walsh and co-workers (16) recently reported that these smaller oligomers tend to hold together mainly by hydrophobic interactions between random coil peptide strands and subsequently undergo a conformational change to high β-sheet contents when clustering to larger aggregates.

About a decade ago, a worldwide search began with an ever increasing intensity for small molecules against protein folding diseases. Several new ligands or ligand classes have been identified that intervene with fibril or plaque formation (17–21). However, very few ligands demonstrate a rational design. Those that do are modified peptides derived from key recognition elements of pathological proteins; Tjernberg et al. (22) were the first to discover that small peptides with the peptide fragment KLVFF taken directly from the middle part of Aβ are able to block Aβ aggregation. Soto et al. (23) developed peptides with the LPFFFD sequence that could be used as β-sheet breakers against Aβ. Others have modified these sequences, e.g. by inserting disrupting elements such as KKKKKK or EEEEEE (24) or by adding amino acids DD (25). In a very interesting approach, Meredith and co-workers (26, 27) have introduced N-methylated amino acids in every second position of a key peptide NH4-K(Me-L)F/Me-V/Me-F/Me-AECONH4 and obtained inhibitors for Aβ aggregation with ester instead of amide bonds. However, nonpeptidic inhibitors with a rational design are unknown to date. We present in this report 3-aminopyrazole derivatives and their oligomers as rationally designed nonpeptidic β-sheet templates that block Aβ aggregation. By

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fluorescence correlation spectroscopy (FCS), ultracentrifugation, and ES-MS measurements we have studied this inhibition process with respect to its concentration dependence, the size distribution of β-aggregates, as well as its kinetics.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling Experiments**—Force field calculations were performed on a Silicon Graphics O2 workstation with MacroModel 7.0 from Schrodinger Inc. using the AMBER* force field. The simulations were conducted in water applying the generalized born/surface area (GB/SA) approach of Biochemistry, Charité Berlin, Germany). Prior to incubation, the system was dissolved in 100% water-free Me$_2$SO at 3.4 mM. The ligand was added to 100% acetonitrile and dissolved at 50 °C in a screw cap vial for 1 day by repeated vortexing and sonification. Centrifugation at 14,000 rpm for 10 min removed a minor portion of undissolved particles from the saturated solution. Concentration of the solution was estimated from the weighed out content. The 5 mM stock solution was stored until use in the dark at room temperature. To start the aggregation process 250 μM Aβ-(1–40) was incubated with or without 750 μM Amplex (see Fig. 2, ligand 2) or 600 μM Trimer (see Fig. 2, ligand 9) in 10 mM sodium phosphate, pH 7.2, 7.4% Me$_2$SO, and 15% acetonitrile at 37 °C. The assay volume was 300 μl, enough for 3 aliquots of 100 μl each after different incubation times to be analyzed by density gradient centrifugation.

**Density Gradient Centrifugation**—A discontinuous gradient of iodixanol was preformed by layering 260 μl of 50% Optiprep™ (AXIS-SHIELD, Oslo, Norway) at the bottom of an 11 × 34-mm Polycollamn centrifuge tube, overlayed by 260 μl of 40%, 260 μl of 30%, 780 μl of 20%, 260 μl of 10%, and 100 μl of 5% Optiprep™. The total volume of the phosphate-buffered non-linear gradient was 1920 μl. The top of the gradient was overlayed by a 100-μl aliquot of incubated Aβ-(1–40). The tubes were spun at 259,000 × g at 4 °C at 1 °C in a TL 100 ultracentrifuge (Beckman Instruments) with a TLS-55 rotor after 10 min, 1 day, and 5 days of incubation. After the centrifugation 14 fractions of 140 μl and one last fraction of 60 μl were harvested with a pipette by upward displacement. Fraction 1 from the top of the gradient was the least dense, and fraction 15 from the bottom was the most dense fraction. The fractions were subsequently analyzed with respect to their peptide content by Tris-Tricine SDS-PAGE.

**SDS-PAGE with Silver Staining**—The harvested fractions were analyzed by a denaturing discontinuous Tris-Tricine SDS-PAGE optimized for the detection of small proteins (28). A separating gel containing 6% acrylamide was overlaid with a 7.2% spacer gel and a stacking gel with 2.8% acrylamide in a Hoefer Mighty Small S.E. 250 (Hoefer Scientific Instruments, San Francisco, CA). A protein wide range marker (Mark12™ wide range protein standard, Invitrogen) with molecular weights between 2500 and 20000 was used as a size standard. The protein amounts/fraction were determined by silver staining (29).

**Fluorescence Correlation Spectroscopy**—Measurements were performed with the Confocor I instrument (Zeiss, Jena, Evotec, Hamburg, Germany) equipped with an argon laser. The pinhole diameter was 45 μm, and the focus was set 200 μm above the cover glass. Adjustment of diffusion times was achieved by comparing with rhodamine 6G. Measurements were made on Lab-Tek chambered borosilicate cover glasses (Nalge Nunc Int., Naperville, IL). The percentage of aggregated signal was determined as described in the legend to Fig. 2. Fluorescence probe Aβ-(1–42) was synthesized in solid phase using Fmoc chemistry and labeled directly at the N terminus with OregonGreen™ (Molecular Probes, Leiden, The Netherlands). The peptide was purified by reverse-phase HPLC. Purity was >95% as estimated by reverse-phase HPLC and mass spectrometric analysis (Dr. P. Henklein, Institute of Biochemistry, Charité Berlin, Germany). The stock solution containing 10 μM OregonGreen-labeled Aβ-(1–42) was prepared monomeric Aβ-(1–40) in 10 mM sodium phosphate, pH 7.2, and was filtered through 0.45-μm pore nylon filters. Although originally chosen for combination with unlabeled Aβ-(1–40), it proved to be more advantageous to use this probe with the less aggressively aggregating Aβ-(1–40). The unlabeled Aβ-(1–40) (Sigma) was dissolved at 500 μM in 100% water-free Me$_2$SO. Comparative measurements with different ligands were performed with trifluoracetic salts of the ligands, which rendered the sample preparation more convenient because of their higher solubility. In these experiments the ligands were dissolved at a concentration of 5.4 mM in 100% Me$_2$SO. The final incubation assay contained 20 μM Aβ-(1–40), 10 nM OregonGreen™-labeled Aβ-(1–42), with or without 100 μM ligand in 10 mM sodium phosphate and 6% Me$_2$SO in a 50-μl final volume. All solutions were steril filtered except the Aβ-(1–40) stock solution. For each sample the fluorescence intensities were recorded 10 times for 60 s directly after mixing and again after 1 day of incubation at room temperature. It should be noted that the sample holder of the instrument is not thermostated, so experiments were performed at ambient temperature.

The concentration dependence was analyzed by repeated measurement cycles beginning after 2 h of incubation at room temperature and lasting for 8 h. In each cycle fluorescence fluctuations for each sample position were measured 20 times for 30 s with a resolution of 16.7 data points/s. All samples contained 10 mM sodium phosphate, pH 7.2, 50 mM NaCl, 33 μM Aβ-(1–40) (Bachem Biochemica, Heidelberg, Germany), 8% Me$_2$SO, and 5.6 mM OregonGreen™-labeled Aβ-(1–42). The concentration of the ligand varied from 1.35 to 108 μM.

**ESI-MS**—For ESI-MS experiments an acidic buffer (buffer A, NH$_4$Ac/HCOOH, pH 4.2) or a neutral buffer (buffer B, Me$_2$NaCl, pH 6.8) was used. Samples containing 25 μM Aβ-(1–40) (Bachem Biochemica, Heidelberg, Germany) with or without inhibitors (Amplex, or Trimer at concentrations of 250 μM) in 10 mM buffer with 4% Me$_2$SO were freshly prepared. At last the stock solution of Aβ-(1–40) (2.5 mM in Me$_2$SO) was added to the different samples, and the mixtures were subsequently incubated at 25 °C. The time of Aβ addition to the samples was set as the starting point of the aggregation experiments. At defined time points, 25-μl samples were injected into a QuadPulsar i ESI-time-of-flight mass spectrometer (Applied Biosystems, Darmstadt, Germany) with the standard ESI source mounted. Masses were measured between 700 and 2000 atomic mass units. Buffer A or buffer B was used as a transport solvent at flow rates of 50 μl/min. For ESI-MS experiments in the positive ion mode the following settings were applied: declustering potential (DP1), 85; focusing potential (FP), 225; declustering potential 2 (DP2), 15; ion energy (E1), 1.2; nebulizer gas (GS1), 40; curtain gas (CUR), 25; and ion spray voltage (IS), 5400. The amount of monomeric Aβ was calculated by adding the peak heights of the monoisotopic M$^+$ = 1443.383 m/z, M$^{+2}$ = 1082.787 m/z, and M$^{+3}$ = 866.430 m/z peaks.

Because all internal standards would potentially interfere with the aggregation process, the use of an internal mass standard was not possible in our case. Care was therefore taken to ensure that the four samples were injected under comparable ion source conditions for each time point measured. To this end, the different aggregation experiments (β-peptide dissolved in buffer or in buffer containing melatonin, Amplex, or Trimer, respectively) were all performed in parallel with a time lag of 5 min. Although the ESI source is normally stable over several days within an acceptable range, the experiments were repeated several times, assuring that temporary fluctuations in the ESI spray efficiency did not influence the observed signal intensities.

At the beginning of these experiments, we observed a somewhat unexpected effect; obviously the ESI source capillary adsorbed Aβ to a certain extent until saturation was reached. Flushing the source over a prolonged period (1 h) with buffer inverted this effect. Therefore we saturated the source before injection of our samples with freshly prepared monomeric Aβ solution.

**RESULTS**

**Concept and Structures**—In recent years, considerable effort has been invested in the synthesis of small soluble β-sheet ligands (30–33). Simple β-sheet templates have also been incorporated into drugs that bind to a peptide region with β-sheet conformation (34). However, external templates, which force a peptide strand into the β-sheet conformation, are very rare (35–37). Meredith and co-workers (26, 27) very recently demonstrated the paramount importance of backbone hydrogen bonding for the formation of β-amyloid fibrils; replacing the amide bonds by ester linkages completely eliminated any aggregation propensity of the respective peptide-like compound taken from the Aβ sequence (26, 27). Some years ago, our group presented the first template molecule that is able to induce the formation of the β-sheet conformation in small peptides (14).

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1 The abbreviations used are: FCS, fluorescence correlation spectroscopy; ESI, electrospray ionization; MS, mass spectrometry; Fmoc, N-(9-fluorenylethoxycarbonyl); HPLC, high pressure liquid chromatography; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl))ethylglycine.
favorable multipoint hydrogen bond interaction with β-sheets. The new classes of ligands have some common promising features; they are easily synthesized, contain non-toxic components, have low molecular weights around 300, and are neutral, stable molecules in their biologically active form.

**Experimental Analysis**—Prior to systematic studies on the inhibition of protein aggregation by the designed β-sheet binders, two major experimental difficulties had to be overcome. These are (i) the low reproducibility of the Aβ aggregation, which is a typical difficulty with irreversible reactions, and (ii) the low solubility of the aminopyrazole derivatives in aqueous buffers. Induction of Aβ aggregation in the absence and presence of aminopyrazoles was achieved by decreasing the Me2SO concentration from 100% in the Aβ stock solution to a maximal 10% concentration. The details of the procedure are described under “Experimental Procedures.” We used two different approaches to analyze the interaction of the Aβ peptide with the aminopyrazole derivatives and its inhibitory effect on the peptide aggregation.

**Analysis of Aβ Aggregation by Density Gradient Ultracentrifugation**—The size distribution of the protein aggregates formed during incubation with and without ligand was determined by a sedimentation analysis on a preformed gradient of iodixanol (OptiPrep™) (42). The Aβ-(1–40) peptide was used because it was less prone to irreversible aggregation than the longer Aβ-(1–42). The incubation mixtures contained 250 µM peptide to guarantee a sufficiently sensitive protein detection in the different fractions. A volume of 100 µl of aggregation assay was incubated for 10 min, 1 day, and 5 days at 37 °C, respectively, and overlaid on a step gradient of 5–50% OptiPrep™. After centrifugation 15 fractions of 140 µl each were harvested by pipetting from the top of the gradient; fractions were analyzed by SDS-PAGE and silver staining (Fig. 3). The uncharged ligands with molecular weights below 500 were not stained in the gels. Without addition of an aminopyrazole compound the Aβ peptide was highly aggregated already at 10 min of incubation time; i.e., most of the protein was found within the bottom five fractions containing 30–50% OptiPrep™ with a density from 1.14 to 1.36 g/cm³ as determined by refractometric measurements. By comparison with gradients loaded with proteins of known s-values, these fractions contained aggregates with s-values of at least 18 S. In the bottom fraction Aβ peptide has reached its buoyant density; i.e., it does not sediment further. Some peptide, however, was left in the first three or four fractions (corresponding to s-values between 3 and 5 S). Aggregation assays without added ligand analyzed after 1 day of incubation at 37 °C showed a very narrow distribution over only two fractions (fractions 12 and 13) at the bottom of the gradient. After 5 days of incubation Aβ concentrates in fractions 13 and 14. The narrow distributions after 1 day and particularly after 5 days of incubation represented large aggregates. By contrast aggregation assays containing 750 µM Ampox or 600 µM Trimer showed an enrichment of the Aβ peptide in the upper half of the gradient, whereas nearly no peptide could be detected in the bottom fractions at the first time point. Only Ampox and Trimer were analyzed by density gradient (Fig. 3) because only these showed marked inhibition of aggregation during screening of all components by FCS (see the next paragraph). For Ampox the observed shift to lower s-values was still visible after 1 day of incubation, although after 1 and 5 days of incubation larger Aβ aggregates appeared in fractions 12, 13, and 14 similarly to the control experiment. Even after 5 days of incubation, however, some peptide could still be detected in the upper half of the gradient. For Trimer the larger aggregates were still missing after 1 day of incubation but appeared after a 5-day incubation period. The fractions in the

![Fig. 1. Hydrogen bond multiplication by covalent oligomerization of the ligands: recognition of the hexapeptide KKLVFF by a trimeric aminopyrazole ligand. Shown is a MonteCarlo simulation (MacroModel 7.0, optimized potentials for liquid simulations – all atom (OPLS-AA), water, 3000 steps). a, front view. b, side view. (Phe residues involved in hydrophobic interactions are shown in light gray).](image-url)
upper half of the gradient correspond to s-values between 3 and 5 S; i.e., the size of aggregates is below 15-mers. Thus by density gradient centrifugation we demonstrated that the aminopyrazoles Ampox and Trimer drastically influenced the size distribution of formed Aβ/H9252-(1–40) aggregates by increasing the number of small oligomeric species. Because larger aggregates as found in the control also appeared in the presence of the ligands after longer incubation times it looks rather like a retardation of the aggregation process than a prolonged prevention of aggregate formation caused by the ligands.

Counting of Aβ Aggregates by Fluorescence Correlation Spectroscopy—With FCS usually the diffusion time of a molecule or a particle with fluorescence activity through a small volume, i.e., the confocal volume of the laser optics (1 fl), can be determined. Protein aggregation is measured either by an increase of diffusion times or by a single fluorescence peak, if a single molecule or particle passes the laser focus (43). These peaks are rare events that cannot be evaluated by autocorrelation to give diffusion times. Large fluorescence bursts are produced when large and therefore highly labeled aggregates pass through the focus. The method offers the possibility to monitor the aggregation process of Aβ in a time range of min to several h; only a very small amount of peptide is required. The Aβ/H9252-(1–42) peptide was labeled directly after solid phase synthesis at the N-terminal.
A final concentration of 10 nM fluorescent probe added to 20 μM Aβ-(1–40) was sufficient for FCS measurements with a high signal to noise ratio. After the induction of aggregation the effect of aminopyrazole derivatives on the Aβ aggregation process was tested as described under “Experimental Procedures.” The assay mixtures were incubated from less than 30 min up to several days at constant temperature prior to the FCS analysis. In all experiments the aggregation assays were carried out in the absence of aminopyrazoles as a control and in the presence of aminopyrazoles to test their inhibitory efficiency. In a more qualitative type of screening experiments seven substances of the second generation (two aminopyrazoles connected via their amino group by different linkers), one substance from the third generation (a trimer of head-to-tail connected aminopyrazole moieties), and one compound of the fourth generation (modified N or C terminus) were tested. The dimers Ampox, AmpMal, AmpPht, AmpPhos, and the Trimer (Fig. 2) inhibited the aggregation of Aβ peptide, whereas addition of the dimers Amp-Pyr and Gly Dimer did not reduce the aggregation. Ampox and Trimer were identified as the most effective inhibitors in this assay and were analyzed by FCS more quantitatively. The original data are shown (Fig. 4). It is clearly seen that the number of countable Aβ aggregates is markedly reduced because of the addition of a 5-fold molar excess of ligand Ampox (Fig. 4I, b) or Trimer (c). In each graph 10 single registrations for 60 s are superimposed.

To prove that the measured inhibitory effect of Aβ aggregation is indeed ligand-dependent we determined the concentration dependence of the effect. Seven aggregation assays were started and analyzed by FCS in parallel. To quantify the aggregation of the different samples the number of all of the...
measured spikes as well as their height had to be considered. A few large aggregates should count the same as many small sized aggregates if the same number of fluorescent Aβ peptide monomers were involved. The peaks were characterized by a minimum deviation from the mean value of the fluorescence fluctuation set at 5× the standard deviation (Z-score) and multiplied by their heights. It should be noted that the nearly Gaussian distribution between Z-scores −5 and +5 represents the fluctuation signal of mono- and oligomers, and only the signals with a Z-score higher than +5 are characteristic of larger aggregates (Fig. 5a). After an incubation time of 2 h the samples were measured repeatedly for 20 times and 30 s over a period of 8 h. Trimer was used in concentrations from 1.35 to 108 μM, and the ratios of Aβ to ligand varied from 22 to 0.27 (Fig. 5). In sample 1 the number of peaks in the fluorescence-labeled 5.6 nM Aβ peptide alone was measured as a negative control. Samples 2 and 3 were two independently measured positive control assays and showed the degree of peptide aggregation without the presence of an inhibitor. The number of peaks counted in sample 4 containing 1.35 μM Trimer was the same as measured in the positive control assays. Ligand concentrations from 27 to 108 μM in samples 5–7 resulted in an increasing inhibitory effect. At 27 μM Trimer 50% inhibition was achieved. Inhibition with 108 μM Trimer was nearly complete as compared with the negative control, sample 1. During the first 10 h after inducing aggregation the number of counted peaks showed a definite dependence on the amount of added ligand.

**Determination of the Residual Concentration of Aβ Monomer during the Aggregation by ESI-MS**—Recently, it was shown that complexes of the well known β-peptide aggregation inhibitor melatonin with Aβ can be detected by ESI-MS (44). However, all of these experiments used acidic buffers (pH 4.2) (45). The buffer conditions used for FCS and ultracentrifugation measurements were not suitable for ESI-MS because a volatile buffer is necessary to transfer sizeable amounts of the peptide into the gas phase. As a positive control, we prepared a sample of Aβ containing melatonin and indeed obtained molecular ion peaks corresponding to the Aβ-melatonin 1:1 complex (data not shown). However, we were not able to discover any Aβ-inhibitor complex with Ampox or Trimer under these acidic conditions. To approach the conditions of the aggregation studies described above, we switched to a tetramethylammonium acetate buffer with a physiological pH of 6.8. As presented in Fig. 6, multiply charged ions of the monomeric Aβ (calculated, Mz+ = 1443.383 m/z, M3z+ = 1082.787 m/z, M4z+ = 866.430 m/z, and M5z+ = 722.1914 m/z (weak)) and additional sodium and potassium peaks were obtained, whereby the M4z+ signal was the most intense one. Very weak signals were obtained corresponding to Aβ dimers (calculated, M2z+ = 1731.859 m/z and Mz+ = 1237.328 m/z), and none were obtained for higher aggregates. However, again no complexes with Ampox or Trimer were detected under these conditions.

Very interestingly, in aggregation experiments, the neutral buffer displays a time-dependent decrease in the signal intensities for monomeric Aβ within 24 h. Although the aggregates cannot be detected by ESI-MS, their formation can thus be monitored indirectly by way of the Aβ monomer decrease. By contrast, in the acidic buffer, signal intensities decreased very slowly within a time scale of several days (data not shown). We assume that the aggregation process is slowed down severely under acidic conditions. This is also true if lower concentrations of Aβ are used (data not shown).

The ESI-MS method was used to determine whether the inhibitors Ampox and Trimer lead to a retarded decrease in the concentration of monomeric Aβ. Aggregation assays were prepared as described under “Experimental Procedures.” However, the Aβ monomer decrease was very similar with and without inhibitors (Fig. 6). The only deviation, which was discovered in a parallel set of experiments, was a less intense MS signal for all samples containing Ampox or Trimer from the beginning on, i.e. after an effective incubation time of 1 min.

**DISCUSSION**

Increasing experimental evidence has been accumulated during recent years, indicating that Aβ peptide aggregation itself is the primary cause for neurodegeneration in Alzheimer’s disease (15). Its pathological relevance has been underlined by recent findings of several groups that mark oligomers (10–100-mers) as the most neurotoxic species (16). In the present work a nonpeptidic drug with a rational modular design was targeted against Aβ peptides in a β-sheet structure. The donor-acceptor-donor pattern ensures selectivity for solvent-exposed β-sheets, which is not necessarily restricted to the pattern of the Aβ peptide (46).

The inhibition of the Aβ peptide aggregation by aminopyrazone was analyzed by independent, partially complementary biophysical methods. On one hand the aggregates were analyzed by FCS and density gradient centrifugation, with FCS...
focusing on larger aggregates and density gradients showing both small and larger aggregates. In FCS the aggregation process can be monitored from the early min of incubation up to several days, whereas the analysis by a density gradient cannot be carried out earlier than 4 h after the start of the aggregation process. On the other hand the Aβ monomers could be detected by ESI-MS, and in particular their concentration decrease could be monitored early after starting the aggregation. All dimeric ligands were screened by FCS, but only two strong hits were identified; a drastic decrease in the Aβ aggregation tendency was found exactly with those ligands (Ampox and Trimer) that were identified as perfectly complementary to a β-sheet by model calculations. For the 1:1 complex between an extended tetrapeptide and the ligands 1 and 3–6 only three to four hydrogen bonds were determined, whereas the complexes with ligands 2 and 7–9 contain the full number of six hydrogen bonds according to the molecular mechanistic calculations. The Trimer 9 even shows the potential for nine hydrogen bonds in
its complex with a hexapeptide. We regard this correlation between β-sheet complementarity and experimentally verified retardation of aggregation as proof of the principle of our rational design. However, these modeling data cannot explain the inefficiency of ligands 7 and 8 with respect to aggregation prevention. The reason is most likely found in the nature of their C-terminus hydrophobic (butyl group) or N-terminus hydrophilic tail (polyethylene stretch), which introduces additional capabilities for interactions and thus eliminates the capping function of the ligand. Based on this assumption the butyl-modified aminopyrazole dimer binds to Aβ but uses additional hydrophobic interactions turning prevention into enhancement of aggregation. We regard as highly improbable that the enhancement effect could be only a consequence of changing the solvent conditions because the concentration of Butyl Dimer is very low. Certain pyridone derivatives found by random screening of a large library of small organic ligands also promote Aβ polymerization (47). Variable temperature experiments with the free ligands as well as their relatively low water solubility indicate a considerable degree of self-association for ligands Ampox and Trimer (48). This problem can in part be circumvented by using their trifluoroacetate salts, which are more soluble and will automatically be neutralized in buffered solution. Thus, self-association should be minimized to achieve an optimized binding efficiency.

The ESI-MS experiments provide further insight into the aggregation process. From the ESI effects outlined above, we conclude that our β-sheet ligands do not prevent the initial oligomerization of monomeric Aβ but rather block the further aggregation of small oligomers. This explains both the absence of molecular ion peaks for 1:1 complexes between Aβ and Ampox or Trimer as well as the comparable decrease of monomeric Aβ in samples with or without inhibitors. Because of the limited accuracy of the kinetic data in Fig. 6b, additional mechanistic details cannot be derived without overinterpretation. The data are, however, in good accordance with the results from density gradient centrifugation; those results demonstrate an accumulation of Aβ oligomers of up to 15 units in the presence of the inhibitor. The finding appears also reasonable from a dynamical point of view; if the ligand binds to an Aβ monomer that is still flexible, its loss of entropy would be enhanced by the binding of the inhibitor. The finding appears also reasonable from a dynamical point of view; if the ligand binds to an Aβ monomer that is still flexible, its loss of entropy would be enhanced by the binding of the inhibitor.

Aggregation by Rationally Designed β-Sheet Ligands

Prevention of Aβ Aggregation by Rationally Designed β-Sheet Ligands

In our study the concept of rationally designed β-sheet ligands led to an effective inhibition of amyloid β-peptide aggregation. The most effective compounds show some features that are promising for future drug development; they are active in low concentrations, their hydrophobic nature might facilitate membrane passage, and they are synthesized from non-toxic building blocks. However issues like passage through membranes and toxicity have to be tested in vitro, i.e. at the first stage in cell cultures, and later in experimental animals. From a relatively large number of compounds synthesized as β-sheet binders only two were found with sufficient inhibitory activity. Thus one might expect a particular degree of specificity of these compounds for the Aβ peptide. In the future protein selectivity has to be tested in more detail because it is intolerable that new Alzheimer’s disease drugs would bind and deactivate also cellular proteins with a β-sheet structure. To increase specificity the aminopyrazoles will be linked to peptide recognition sequences. The segments KLVFF in the central part and VGGVV close to the C terminus are discussed in the literature as cover at least a portion of the interacting surface. Further experiments with neuronal cell cultures and finally with Aβ-expressing transgenic mice have to be performed to complement this in vitro study with required in vivo tests. It is known that after long incubation times Aβ aggregates undergo further conformational transitions, i.e. into fibrils (51), and it might be speculated that fibril growth finally displaces the inhibitory ligands. It remains to be clarified whether hydrogen bond interactions are the dominant players or whether as has been suggested for the early aggregation stages, hydrophobic interactions play the key role. It is conceivable that unpaired portions of the amyloid peptide bind to the aromatic aminopyrazole cores and thus interfere with formation of hydrophobic Aβ clusters.

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