Transient small molecule interactions kinetically modulate amyloid β peptide self-assembly

Axel Abelein, Lisa Lang, Christofer Lendel, Astrid Gräslund, Jens Danielsson

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1. Introduction

Increasing evidence shows a strong link between the self-assembly of the amyloid β (Aβ) peptide and the pathogenesis of Alzheimer’s disease (AD) [1]. Soluble oligomeric Aβ assemblies are thought to be the toxic species causing synaptic and neuronal injury in the patient’s brain [1,2]. Small molecules, such as lacmoid and Congo red (CR), that interfere with the oligomerization and/or fibrillation processes of proteins related to neurodegenerative diseases are frequently reported [3,4]. The effect on Aβ self-association is non-trivial, and both inhibition and acceleration have been suggested. Lacmoid was recently shown to act as an inhibitor of Aβ amyloid formation. CR promotes β-structure formation but its effect on amyloid formation is not yet clear [3,5,6]. With the more hydrophobic 42-residue variant Aβ42, lacmoid in a mixed DMSO/buffer solvent has been shown to reduce Aβ42 toxicity by acceleration of the oligomer to fibril conversion [7]. Also CR has been reported to act both as an inhibitor and promotor of Aβ fibril formation. This may be explained by the recent finding that CR has a high and a low affinity binding to the 40-residue variant Aβ40, where one accelerates and one inhibits fibril formation [8]. Both lacmoid and CR have been found to interact with monomeric Aβ40, giving rise to reduced peak intensities of NMR signals throughout the peptide sequence [5,6]. A strikingly similar behavior was shown for binding of Aβ40 to detergents [9] as well as binding of α-synuclein (αSN), a protein associated with Parkinson’s disease, to lipid vesicles [10]. The loss of NMR signal due to the formation of an “NMR dark state” has made kinetic and structural characterization difficult. Similar NMR behavior has previously been reported for the binding of lacmoid and CR to αSN [11]. In a recent study, rapid exchange between the free and CR-bound state of αSN with a population around 2% was proposed to cause attenuation of the peak intensities [12].

It is known that lacmoid and CR form supramolecular structures in aqueous solutions [5,6,12]. A polydisperse size distribution has been found, including large particles with sizes of the order of hundreds of nanometers as well as smaller species with a hydrodynamic radius of a few nanometers.

In the present study we quantitatively characterize the kinetics of the binding process between Aβ and the dyes lacmoid and CR by NMR relaxation dispersion and intrinsic tyrosine fluorescence measurements. Furthermore, the cell toxicity exerted by the Aβ:lacmoid complex is investigated.

2. Materials and methods

2.1. Materials

15N-labeled and unlabeled Aβ40 peptides were purchased from Alexo-Tech (Umeå, Sweden). Peptides were dissolved in 10 mM...
NaOH and diluted in 10 mM sodium-phosphate buffer, pH 7.4, to obtain the final concentrations as previously described in detail [5]. For NMR experiments samples with a peptide concentration of 50 μM Aβ_{40} and 10% D_{2}O were used. Fluorescence experiments were performed with 100 μM peptide stock solutions. All solutions were kept on ice throughout the whole preparation and stored at 4 °C. Lacmoid and CR were purchased from Sigma (Stockholm, Sweden) and dissolved in the same buffer used for the peptide preparation to obtain 5 mM stock solutions.

2.2. Fluorescence spectroscopy

A 100 μM peptide solution was diluted to 9 μM Aβ concentration during the fluorescence stopped-flow measurement in a 20 μM lacmoid solution to obtain a molar ratio of Aβ:lacmoid close to 1:2. The change in tyrosine fluorescence at wavelengths above 330 nm was followed as a function of time in five consecutive runs 1:2. The change in tyrosine fluorescence at wavelengths above 200 nm was excited at 280 nm and recorded in the range of 295–400 nm.

2.3. Cell viability

SH-SY5Y cells were plated at a density of 30 000 cells per well in a 96-well plate and incubated in media supplemented with 1% fetal bovine serum for 24 h at 37 °C. Media was added to reduce fetal bovine serum to 0.5%. Aβ, Aβ:lacmoid and lacmoid were added in triplicate to final concentrations of 10, 5, 2.5, 1.25 and 0.625 μM. After incubation for 72 h at 37 °C viable cells were quantified by their capacity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) [13] and resazurin [14] in parallel experiments. MTT was added to a final concentration of 0.5 mg/ml and incubated for 4 h. The formazan product was added to a final concentration of 0.1 mg/ml. After 3 h incubation fluorescence was measured (excitation 530 nm, emission 590 nm).

2.4. Nuclear magnetic resonance

NMR data were acquired at 3 °C on a Bruker Avance 700 MHz spectrometer with a cryogenic probe. 1H–13N heteronuclear single quantum coherence (HSQC) spectra were recorded using 1748 × 64 complex points and 4 scans per transient. Lacmoid and CR were titrated into 50 μM 15N-Aβ_{40} samples to obtain final molar ratios of 1:2 Aβ_{40}:lacmoid and 3:10 Aβ_{40}:CR, respectively. NMR data were processed with NMRPipe [15] and spectra were analyzed with Sparky [16]. Cross-peak intensities were evaluated as signal amplitudes. Relaxation rates were measured at different CP between the 180° pulses in the CPMG pulse train. Relaxation rates were calculated by R_{2}^{obs} = 1/T_{2}^{CP}(ln(1/I_{0})) (Eq. 1) using a reference intensity, I_{0}, with T_{2} = 0 ms, a mixing time T_{CP} of 60 ms and peak heights I for different CPMG frequencies. The fit of equation (Eq. 1) to relaxation dispersion data yields the chemical exchange rate ker, the states’ populations p_{a} and p_{b}, the relaxation rates R_{2}^{calc} and the chemical shift changes [Δδ]. Details for the fitting of relaxation dispersion data and estimation of the co-aggregate size can be found in Supplementary material.

3. Results and discussion

3.1. Both lacmoid and Congo red inhibit Aβ_{40} aggregation at near equimolar conditions

1H–13N heteronuclear single quantum coherence (HSQC) NMR experiments of the 1:1 and 1:2 complexes of Aβ_{40} and lacmoid show a concentration-dependent decrease of all cross-peak intensities throughout the whole peptide sequence (Supplementary Fig. S1) as previously reported for these dyes [5,6]. Conditions with a molar ratio of 1:2 Aβ_{40}:lacmoid and 3:10 Aβ_{40}:CR, where approximately 50% of the signal intensities remain, were chosen for the further analysis. We find that lacmoid as well as CR significantly reduces Aβ_{40} aggregation and after >100 days incubation at below 8 °C still more than 50% of the initial monomeric NMR signal is detected in the Aβ_{40}:lacmoid mixture as compared to approximately 10% remaining signal for Aβ_{40} in buffer alone after 72 days (Fig. 1). CR was also found to keep Aβ_{40} in solution, and after >80 days in refrigerator 40% of the initial monomeric Aβ_{40} NMR signal was still detected.

These findings show that the small molecule interactions effectively keeps the aggregation prone Aβ_{40} soluble and in order to understand the mechanism this interaction between Aβ_{40} and both lacmoid and CR was further studied by NMR spectroscopy.

3.2. Aβ_{40} forms transient dynamic co-aggregates with lacmoid

Fig. 2 shows the results of 15N Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments on 1:2 Aβ_{40}:lacmoid and 3:10 Aβ_{40}:CR samples. 15N CPMG relaxation dispersion profiles can be fitted to a two-site exchange model [17–19] and yield the exchange rate ker, the free and bound populations p_{a} and p_{b}, the relaxation rates R_{2}^{calc} and the chemical shift difference between the two states [Δδ] (Supplementary Table S1). Assuming a two state process involving the whole peptide, the parameters ker and p_{a} are kept fixed for all residues, while the parameters [Δδ] and R_{2}^{calc} are specific for each residue. All dispersion profiles were fitted to a constant value as well as to the two state exchange model and only data with F-test values p < 0.01 were used for the relaxation dispersion profile fitting (Supplementary Fig. S2). The analysis yields an exchange rate of ker = (2400 ± 150) s^{-1} between the free and lacmoid-bound state and the fraction bound p_{a} is (1.5 ± 0.1)%.

The exchange dynamics at a lower dye concentration, Aβ_{40}:lacmoid 1:1 ratio, was also studied in order to further characterize the...
exchange reports on a general Aβ40 aggregation process. To obtain information on this we performed relaxation dispersion experiments on Aβ40 alone, in buffer. No exchange occurs within the examined time frame, which is displayed by straight relaxation dispersion profiles, suggesting that, at this low concentration, no significant oligomeric population, corresponding to that found at higher concentrations by Fawzi et al. [20,21], can be detected (Supplementary Fig. S2 and Fig. 2D). Furthermore, we performed measurements on the remaining signal on an aggregated sample after 72 days incubation (Fig. 1). No detectable exchange was found indicating that these relaxation dispersion experiments do not report on any exchange between pure Aβ40 aggregates and monomeric Aβ40 at the chosen conditions. Calculated transverse relaxation rates of Aβ40 alone correlate well with experimental values presented previously (Supplementary Fig. S4) [22].

3.3. Size estimation of the Aβ40:lacmoid co-aggregates

The calculated relaxation rates of Aβ40 in presence of lacmoid consist of the weighted average relaxation rates of the unbound and bound population and, hence, show higher values than the experimental $R_2$ rates measured on the unbound state only. Furthermore, the asymptotic $R_2$ values are dependent on lacmoid concentration, showing lower $R_2$ values at lower lacmoid concentration (Supplementary Table S1), again reporting on the reduced peptide fraction bound to the co-aggregates. Chemical shift changes are similar along the whole peptide sequence but most significantly involving the two hydrophobic parts of the peptide as well as the hydrophilic N-terminus (Fig. 2). This indicates that the hydrophobic parts of the peptide bind the hydrophobic dye. The structural change of the hydrophilic N-terminus, which is monitored by the chemical shift changes, could be caused by restrictions of the conformational space for the N-terminus in the complex due to steric clashes.

The long-term solubilization of Aβ40 induced by lacmoid [5] and CR suggests a complex where the hydrophobic, aggregation prone, regions of Aβ40 are buried into the core of the aggregate. Such a complex may be pictured as a generalized micelle. Assuming a spherical shape of this co-aggregate the complex' size may be estimated using the relaxations rates $R_2^{calc}$ from the fitting of free and bound Aβ40 and the population $p_B$ of the bound state. The general relation between the transverse relaxation rate and the overall correlation time ($\tau_c$) [23–25] is applied and from the calculated $\tau_c$ the hydrodynamic radius was estimated to 4.0 nm for 1:2 Aβ40: lacmoid co-aggregates (details in Supplementary materials). This calculated radius is not affected by reducing the lacmoid concentration, underlining that the co-aggregate has an overall morphology that is not modulated by dye concentration. This radius coincides with the small size fraction of particles monitored by dynamic light scattering (DLS) [5], and corresponds to approximately 42 Aβ40 molecules per oligomer, assuming a molar ratio of 1:2 Aβ40:lacmoid.

3.4. Initial formation of Aβ40:lacmoid co-aggregates is slow compared to chemical exchange between free and bound Aβ40

Fig. 3 shows the kinetic traces of mixing Aβ with lacmoid. Here, the fluorescence stopped-flow experiments reveal a second, slow rate constant on a time scale of 11 ± 2 s⁻¹ and, thus, show another kinetic process than that monitored by NMR. The tyrosine fluorescence intensity displays an exponential increase after mixing the compounds and may report on the $k_{agg}$ rate for the formation of Aβ40:lacmoid co-aggregates. This co-aggregate formation rate is in contrast to the significantly faster exchange rate determined from NMR data, and suggests two separate processes, where the slower process is the initial formation of macroscopically ordered aggregates while the faster process corresponds to the exchange process.
between bound and free Aβ40. Although the estimated size of the co-aggregates coincides with the lacmoid assemblies detected with DLS the kinetic data suggest formation of a co-aggregate, different from that of lacmoid alone.

3.5. The dynamic co-aggregates are non-toxic to cultured human cells

Aβ40 forms dynamic soluble co-aggregates with lacmoid and in order to determine whether these aggregates mimic the toxic Aβ oligomers we measured the cell culture toxicity of Aβ40: lacmoid 1:2 mixture using the resazurin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. CR has been reported to decrease the cell toxicity induced by Aβ [26–28]. MTT has been proven difficult to interpret for Aβ40 toxicity [29] but the combined analysis of resazurin and MTT on cell viability shows that addition of monomeric Aβ40 has no or very moderate toxic effect on cultured cells (Supplementary Fig. S5). Addition of the co-aggregate Aβ40: lacmoid is equally non-toxic, showing that these soluble aggregates are not similar to the highly toxic Aβ oligomers [30,31]. In the MTT assay lacmoid even acts as weak scavenger of toxicity, while the resazurin assay does not report the same effect. Lacmoid alone shows no toxicity on the cultured cells. We conclude that the dynamic co-aggregates are not toxic.

3.6. Congo red and Aβ40 forms kinetically similar co-aggregates as Aβ40: lacmoid

Relaxation dispersion measurements on Aβ40 in presence of CR with a molar ratio of 3:10 Aβ40: CR, a state where about 50% of the initial NMR signal is present, show an exchange rate of (2100 ± 210) s⁻¹ between bound and unbound peptide with a complex-bound population of (1.6 ± 0.2)% (Fig. 2D and Supplementary Fig. S2), very similar kinetic parameters as in the lacmoid case. The chemical shift differences, however, display much higher values and a less uniform profile compared to Aβ40: lacmoid exchange (Fig. 2C). As described for Aβ40: lacmoid the hydrodynamic radius was also estimated for 3:10 Aβ40: CR co-aggregate to 3.8 nm which corresponds to approximately 29 Aβ40 molecules per oligomer. This is consistent with the small size fraction detected by DLS on CR alone [8]. Thus, the 3:10 Aβ40: CR co-aggregate size is somewhat smaller than for 1:2 Aβ40: lacmoid co-aggregates. In the Aβ40: CR complex the hydrophobic regions of Aβ40 are more strongly affected, and this may be due to that CR promotes secondary structure of Aβ40 when it is complexed to the co-aggregate. Indeed, CR has been reported to promote a β-structure formation in Aβ40 as determined by circular dichroism spectroscopy [6]. Hence, although a similar dynamic system describes the Aβ40: CR and Aβ40: lacmoid exchange mechanism the structural
states of the co-aggregated Aβ40 in the two cases are not the same. Although the β-structure formation induced by CR is on the amyloid formation pathway the exchange rate between the monomeric Aβ40 and the co-aggregate kinetically redirects the Aβ40 self-association from amyloid formation. Lacmoid has been shown to favor a less structured state of Aβ40 and this serves as an additional explanation for inhibition of the aggregation process to insoluble aggregates. The exchange parameters, $k_{ex}$ and $p_b$, coincide well with the values reported for the αSN:CR exchange and, thus, the underlying mechanism could be assigned to dynamic exchange of amyloidogenic proteins in presence of amyloid modulators. The exchange favors the soluble monomeric state and thus shifts the population from the aggregation precursors. Yet, the specific interaction, structural composition and modulation of the aggregation process may differ for different compounds.

4. Concluding remarks

Our studies reveal two kinetic processes for Aβ40: lacmoid interaction (Fig. 4). The slow process monitored by fluorescence stopped-flow experiments describes the initial formation of Aβ40: lacmoid co-aggregates, characterized by $k_{ex}$, where monomeric Aβ40 and lacmoid assemble and build up Aβ40: lacmoid complexes. The fast exchange process, characterized by $k_m$ and observed by NMR relaxation dispersion measurements, corresponds to exchange between complex-bound and unbound peptide. Although our NMR data do not provide definite information about the type of structure induced in the peptide, chemical exchange is found in the two hydrophobic parts as well as the hydrophilic N-terminus. In a recent study, Fawzi et al. showed that Aβ40 alone undergoes exchange between monomeric and oligomeric states on a significantly slower time scale ($k_{ex} = 73 s^{-1}$) than the exchange rate between free and co-aggregated Aβ40 found in this study [20,21]. This points at the faster kinetics of the lacmoid interaction kinetically redirects Aβ40 away from the on-pathway fibrillation, although only a very small population (1.5%) at any given time is involved in the co-aggregates. The structural state of the complexed Aβ40 seems to be of less importance as CR kinetically drives Aβ40 into a co-aggregate state featuring a β-structure, usually prone to form amyloid structures, but here kinetically favors a soluble monomeric state.

Taken together, the present results provide a detailed characterization of the dynamic exchange process of Aβ40 and two aggregation-modulating compounds. We show that the self-assembly of amyloidogenic polypeptides, such as Aβ40, can kinetically be redirected from aggregation by transient interactions with dynamic co-aggregates. This result suggests that also weakly binding molecules might be effective as therapeutics against pathogenic protein aggregation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fbelets.2012.09.035.

References

[5] Abelein, A., Bolognesi, B., Dobson, C.M., Gräslund, A. and Lendel, C. (2012) Amyloidogenic polypeptides, such as Aβ40, can kinetically be redi-