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

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ABSTRACT

Small organic molecules, like Congo red and lacmoid, have been shown to modulate the self-assembly of the amyloid β peptide (Aβ). Here, we show that Aβ forms NMR invisible non-toxic co-aggregates together with lacmoid as well as Congo red. We find that the interaction involves two distinct kinetic processes and at every given time point only a small fraction of Aβ is in the co-aggregate. These weak transient interactions kinetically redirect the aggregation prone Aβ from self-assembling into amyloid fibrils. These findings suggest that even such weak binders might be effective as therapeutics against pathogenic protein aggregation.

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 $A\beta_{42}$, lacmoid in a mixed DMSO/buffer solvent has been shown to reduce $A\beta_{42}$ 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 $A\beta_{40}$, where one accelerates and one inhibits fibril formation [8]. Both lacmoid and CR have been found to interact with monomeric $A\beta_{40}$, giving rise to reduced peak intensities of NMR signals throughout the peptide sequence [5,6]. A strikingly similar behavior was shown for binding of $A\beta_{40}$ 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 polydispersed size distribution has been found, including large particles with sizes of the order of hundred 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

^{15}N-labeled and unlabeled $A\beta_{40}$ peptides were purchased from Alexo-Tech (Umeå, Sweden). Peptides were dissolved in 10 mM

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β; CR, Congo red; αSN, α-synuclein; HSQC, heteronuclear single quantum coherence; DLS, dynamic light scattering

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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 $A\beta_{40}$ and 10% D_2O 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 1:2. The change in tyrosine fluorescence at wavelengths above 330 nm was followed as a function of time in five consecutive runs at 5 °C. As a reference the kinetic trace of Aβ diluted into buffer without lacmoid was recorded and a change in fluorescence was indeed detected but with opposite sign and k_{obs} 0.88 ± 0.01 s^{-1}, suggesting a dilution effect on the tyrosine fluorescence (Supplementary Fig. S6).

For static fluorescence intensity measurements 100 μM Aβ was titrated into a 20 μM lacmoid solution. Fluorescence intensity was measured for the pure compounds and their mixture with molar ratios of 1:4, 1:2 and 1:1 Aβ:lacmoid. The fluorescence signal 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 dissolved by addition of 20% sodium dodecyl sulfate (SDS) in 50% dimethylformamide (DMF) pH 4.8. Samples were further incubated 24 h at 37 °C before absorbance was measured at 570 nm. Resazurin 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. $^1H–^{15}N$ 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 ^{15}N-$A\beta_{40}$ samples to obtain final molar ratios of 1:2 $A\beta_{40}$:lacmoid and 3:10 $A\beta_{40}$: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 delays τ_{CP} between the 180° pulses in the CPMG pluse train. Relaxation rates were calculated by $R_2^{obs} = 1/T_{CP} \ln(I/I_0)$ (Eq. 1) using a reference intensity, I_0, with T_{CP}=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 k_{ex}, 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 $A\beta_{40}$ aggregation at near equimolar conditions

$^1H–^{15}N$ heteronuclear single quantum coherence (HSQC) NMR experiments of the 1:1 and 1:2 complexes of Aβ40 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 $A\beta_{40}$:lacmoid and 3:10 $A\beta_{40}$: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 $A\beta_{40}$ aggregation and after >100 days incubation at below 8 °C still more than 50% of the initial monomeric NMR signal is detected in the $A\beta_{40}$:lacmoid mixture as compared to approximately 10% remaining signal for $A\beta_{40}$ in buffer alone after 72 days (Fig. 1). CR was also found to keep $A\beta_{40}$ in solution, and after >80 days in refrigerator 40% of the initial monomeric $A\beta_{40}$ NMR signal was still detected.

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

3.2. $A\beta_{40}$ forms transient dynamic co-aggregates with lacmoid

Fig. 2 shows the results of ^{15}N Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments on 1:2 $A\beta_{40}$:lacmoid and 3:10 $A\beta_{40}$:CR samples. ^{15}N CPMG relaxation dispersion profiles can be fitted to a two-site exchange model [17–19] and yield the exchange rate k_{ex}, 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 k_{ex} and p_B 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 $k_{ex} = (2400 \pm 150)\ s^{-1}$ between the free and lacmoid-bound state and the fraction bound p_B is (1.5 ± 0.1)%. The exchange dynamics at a lower dye concentration, $A\beta_{40}$:lacmoid 1:1 ratio, was also studied in order to further characterize the

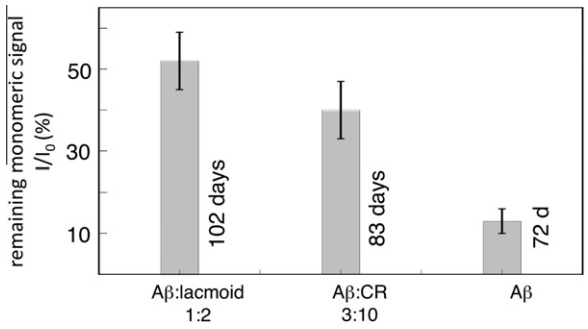

Fig. 1. Relative changes of $^1H–^{15}N$ HSQC cross-peak intensity (amplitude) of 50 μM $A\beta_{40}$ acquired at 3 °C as function of added compound and time. Remaining intensity ratio of 1:2 $A\beta_{40}$:lacmoid and 3:10 $A\beta_{40}$:CR 72–102 days after mixing are displayed. $A\beta_{40}$ alone shows significantly less monomeric signal after 72 days in refrigerator. Error bars are calculated as standard deviation of the individual cross-peaks.