Molecular Mechanism of β-amyloid Inhibition By Inositol

by

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Contents

List of Figures

List of Symbols

$\mathring{A}$	Angstrom
s	Microsecond
Aβ	β-Amyloid
AD	Alzheimer's Disease
ADP	Alanine dipeptide
CNS	Central Nervous System
MD	Molecular dynamics
mM	Millimolar
PME	Particle Mesh Ewald
STDR	simulated tempering distributed replica sampling
TMAO	trimethylamine N-oxide
VMD	Visual Molecular Dynamics

Chapter 1

Introduction

1.1 Amyloid disorders

Many diseases, most of them neurodegenerative, have been linked with the presence of amyloid: Prion-related diseases, Parkinson's, Huntington's disease, Type II diabetes etc.

1.1.1 Alzheimer's Disease

1. More than a hundred years have pass since Dr. Alois Alzheimer first presented the connection between the presence of neuronal plaques and the clinical symptoms of presenile dementia characteristic of Alzheimer's disease (AD).

2. Today, AD is known to be the most common cause of dementia in persons of age 65 or older. With the increasing longevity of our population, AD is approaching epidemic proportions with no cure or preventative therapy available.[1]

3. The discovery of amyloid plaque deposits in brains of deceased dementia patients led to the formulation of the amyloid hypothesis, which posits that amyloid aggregates initiates the pathogenesis of AD and that the other pathological symptoms such as neurofibrillary tangles are secondary.

1.2 Amyloid: formation and mechanism of toxicity

1. Finding a treatment for AD and other fatal neurodegenerative diseases motivated many biochemical and biophysical studies of the amyloid state.

2. In vitro, a variety of proteins and peptides, folded or intrinsically disordered, have been shown to be able to aggregate to form amyloid fibrils under certain solution conditions. Currently, it is thought that amyloid fibrillar state may be the globally stable folded state for all proteins.

3. Amyloid fibrils are formed via a complex aggregation pathway. Initially, monomers aggregate to form oligomers with different morphologies which exists in equilibrium

Figure 1.1:

with amyloid fibrils. Some of these oligomers are on-pathway to fibril formation, while others themselves may be end-points of the aggregation pathway. Amyloid fibrils, typically the visible endpoint of aggregation, has a cross-β structure.

4. Fibrils are protease resistant and are insoluble in the presence of SDS.

1.2.1 Fibrils

Structure

- Decades after the initial discovery by Alois Alzheimer, Aβ, the central protein component of neuronal plaques, was synthetically produced in the laboratory. In vitro, Aβ was found to precipitate out of solution almost immediately.

  – Describe the molecular structure of Aβ amyloid fibrils. Briefly mention the techniques that can be used to obtain structural information of amyloid fibrils. Early X-ray diffraction studies show that fibrils have a regular structure. This structure is defined by a 4.8Å interpeptide, and 9.8Å intersheet spacing. This defines the cross-β structure. (Figure 1.1)

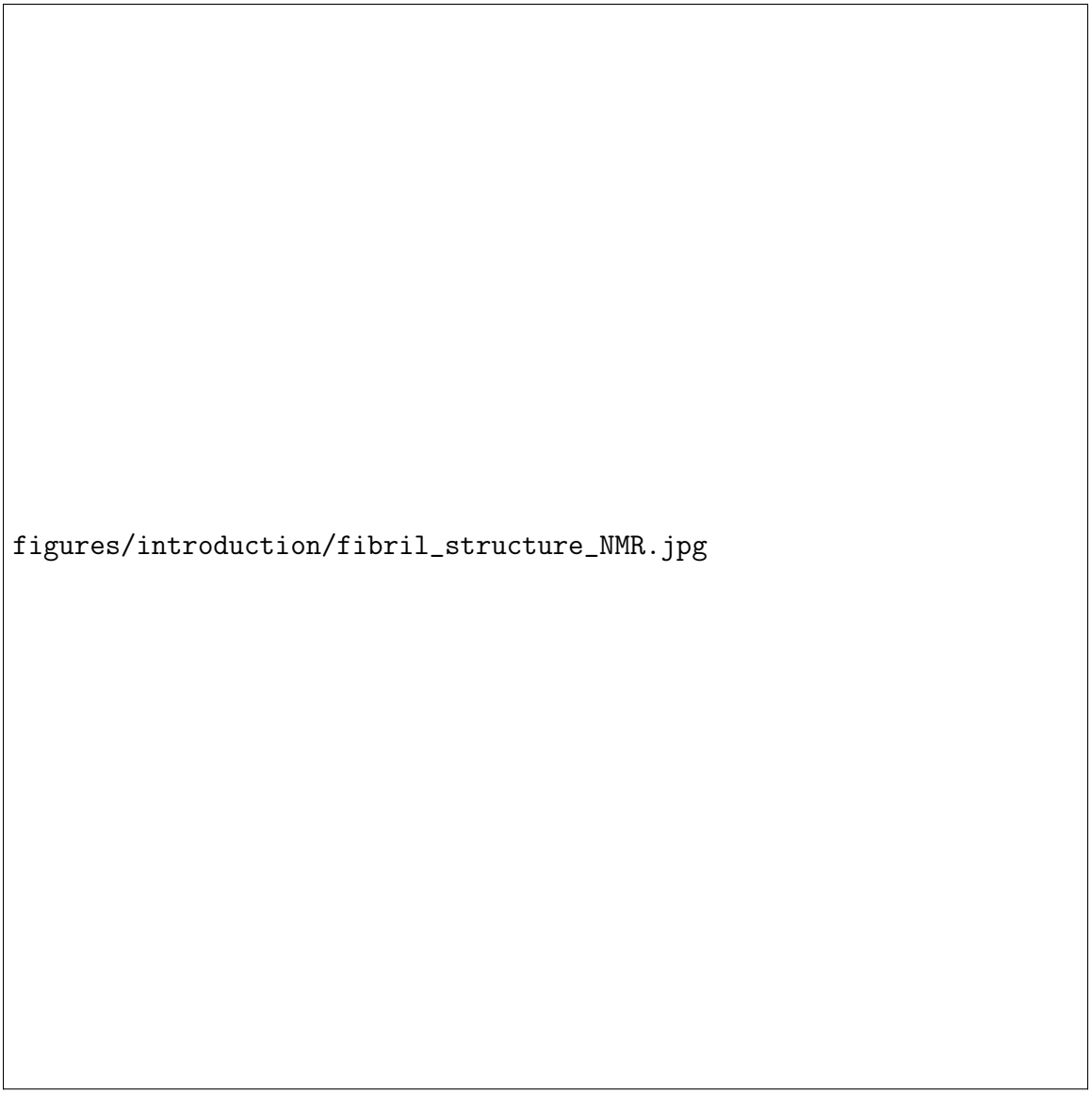

Figure 1.2: NMR model of the core Aβ amyloid fibril consistent with MPL from EM, STEM and the cross-β structure

1.2.2 Structure of Non-fibrillar oligomers

Due to their structural disorder and their insolubility, structural determination of oligomers have posed challenging experimentally.

1.2.3 Kinetics

Amyloid fibrils have been observed to form via a two-step nucleation-polymerization process. In the nucleation phase, energetic barriers of aggregation must be overcome to form the initial aggregation nucleus or seed. Following nucleation, free monomers bind to the nucleated aggregates and polymerize into mature fibrils.[2]

1.2.4 Toxicity

- Multiple lines of research have identified oligomers as a likely causative agent for neuronal cell death. By contrast, the monomeric and fibril forms are thought to be less toxic than oligomers. It is hypothesized that soluble oligomers may cause toxicity by perturbing the integrity of cellular membranes through binding and disrupting the lipid bilayer (perhaps by making them ion permeable).[3]
- Include a paragraph about amyloid formation and lipid membranes (?)

1.3 Amyloid Inhibition: A promising treatment for amyloid disorders

- In this section, I will provide an overview of some of the challenges to overcome when developing a small molecule therapeutic for Alzheimer's disease. Furthermore, using this information, I will motivate why inositol is an exciting avenue to explore.
  - scyllo-Inositol is able to cross the blood brain barrier. It has high bioavailability. Because it is not broken down in the gut, it can be taken orally.
  - Inositol is not toxic to the human body. Inositol is used in signaling pathways.
- Briefly mention non-small molecule putative therapies which also acts via amyloid inhibition. The focus of this thesis will be on small-molecule amyloid inhibition.

1.3.1 Molecular mechanisms of amyloid inhibition by small molecules

1. Amyloid inhibition as a treatment for Alzheimer's disease and related amyloid disorders. Amyloids are attractive drug targets. Small molecules may be one effective way to develop a treatment for amyloid disorders because they have the potential to be able to treat the underlying disease. Through in vitro screening, many small molecules have been found to effect the amyloid aggregation pathway.

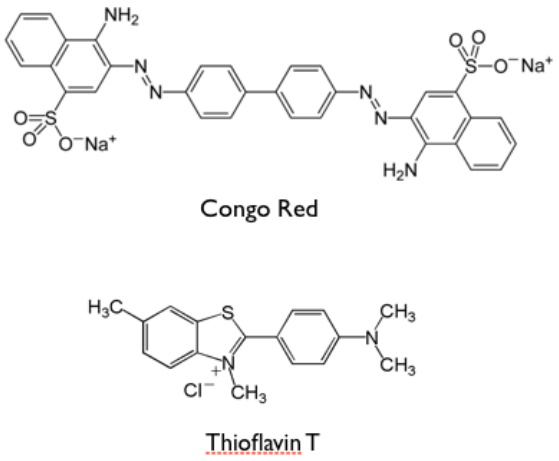

Figure 1.3: Amyloid binding dyes Congo Red and Thioflavin T

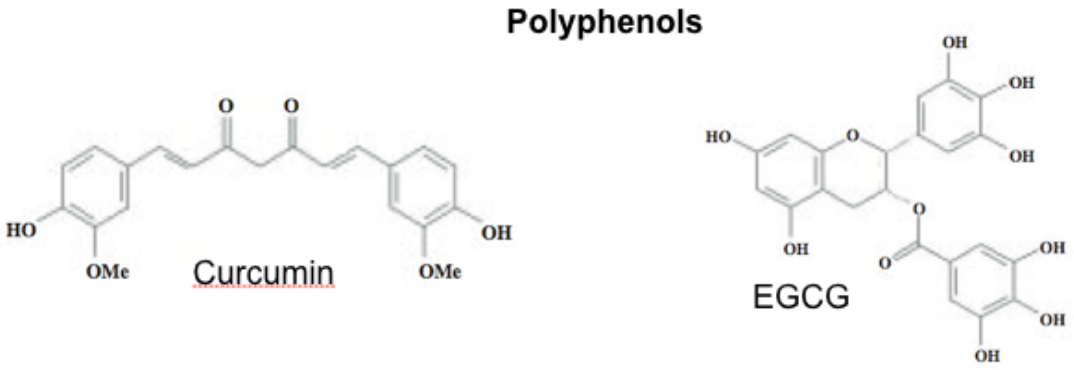

Figure 1.4: Polyphenols

Some were demonstrated to inhibit amyloid fibrils, where as others were shown to arrest or reduce oligomer formation.

(a) Pharmacological perspective of the challenge of developing an Alzheimer's drug. In order to effectively treat Alzheimer's and other neurodegenerative diseases, small molecule drug candidates must pass the blood brain barrier at sufficient concentrations for inhibition. This is difficult to achieve.

(b) In vitro screening has led to the discovery of a large number of small-molecules which were found to affect the amyloid aggregation pathway. Many of these small molecules are thought to act by directly binding to amyloidogenic peptides and aggregates.

   i. Thioflavin T and Congo red are dye molecules used to identify the presence of amyloid. Both molecules bind to mature amyloid fibrils and have been shown to affect fibril formation.(Fig. 1.3)

   ii. Polyphenols, is a large group of natural and synthetic molecules. (−)-epigallocatechin-3-gallate, curcumin, and a polyphenolic grape seed ex-

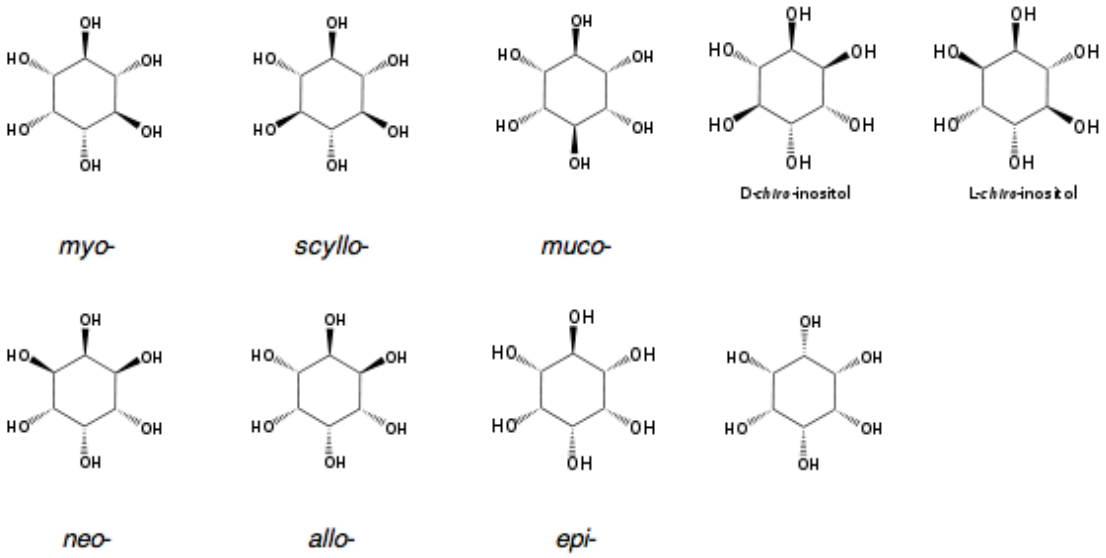

Figure 1.5: Inositol stereoisomers

tract, known for their anti-oxidant properties, were recently discovered to be capable of affecting amyloid formation.(Fig. 1.4)

(c) Small molecule inhibitors share common chemical features and groups. They are typically planar in geometry, have many aromatic rings, and polar functional groups (hydroxyl groups) around the edge of these aromatic rings.

(d) Mechanism of action. Some small molecules inhibit fibril formation, where as others may prevent oligomerization, but not fibrillation. A high concentration is often required to observe activity (micromolar to millimolar), which suggests that they may be non-specific inhibitors. EGCG, one such polyphenol, is known to have the lowest IC50.

   i. Molecular mechanism of binding of dye molecules. Thought to bind flat on on the surface grooves of amyloid fibrils where they interact with hydrophobic groups exposed at the surface.

1.3.2 Inositol molecules

(e) Inositol stereoisomers.(Fig. 1.5) Role of inositol in the human body.

   i. *myo*-inositol. Use the physiological role of myo-inositol as a lead to transition into the

(f) Where is inositol found. Present in human body tissues. Myo- is present in certain grains, grape fruit, but scyllo- is only found in small quantities in food sources.

(g) Role of inositol in amyloid inhibition. Include the background on how inositol was discovered as an Aβ amyloid fibril inhibitor.

i. In vitro and In vivo studies (mouse)
ii. Include some data on human clinical trials (?)

1.4 Analogy to Sugar-protein binding

1.4.1 Experimental techniques to study sugar-binding modes

1.4.2 Sugar Binding modes

1.5 Protein-ligand interactions

1.5.1 Forces involved in binding

- Protein-ligand non-covalent interactions that are important for ligand binding and recognition
  - Electrostatic interactions. Polar (hydrogen bonding) and charge-charge interactions
  - Nonpolar (hydrophobic) interactions
    * Van der Waals

1.5.2 Binding equilibria

- Enzyme and its putative ligand typically bind specifically (high affinity binding). We want to optimize binding specificity to increase the efficacy of the putative drug, and decrease adverse side effects (toxicity) in the human body.
- The dissociation constant, K_d, is a measure of the affinity of a ligand for its binding site on the host protein. Pharmacologically, it can be interpreted as the concentration at which 50% of the drug is bound to the protein. In experimental studies, K_d is often used to quantitatively screen for potential drug candidates.
- Binding equilibrium

$$[Protein \cdot Inositol] \rightleftharpoons [Protein] + [Inositol] \tag{1.1}$$

- The binding free energy of a ligand to a protein is directly related to its dissociation constant, K_d, the equilibrium constant of the above reaction

$$K_d = f_{ub} \frac{[Protein]\,[Inositol]}{[Protein \cdot Inositol]}, \tag{1.2}$$

- Experimental techniques for estimating K_d
  - What experimental techniques are used to estimate binding affinity? (May need to study up on this)

- Isothermal titration calorimetry (ITC) is a technique which can be used to measure energetics of ligand binding to peptides.

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[3] Dominic M Walsh and Dennis J Selkoe. A beta oligomers - a decade of discovery. *J Neurochem*, 101(5):1172–1184, 2007.

Chapter 2

Methods

- Molecular dynamics simulations are a useful tool to study the structure, dynamics, and interaction of biomolecules. MD simulations employ an empirical mathematical function to describe the atomic interactions in a molecular system, and together with classical laws of Newtonian mechanics, atomic trajectories of motion are generated. Thermodynamic and kinetic properties can then be extracted as time averages from these trajectories and used to make a number of predictions that are often experimentally challenging to observe or measure.

- MD simulation studies have been useful in studying many existing fundamental problems of biology and biochemistry, including protein dynamics and function, protein folding, biomolecular self-aggregation, and protein-ligand binding.

2.1 Methodological Details

- Why is MD correct? Describe the fundamental assumptions of MD. Here, I want to give the readers who aren't familiar with the methodological details of MD a sense of the rigorousness of MD.

- The Born-Oppenheimer approximation: electronic and nuclear motions are uncoupled, and therefore can be treated separately.

- MD does not account for the movement of electrons. Although electrons are not taken into account in MD simulations, their presence is implicitly accounted for via the use of potential energy functions. Atomic nuclei can be treated as classical particles.

- Application of an empirical force field can be used approximate atomic interactions in the system. A force field typically has many parameters which need to be calculated. One approach to do this is to fit to quantum mechanical calculations. Often, force fields are iteratively improved by predicting experimentally observable quantities for small compounds, and adjusting the fit based on comparisons of these computationally predicted quantities with experimental measurements.

- There many different force fields (AMBER, Gromos etc), each differing slightly in the potential energy function and its parameterization. In this thesis we performed all of our simulations using the force field OPLS-AA/L.

- Force field potential energy function

$$\begin{aligned} E = & \sum_{bonds} k_b(b-b_0)^2 + \sum_{angles} k_\theta(\theta-\theta_0)^2 \\ & + \sum_{dihedrals} k_\chi(1+cos(n\chi-\delta)) + \sum_{impropers} k_\gamma(\phi-\phi_0)^2 \\ & + \sum_{nonbonded} \frac{q_1 q_2}{er} \\ & + \sum_{nonbonded} \epsilon[(\frac{r_{min}}{r})^{12} - 2(\frac{r_{min}}{r})^6] \end{aligned} \tag{2.1}$$

2.2 Challenges and limitations of MD simulations

- MD is computationally challenging because of limitations in length and time scales.
  - Length scale. Large systems are too complex to obtain statistics and quantitative predictions.
  - Time scale. Relevant biochemical reactions such as protein folding happens on the time scale of milliseconds, hours, and days. Currently with MD simulations, we are routinely able to approach the microsecond time scale, massive computing power is still insufficient to observe phenomena on the millisecond timescale.
  - Obtaining convergence. Explain why this is difficult, in particular for systems with disordered peptides.

- Limitations in the accuracy of current force fields.

2.3 Application of MD: structure-based drug discovery

- A broad application of simulations of proteins is to computationally design drugs and combining that with structure-based drug design. In recent years structure-based computer modeling of protein-ligand interactions have become a core component of modern drug discovery.

- Current drug discovery platform. Typically, the first step in drug discovery is to identifying a target, a putative binding site. Then, solve the X-ray crystal structure of the target.

- Ligands which may act as potential drugs typically have high binding affinity to the binding site. The goal is to find high specificity inhibitor of a protein (usually an enzyme). The binding free energy is an important quantity which can be used to evaluate how well a ligand binds. One method of estimating binding affinity is by using computational docking methods, where the binding affinity is typically estimated without taking into account of protein dynamics. Although docking is computationally less expensive, it is inaccurate for identifying true drug candidates because binding often involves crucial changes in protein conformation.

- With computer hardware becoming faster and cheaper, MD simulation and modeling can be used to rapidly prototype experimental ideas – for example, one can perform computational alchemy, that is, "mutate" residues to test various hypotheses. Furthermore, simulations may be used to determine whether a chemical change will produce a more potent drug candidate.

- Currently state of the art computational binding studies take into the account of change in protein conformation. MD simulations is an effective method, where the protein and drug is allowed to relax and freely move about in the system.

- However, in the case of understanding a specific binding reaction (eg. when developing an enzyme inhibitor), the ability to observe the relevant binding events is a low probability event on the timescale achievable in our simulations. Therefore, it is impractical in this case to solely apply brute-force sampling techniques to determine binding free energies.

  - Methods used to determine binding free energies using simulations:
    * Thermodynamic perturbation[1]
      · thermodynamic integration
      · free energy perturbation

2.4 Review of MD studies of amyloid inhibition by small molecules

- In recent years, molecular dynamics simulations have been intensively used to investigate the molecular basis of the structure and stability of amyloid fibrils.

- MD simulations of Congo red binding have been done with the protofibril-like crystal structure composed of the segment GNNQQNY.{Wu, 2007 #621}

- A recent simulation study of an N-methylated peptide with Aβ16-22 models of amyloid aggregates has provided insight into the possible mechanism of action of peptide inhibitors of amyloid formation.{Soto, 2007 #597} This peptide inhibitor was shown to preferentially bind monomers to form dimers, possibly acting to inhibit fibril formation by sequestering monomers. However, peptide-based inhibitors have poor pharmacological profiles as they are actively broken down by proteases

in the stomach and are difficult to transport across the blood-brain barrier. In addition, these peptide inhibitors specifically target Aβ and thus do not have the potential to treat multiple amyloid diseases.

2.5 Thesis objectives and rationale

2.5.1 Challenges of amyloid inhibition

- The protein-ligand binding model developed to understand enzyme inhibition cannot be directly applied to understand the molecular mechanism of amyloid inhibition by small molecules.
  - Amyloid inhibitors are found to be very weak binders. How do non-specific inhibitors act as a drug? And how do we approach this with MD simulations?
  - Because the Aβ amyloid aggregate pathway encompasses a variety of species, some of which has no folded structure, a single conformation cannot be assumed for binding. Furthermore, structural information of amyloidogenic species lags behind those of enzymes, which tends to be globular proteins amenable for X-ray crystallography. This means that the putative binding sites are not known.
  - The structural disorder of the peptides involved poses a challenge for obtaining converged properties from MD simulations.
- Aβ peptides are completely disordered. We also do not know what the binding site looks like, where it is located on these structures.
- To date, few studies have attempted to provide statistically meaningful results pertaining to general mechanisms of protein self-aggregation and amyloid formation. Furthermore, despite the abundance of MD studies of Aβ, few studies have systematically examined the mechanism of action of small molecule inhibitors of amyloids
- In AD, there is the added challenge of the drug being able to cross the brain barrier, while remaining non-neurotoxic. What kind of drugs cross the BBB? Typically hydrophobic drugs.

2.5.2 Study Design and Rationale

- Here describe in detail how I designed my study to circumvent the challenges presented by the amyloid inhibition problem, and the limitations of MD simulations. At this point, clearly explain and discuss my study design and rationale. (Fig. 2.1)
- Beginning with the simplest model systems for an amyloidogenic peptide, the alanine dipeptide, we systematically examine binding of inositol with systems of both increasing sequence and structural complexity.

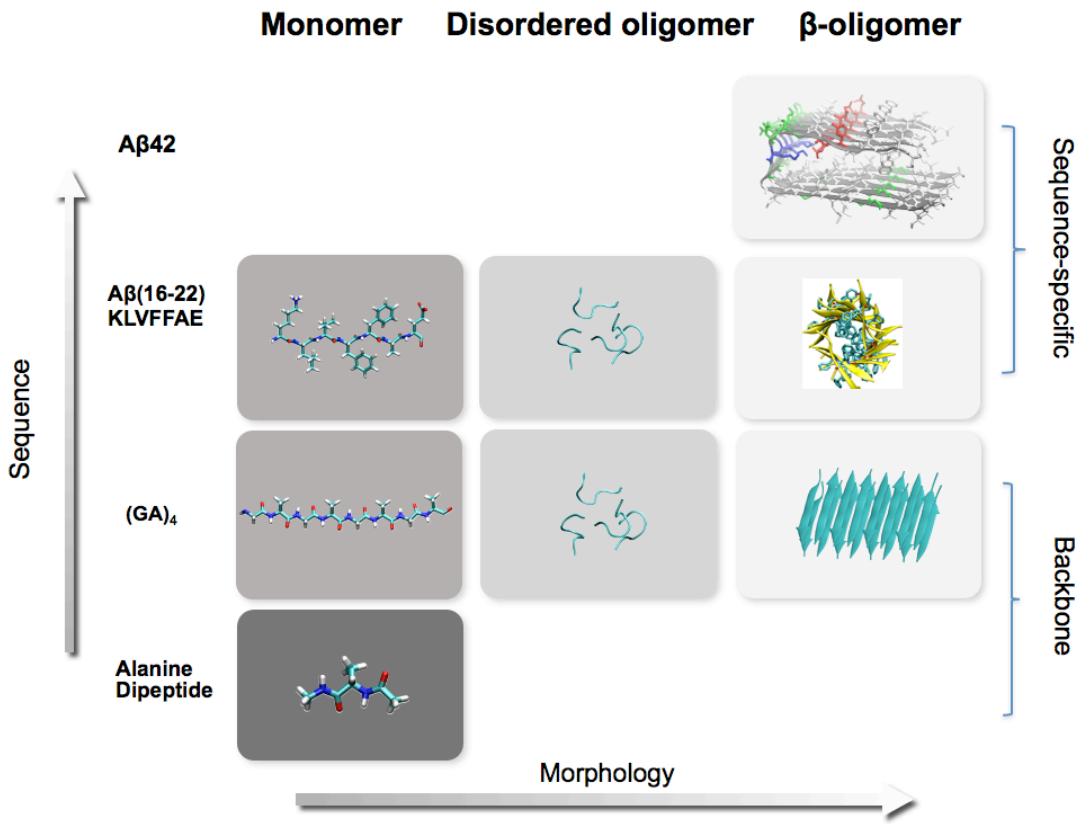

Figure 2.1: Shows the progression from small, model systems to larger and structurally more complex systems involving the full-length Aβ42 peptide.

- We exploit conventional MD simulation techniques because simulation approaches used for understanding enzyme-ligand binding is not applicable.
- Instead, we use conventional MD simulations and repeats of independent simulations to determine the binding modes, and binding equilibria of inositol with amyloidogenic peptides and aggregates of Aβ.

2.6 Thesis Organization

The first chapter introduces the thesis in the context of the field. The second chapter introduces the main methods used in the work in the thesis. Chapters 3, 4, and 5 are the results of simulations of inositol with amyloid like peptides and aggregates. Chapter 6 shows work of the general applicability of our methods developed throughout this thesis to MD simulations to understand protein - carbohydrate binding. Chapter 7 provides discussion, suggestions for future work, and perspectives.

Bibliography

[1] Michael K Gilson and Huan-Xiang Zhou. Calculation of protein-ligand binding affinities. *Annual review of biophysics and biomolecular structure*, 36:21–42, 2007.

Chapter 3

Binding of inositol stereoisomers to model amyloidogenic peptides

The contents of this section were adapted from an article published in the *Journal of Physical Chemistry*.

Reference: Li, G., Rauscher, S., Baud, S., Pomès, R. (2012). Binding of Inositol Stereoisomers To Model Amyloidogenic Peptides. Journal of Physical Chemistry B, 116(3), 1111–1119.

Contributions: Grace Li conducted the research and wrote the section. Régis Pomès provided editorial input and guidance.

3.1 Summary

The self-aggregation of proteins into amyloid fibrils is a pathological hallmark of numerous incurable diseases such as Alzheimer's disease. Scyllo-inositol is a stereochemistry-dependent in vitro inhibitor of amyloid formation. As the first step to elucidate its mechanism of action, we present molecular dynamics simulations of scyllo-inositol and its inactive stereoisomer, chiro-inositol, with simple peptide models, alanine dipeptide (ADP) and $(Gly-Ala)_4$. We characterize molecular interactions and compute equilibrium binding constants between inositol and ADP as well as, successively, monomers, amorphous aggregates, and fibril-like β-sheet aggregates of $(Gly-Ala)_4$.[3] ? nositol interacts weakly with all peptide systems considered, with mM to M affinities, and displaces the conformational equilibria of ADP but not of the $(Gly-Ala)_4$ systems. However, scyllo- and chiro-inositol adopt different binding modes on the surface of β-sheet aggregates. These results suggest that inositol does not inhibit amyloid formation by breaking up preformed aggregates, but rather by binding to the surface of pre-fibrillar aggregates.

3.2 Introduction

Amyloid fibrils formed by various peptides and proteins are known to be associated with neurodegenerative diseases, type II diabetes, and prion-related disorders.[9] In particular, amyloid fibrils of Aβ peptides are found in the extracellular deposits of neuronal plaques and are thought to be central to the pathogenesis of Alzheimer's Disease (AD),[9;19] a common and incurable neurodegenerative disease causing dementia and eventual death.

In recent years, amyloid fibril formation was discovered to be a common phenomenon among many proteins in vitro; that is, under certain misfolding and denaturing conditions, proteins can self-aggregate to form amyloid fibrils.[9] When viewed with negatively-stained transmission electron microscopy, amyloid fibrils appear as elongated, rope-like structures that are often 100 nm in length.[9] The core structure of all amyloid fibrils is the cross-β sheet.[9;60] At the molecular level, NMR[3;45] and X-ray crystallography[56] studies have revealed that the cross-β structure is comprised of extended polypeptides organized in highly-ordered, in-register β-sheets. Although amyloid fibrils are a pathological hallmark of amyloid-based diseases, smaller nonfibrillar oligomers as little as three or four peptides in size have been demonstrated to display higher cytoxicity than mature fibrils.[6;8;18;26–28;59]

An important strategy to finding a cure to AD and other amyloid diseases is to derive new therapeutic candidates through the rational design of effective small-molecule inhibitors of amyloid formation. In recent years, a number of small molecules capable of preventing aggregation and/or fibril formation have been discovered and have emerged as potential therapeutic approaches for protein misfolding diseases.[17;20;31;42;58;61] Interestingly, many of these small molecules share common chemical structural features, such as aromaticity and the presence of multiple hydrogen-bonding groups.[13;32;33;46] However, the molecular basis of the structure-activity relationship of these small molecules is not understood, thus hindering drug development efforts for amyloid-based diseases. Recently, one such small molecule, scyllo-inositol, has shown promise as a therapeutic for

AD.[36;38] Scyllo-inositol is one of nine stereoisomers that belongs to a class of cyclic polyols called cyclohexanehexols. Four stereoisomers, myo-, epi-, scyllo- and chiro-inositol (Fig. 1) are physiologically active.[15] Myo-inositol, the most abundant stereoisomer, plays an important role in signal transduction as precursor of phospholipid headgroups: once phosphorylated, myo-inositol phosphatides act as second messengers in intracellular signal transduction pathways.[15] Importantly for its therapeutic potential, inositol readily crosses the blood-brain barrier. Myo- and scyllo-inositol are found in tissues of the human central nervous system (CNS), with approximate concentrations of 5 mM and 0.1 to 0.5 mM, respectively.[39] Accordingly, they are also important osmolytes in the CNS, where alterations in their concentration have been associated with neuropathological conditions.[15;40]

In vitro, inositol stereoisomers stabilize nonfibrillar β-structure and prevent the formation of amyloid fibrils in a stereochemistry-dependent manner: scyllo-, epi- and myo-inositol inhibit Aβ fibril formation, but not chiro-inositol.[35;36;44;65;68] Moreover, scyllo-inositol was also demonstrated to be the most effective stereosiomer in preventing and reversing AD-like symptoms in transgenic mice while reducing their brain plaque load.[38] Despite this progress, the molecular basis of amyloid inhibition by inositol is not understood. In vitro studies suggest that inositol stereoisomers affects aggregation through direct interaction with Aβ peptides.[35;36;44;65] However, it is not known whether inositol acts on monomeric peptides, non-fibrillar oligomers, or fibrillar aggregates.

Some small molecule inhibitors, including the osmolytes glycerol and trimethylamine N-oxide (TMAO), are known to interfere with in vitro aggregation of amyloidogenic peptides with different sequences,[5;12;13;37;57;71] suggesting that generic interactions common to all amyloid-forming peptides and proteins may play a role in the inhibition of amyloid formation. Indeed, small organic osmolytes are hypothesized to modulate protein folding equilibria by interacting with the peptidic backbone, the chemical group common to all polypeptides.[2;21;64] Accordingly, the role of backbone solvation in the modulation of protein folding[2;53] and aggregation equilibria has recently been highlighted.[50] Furthermore, several studies have suggested that N-methylation of the backbone of amyloidogenic peptides can abolish the formation of amyloid fibrils by preventing intermolecular backbone hydrogen bonding.[62;67]

Experimental efforts to characterize the molecular interactions of small molecules with amyloid oligomers and fibrils are often impeded due to the non-crystalline, transient, and disordered nature of the aggregates involved. By contrast, molecular simulations are well-suited for studies of proteins involving disorder.[51] Although several molecular dynamics (MD) simulation studies have begun to examine the effect of small molecules on aggregation and fibril formation,[30;32;47;66] the role of backbone binding has not been considered systematically.

In this article, we present an MD simulation study of the interaction of inositol with simple model peptides to investigate its stereochemistry-dependent effect on amyloidogenic peptide aggregation and morphology. In a systematic approach, we first characterize the binding equilibria of myo-, epi-, scyllo- and chiro-inositol with alanine dipeptide, a model of the peptidic backbone. Next, to probe the stereochemistry-dependent effect of inositol binding on amyloid aggregation, we study the interaction of scyllo- and chiro-inositol, respectively active and inactive stereoisomers in Aβ amyloid inhibition, succes-

sively with monomer, disordered, and fibrillar aggregates of $(Gly-Ala)_4$ or $(GA)_4$. $(GA)_4$ is one of the simplest and shortest amyloidogenic peptides that is known to adopt an extended β-sheet structure both synthetically,[48] as a metallocopolymer,[69] and in nature, in crystalline domains of spider silks.[16;25] The repetitiveness and simplicity of the peptide sequence allow us to achieve statistically-significant estimates of the binding equilibrium from conventional sampling methods while focusing on the effect of backbone interactions in polypeptide self-aggregation.

3.3 Methods

3.3.1 Simulation Parameters and Protocol

Alanine dipeptide (ADP) was methylated at both the N- and C-terminii. The $(GA)_4$ peptide was acetylated and amidated at the N- and C-termini, respectively. The peptides were built using PyMol[1] and modelled using the OPLS-AA/L force field[23]. The extended OPLS-AA force field for carbohydrates[11] was used to model inositol stereoisomers and the TIP3P water model[22] was used to represent the solvent. Versions 3.3.1 and 3.3.3 of the GROMACS software package[63] were used to perform unrestrained all-atom MD simulations with the leap frog algorithm using an integration timestep of 2 femtoseconds. Unless otherwise noted, the following parameters were used for all simulations in this study. Electrostatic interactions were calculated using Particle Mesh Ewald (PME) summation with a grid size of 0.15 nm and a real-space cutoff of 1.45 nm.[14] The Lennard-Jones potential was computed up to 1.3 nm and was switched to zero at 1.4 nm using the GROMACS switch function. The temperature and pressure were controlled at 300 K and at 1 atm using the Berendsen thermostat and pressure coupling scheme, respectively.[4] Covalent bonds involving hydrogens were constrained using the SHAKE algorithm.[55] All resultant simulation systems were first subjected to energy minimization and equilibration with isotropic pressure coupling. Replicas of every system were generated with different random seeds for the choice of initial velocities. A trajectory frame was written to disk every picosecond and all frames were used in the final data analysis. Additional details of simulation setup and total sampling time for all systems performed in this study are listed in Table **??**.

Five initial starting conformations of ADP were obtained by taking a frame every 20 ns from a 100-ns-long simulation of ADP in water. Sets of five independent simulations were carried out successively in the presence of myo-, epi-, chiro- and scyllo-inositol. The initial conformations of monomeric $(GA)_4$ were taken from an ensemble of monomeric structures generated in water at 296 K by simulated tempering distributed replica sampling (STDR) from a previous study.[43] STDR is a generalized-ensemble simulation method developed in our laboratory, which allows each replica in the simulation to undergo a random walk in temperature to enhance conformational sampling.[52] The STDR algorithm and implementation are described elsewhere.[49] A representative set of 1117 structures were chosen from the STDR ensemble at 296 K such that the end-to-end distance probability distribution of this selected subset is similar to the distribution of the entire STDR ensemble of structures (about 12,000 structures in total). These conformations were then

Table 3.1: XXX

System	””
$N_{inositol}$	$c_{peptide}$ (mM
$N_{replicas}$	Time per replic
alanine dipeptide	””
0	61.5
5	0.1
with myo- or epi- or chiro- or scyllo-inositol & 1 & 4 & 61.5 & 246 & 5 & 0.1 & 0.5	
(GA)4 monomer	
0	61.5
1117	0.005
with chiro- or scyllo-inositol	1
61.5	123
0.005	5.585
(GA)4 disordered aggregate (preformed)	
0	246
5	0.1
with chiro- or scyllo-inositol	4
246	123
0.08	0.4
(GA)4 disordered aggregate (dispersed)	
0	246
5	0.1
with scyllo-	4
246	123
0.08	0.32
with chiro-	4
246	123
0.08	0.24
(GA)4 fibrillar aggregate	
0	437
3	0.1
with chiro- or scyllo-inositol	16
437	109
0.1	0.3

used as starting points for simulations at T = 300 K in the presence of two molecules of either scyllo- or chiro-inositol. A total of 5 μs of simulation time was generated for the monomeric systems with either scyllo- or chiro-inositol (Table 1). The initial peptide conformations of disordered oligomeric systems were either dispersed monomers drawn from the STDR ensemble at 296K or a preformed β-sheet oligomer of $(GA)_4$ composed of four peptides. The $(GA)_4$ peptide in the extended conformation was constructed using PyMol and was used to create the fibril-like β-sheet model. An eight-stranded antiparallel β-sheet was constructed by first creating an antiparallel dimer of $(GA)_4$. The principal axis of the dimer was then aligned with the x-dimension of the box and translated along the y-axis to form a single 8-stranded β-sheet. Two of these 8-stranded sheets were stacked in parallel in a "face-to-back" manner (with all Ala methyl groups facing up in the z direction) and placed in the simulation box such that the first strand at the edge of the β-sheetswas hydrogen-bonded in-register to the nearest periodic image of the eighth strand. The fibril-like systems were first subjected to energy minimization and a 500 ps equilibration stage. Production simulations were performed in the NVT ensemble with final box dimensions of 4 nm x 3.8 nm x 4 nm. Three independent simulations of the $(GA)_4$ fibril-like systems were performed for 100 ns each, successively in the presence and absence of scyllo- and chiro-inositol (see Table 1).

3.3.2 Analysis Protocol

The DSSP geometry criteria[24] were used to determine the presence of a hydrogen bond: (1) the distance between donor (D) and acceptor (A) atoms is less than 0.35 nm; (2) the distance between the hydrogen and A is less than 0.25 nm; and (3) the angle formed by D-H-A is greater than 120°. Nonpolar contacts between inositol and peptide were defined by a separation between the center of mass of inositol and the Cβ atom of alanine less than 0.45 nm. The same cut-off was used to compute protein-protein nonpolar contacts between Cβ atoms. All of the dissociation constants for inositol were calculated based on the presence of intermolecular contacts as defined above. Then, assuming that the binding equilibrium of inositol is

$$[Protein \cdot Inositol] \rightleftharpoons [Protein] + [Inositol] \tag{3.1}$$

the dissociation constant is the equilibrium constant of this reaction and is given by

$$K_d = f_{ub} \frac{[Protein]\,[Inositol]}{[Protein \cdot Inositol]}, \tag{3.2}$$

where fub is the fraction of unbound over bound peptide states. The DSSP algorithm was used for the analysis of secondary structure of the disordered oligomer with N- and C-termini of the peptides excluded. The end-to-end distance for a $(GA)_4$ peptide was calculated as the distance between Cα atoms of the N and C-terminus of the peptide. The spatial probability density of inositol is the average spatial occupancy of the atoms of inositol and was computed using the VolMap tool from the Visual Molecular Dynamics (VMD) software package. The planar angle between inositol and the fibrillar model of $(GA)_4$ was computed using the g_sgangle program from GROMACS analysis tools. All

planar angles were corrected to a value between $0 and 90$, using the rule $\alpha = f(\theta) = 180 - \theta$, if θ ¿ $90, otherwise$,f$(\theta) = \theta$. The probability distribution of the planar angles, P(α), was determined for inositol molecules with atoms within 0.25 nm of the fibril. Average nonpolar and hydrogen bonding contacts in disordered aggregates were computed using the last 70 ns of each trajectory. Error bars were determined by computing the standard deviation of the averages obtained from trajectories of independent replicas. Block averaging was used whenever a single trajectory was available.

3.4 Results

Inositol was found to bind weakly and reversibly to all the peptidic systems considered in our simulations, allowing us to characterize binding equilibria from unbiased sampling.

3.4.1 Alanine dipeptide

Inositol stereoisomers bound weakly and reversibly to alanine dipeptide with a molar Kd. A list of computed dissociation constants for each stereoisomer is shown in Table 2. Because all of the results for myo- (1.0 ± 0.1 M), epi- (1.2 ± 0.2 M), chiro- (1.0 ± 0.1 M) and scyllo-inositol (1.1 ± 0.1 M) were within error bars of one another, in this section we provide detailed descriptions and data only for scyllo-inositol. A single molecule of scyllo-inositol can bind the peptidic backbone in either monodentate or bidentate fashion, as defined by the number of hydrogen bonds between hydroxyl groups of inositol and peptide groups. At a concentration of 250 mM, scyllo-inositol was bound in the monodentate and bidentate modes about 14 ± 1% and 1.1 ± 0.1% of the time, respectively. The dominant bidentate binding modes of scyllo-inositol involved hydrogen bonds formed with the peptide main chain either in the mean plane of the inositol ring (Fig. 2A, panels I-III) or in a "face-to-edge" fashion, where the mean plane of the inositol ring is perpendicular to the plane of the peptide groups (Fig. 2A, panel IV). The Ramachandran map of ADP is characterized by four dominant basins representing the α-helical, polyproline II (PPII), and β-sheet conformations.[41] As shown in Fig. 2A, bidentate-bound ADP adopts backbone dihedral angles that fall within the dominant basins on the ramachandran map, demonstrating that scyllo-inositol is able to bind both helical and β-sheet conformations. Notably, the conformational equilibrium of ADP is shifted in favor of the β-conformer when scyllo-inositol is bound to the peptide backbone in bidentate fashion (Fig. 2B); in contrast, the relative populations of dominant conformers remained unchanged when inositol is unbound or bound in monodentate form. Taken together, our results show that although binding is weak, inositol may influence peptide conformations by binding to the peptidic backbone. In the next sections, we examine the binding of inositol to monomer and aggregates of a simple β-sheet forming peptide, $(GA)_4$.

3.4.2 $(GA)_4$ peptide

In this section, we characterize the binding modes and binding affinity of inositol systematically, first with a peptide monomer, and then with oligomeric and fibrillar aggregates

of $(GA)_4$. Here we examine only the active and inactive stereoisomers scyllo- and chiro-inositol. A summary of the equilibrium binding constants computed for all $(GA)_4$ systems is shown in Table 2.

Monomer

In its monomeric state in solution, $(GA)_4$ is an intrinsically disordered peptide.[43] An example of scyllo-inositol binding to a monomer is shown in Fig. 3A. Similar to ADP, binding is weak: the computed dissociation constants were Kd,chiro $\approx 362 \pm 16$ mM and Kd,scyllo $\approx 376 \pm 10$ mM. Most bound states, 95% for scyllo-inositol and 94.4% for chiro-inositol, involved hydrogen bonds to the backbone (Fig. 3B). At a concentration of 123 mM, inositol molecules formed a single hydrogen bond about 9% of the time, whereas two or more hydrogen bonds were formed about 4 to 5% of the time. In contrast, nonpolar contacts are less frequent and, alone, account for only 3% of bound scyllo- and chiro-inositol (Fig. 3B). In total, the peptide monomer is bound to at least one molecule of inositol approximately 25% of the time, 23% of the time with a inositol:peptide stoichiometry of 1:1 and only 2% of the time with a 2:1 stoichiometry. Contrary to ADP, the presence of inositol did not have a significant effect on the conformational equilibrium of monomeric $(GA)_4$ (Figs. 3C-D).

Disordered Oligomer

To probe whether inositol affects the structure and aggregation of small oligomers of $(GA)_4$ in solution, we performed sets of simulations involving two distinct starting states of four $(GA)_4$ peptides: (1) initially monodispersed peptides and (2) a preformed β-sheet aggregate. In the initially dispersed systems, the peptides rapidly aggregated to form a disordered oligomer (Fig. 4A) in which the majority of the residues (60%) retained a coil conformation (Fig. 4B). Similarly, systems initiated with a preformed 4-stranded β-sheet also evolved into a disordered oligomer over the course of the simulation. Only about 5% and 10% of the residues participated in a β-sheet or in a β-bridge, respectively (Fig. 4B). Despite different initial conditions and independently of the presence of inositol, all aggregates evolved to a similar morphology. The total number of peptide-peptide nonpolar and polar contacts formed within the oligomer converged to similar values for both the dispersed and preformed oligomers and did not change with time (Fig. 5). As shown in Fig. 5 (top panels), the average total number of intermolecular hydrogen bonds (8 ± 1) was consistently β-bridgehigher than the number of intramolecular hydrogen bonds (2.1 ± 0.3). On average, about 4.3 ± 0.4 nonpolar contacts were formed upon aggregation in the absence of inositol compared to 4.4 ± 0.5 contacts for scyllo-, and 5.0 ± 0.4 for chiro-inositol (data not shown for the preformed oligomer). When taken together, the above results show that the presence of scyllo- and chiro-inositol neither prevented aggregation nor disrupted the preformed oligomer. Dissociation constants of about 80 mM to aggregates of type 1 and 2 were obtained for both scyllo- and chiro-inositol. The Kd calculated for each aggregate type is shown in Table 2. In the presence of multiple aggregated chains, a single molecule of inositol was found to cross-link multiple peptides by simultaneously hydrogen bonding to their backbones (Fig. 4A). Similar to monomers,

at a concentration of 123 mM, chiro- and scyllo-inositol were bound predominantly to the backbone: 96% of bound scyllo-inositols formed only hydrogen bonding contacts (94% for chiro-inositol), whereas 2% (3% for chiro-inositol) were involved in nonpolar contacts only (Fig. 4C).

Fibril-like oligomer

In order to probe the binding modes of inositol with a fibril-like aggregate of $(GA)_4$, we constructed an 'infinite β-sheet', where the β-strands at the edge of an octameric β-sheet are hydrogen-bonded to each other's nearest periodic image. A single unit of this periodic model consisted of a stack of two antiparallel and in-register β-sheets, with eight strands per sheet (Figs. 6A,B). Although some of the hydrogen bonds defining the β-sheet structure occasionally broke, in the absence of inositol the protofibril remained approximately planar and aggregated as an infinite fibril throughout the simulation. The spatial distribution of bound inositol molecules shows that both chiro- and scyllo-inositol bind at the surface of the fibril (Fig. 6C, D). Chiro- and scyllo-inositol bound fibrillar aggregates of $(GA)_4$ with a Kd of 36 $\pm$ 15 mM and 51 $\pm$ 3 mM, respectively. The apparent increase in affinity compared to amorphous aggregates can be attributed to the following reasons. First, the fibrillar aggregate presents a much larger effective surface area than both the monomer and the disordered oligomer (Figs. 6C, D). Moreover, a larger fraction of the alanine side chains are completely solvent-exposed in the fibril-like aggregate, increasing the fraction of bound conformations involving only nonpolar contacts by nearly an order of magnitude, from 2% in the disordered oligomer to 12% for scyllo- and 18% for chiro-inositol in the fibrillar aggregate in the presence of 109 mM of inositol (Figs. 3B and 6E). Accordingly, a higher fraction of scyllo-inositol, 83 $\pm$ 1%, versus 77 $\pm$ 1% for chiro-inositol, was found to form hydrogen bonds, where the 6% drop in the hydrogen-bonded-only population of chiro-inositol was compensated by a commensurate increase in the nonpolar-bound-only population of chiro-inositol (Fig. 6E).

Thus, although chiro- and scyllo-inositol have similar binding constants, they have different binding modes to fibrillar aggregates, a feature not previously observed for the monomer and the disordered oligomer of $(GA)_4$. Both scyllo- and chiro-inositol form nonpolar contacts and backbone hydrogen bonds in poses where the mean plane of the inositol ring lies parallel, at an angle, or perpendicular to the plane of the fibril (Fig. 7). Furthermore, two or more molecules of inositol may cluster together and bind at the surface of the sheet (Figs. 7C,D). However, as shown in Fig. 8A, scyllo-inositol adopts specific binding orientations whereas chiro-inositol does not: scyllo- preferentially binds in either nearly flat ($\alpha = 20°$) or upright ($\alpha = 65°$) to the sheet, whereas chiro-inositol does not have such a bimodal preference and binds the fibril at an average angle of $\alpha = 45°$. This stereochemistry-modulated difference in binding specificity explains the somewhat higher fraction of nonpolar binding by chiro-inositol (Fig. 6E): chiro- is more likely than scyllo-inositol to bind at angles of 30° ¡ $\alpha \leq 60°$ (Fig. 8A), where 24% of bound chiro- (versus 16% for scyllo-inositol) is bound by nonpolar contacts only (Fig. 8B). For $\alpha \leq 30°$, the distributions of scyllo- and chiro-inositol bound to polar and nonpolar groups are similar (data not shown). Moreover, because chiro-inositol has a

partially-nonpolar edge whereas scyllo-inositol does not, binding in the upright position also involves more nonpolar interactions for chiro- than for scyllo-inositol (Fig. 8B). Finally, although inositol was observed to bind at the surface, binding did not change the morphology of the fibrillar aggregate.

3.5 Discussion

In the above analysis, we have systematically characterized the association of stereoisomers scyllo-, epi-, myo- and chiro-inositol with alanine dipeptide, a simple model of the peptidic backbone. Furthermore, we examined the binding of scyllo- and chiro-inositol to various aggregated states of $(GA)_4$ to probe the role of backbone binding in amyloid inhibition. Our results show that inositol exhibits weak binding with dissociation constants in the range of 0.04 M to 1 M to the different peptides and aggregation states considered.

Furthermore, the Kd of inositol increases linearly with the number of peptide groups in the system (Table 2), indicating that inositol does not bind cooperatively to the monomer and aggregate states of $(GA)_4$ considered. As expected, inositol binds most weakly to alanine dipeptide, with a value about 4 times smaller than the Kd of urea to N-acetyl alanine reported recently in the literature (0.3 M for urea[29] vs 1.1 M for scyllo-inositol). Taken together, our results indicate that the activity of inositol stereoisomers is similar to that of osmolytes, which typically have binding constants in the millimolar to molar range.[54;64]

Moreover, our results are consistent with the hypothesis that osmolytes influence protein and peptide folding and stability through direct binding rather than by modifying solvent properties.[7;29;64] The spacing of consecutive OH groups of inositol is well-suited to bidentate interactions with adjacent groups of the polypeptide backbone (Fig. 2A). Our findings shown in Fig. 2A are consistent with similar binding modes observed in a recent ab initio simulation and IR spectroscopic study of the binding of glucose epimers to the phenylalanine dipeptide backbone.[10] Furthermore, inositol stereoisomers displace the backbone conformation of alanine dipeptide towards extended β-strand conformations (Fig. 2B). However, neither scyllo- nor chiro-inositol had a significant effect on the conformational equilibrium of the $(GA)_4$ monomer. Taken together, these results indicate that inositol may not act as a drug by directly influencing monomer conformations. However, our results do not preclude the possibility that inositol may block fibril elongation by preferentially binding to monomers that are constrained to extended conformations, such as those at exposed edges of β-sheets.

Independently of the presence of inositol, both the preformed β-sheet oligomer and the monodisperse solution of $(GA)_4$ evolved into a similar morphology (Figs. 4B and 5) with only a small amount of β-structure (Fig 4B), indicating that small aggregates of $(GA)_4$ are likely to be disordered. Unlike the hydrophobic core of the Aβ peptide, $(GA)_4$ is a shorter and more polar peptide that is capable of forming more hydrogen bonds than nonpolar contacts. Our results show that peptide-peptide hydrogen bonding play an important role in the aggregation of $(GA)_4$ peptides in solution (Fig. 5). Because neither stereoisomer disrupted the aggregates of $(GA)_4$, our results indicate that inositol

is unlikely to inhibit fibril formation by breaking up preformed aggregates. Therefore, we conclude that inositol is unlikely to inhibit fibril formation by binding monomers and small disordered oligomers since binding appears to be weak, non-cooperative, and stereochemistry-independent.

By contrast, although the dissociation constants were similar for both scyllo- and chiro-inositol, binding specificity and binding modes involving nonpolar groups of the fibrillar aggregate of $(GA)_4$ were modulated by the stereochemistry of inositol. A significantly higher fraction of chiro-inositol than scyllo-inositol was bound to nonpolar groups of the fibrillar aggregate (Fig. 6E). Moreover, scyllo-inositol exhibited a bimodal distribution of binding orientations, with a significant preference for orientations in which the ring of inositol is either parallel or perpendicular to the mean surface of the β-sheet over chiro-inositol (Fig. 7). As a direct consequence of the presence of axial hydroxyl groups, chiro-inositol is more likely to bind at angles that promote contact with nonpolar groups at the surface of the fibrillar aggregate, whereas the more specific binding modes of scyllo-inositol favors backbone binding. Since this is the only stereochemistry-dependent result of our study, we speculate that scyllo-inositol acts on ordered β-sheet aggregates (as opposed to disordered oligomers or monomers). Moreover, these findings suggest a possible mechanism of action whereby a significant binding affinity to specific side chains on the surface of fibrillar aggregates could lead to the inhibition of β-sheet stacking (and therefore, amyloid fibril growth or maturation) by scyllo-inositol. Similarly, different binding modes observed in MD simulations of Aβ42 fibrils have been proposed to explain differences in binding affinities between Thioflavin T, a well-known amyloid-binding dye, and its chemical analogs.[34;70]

A factor that we have not considered in this study is the influence of inositol:peptide molar ratio on binding and inhibition. In vitro, the inhibition activity of scyllo-inositol was observed at an inositol:peptide ratio of 25:1, where inositol stereoisomers were present in excess of Aβ at concentrations of 0.25 mM to 5 mM.[36] Although our simulations had effective concentration of inositol an order of magnitude higher than in these experiments, it is possible that we have precluded cooperative inositol binding modes by limiting the number of inositol molecules present in the small simulation cell. Furthermore, Kd values obtained from our simulations of $(GA)_4$ were approximately two orders of magnitude higher than measured for Aβ. Based on our results, the predicted Kd of (GA)21, a Gly-Ala repeat peptide similar in length to Aβ, would be 1200 mM/21 = 57 mM, which is still an order of magnitude greater than in vitro inhibitory concentrations. This indicates that scyllo-inositol is unlikely to inhibit β-sheet formation by $(GA)_4$ peptides, and more importantly, that backbone binding by small molecules may not be sufficient for inhibition of amyloid formation. In future studies, elucidating the relationship of binding cooperativity and amyloid inhibition by approaching experimental drug:protein molar ratios, as well as elucidating the sequence specificity of inositol binding to amyloid fibrils, will provide further insight that may be used in the rational drug design of improved inhibitors.

3.6 Conclusions

We have performed systematic simulations of simple amyloidogenic peptide models with both active and inactive stereoisomers of inositol to examine the molecular basis of amyloid inhibition. Our results indicate that although peptide backbone dominates the interaction with inositol, the binding affinity is low and remains in the millimolar range. Moreover, this property is independent of stereochemistry and does not appear to be sufficient to impede peptide dimerization through intermolecular backbone hydrogen bonding. Taken together, our results suggest that amyloid inhibition by inositol cannot be accounted for by generic binding to the peptidic backbone alone and is likely to involve sequence-specific interactions with amino-acid side chains as well as binding to specific aggregate morphologies. Accordingly, although the formation of intermolecular hydrogen bonds is the predominant interaction in protein aggregates composed of $(GA)_4$, amyloidogenic peptides involved in amyloid diseases are often more hydrophobic and in general, self-aggregation is driven largely by the hydrophobic effect.[9] In forthcoming studies, we will examine the role of sequence-specific interactions between inositol and aggregates of pathogenic peptides.

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