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Structural Studies of Amyloid Fibril Polymorphism

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biochemistry and Molecular Biology

by

Angela Binamira Soriaga

2013
ABSTRACT OF THE DISSERTATION

Structural Studies of Amyloid Fibril Polymorphism

by

Angela Binamira Soriaga

Doctor of Philosophy in Biochemistry and Molecular Biology

University of California, Los Angeles, 2013

Professor David Eisenberg, Chair

I began my research in the Eisenberg laboratory by studying the polymorphic nature of amyloid proteins Islet Amyloid Polypeptide (IAPP), a protein whose plaques are implicated in Type II Diabetes, and Amyloid-beta (Abeta), whose aggregates were implicated in Alzheimer’s Disease. Both polypeptides are cleavage products of precursor proteins, are intrinsically disordered, and contain a highly amyloidogenic C-terminus. Later, I expanded the study of polymorphism to tumor suppressor protein p53, whose aggregation has recently been associated with tumor progression. The last part of my
dissertation involves the studies on what may constitute the toxic species of amyloid, involving work with segments from alpha-B-crystallin, IAPP, and paralogs of 53.

This dissertation begins with work on structural and kinetic characterization of IAPP using transmission electron microscopy (TEM) and thioflavin T dye-binding assays. Here, I worked under Jed Wiltzius, who had solved several crystal structures of segments of the polypeptide each of which formed in-register steric zippers. I aided in his studies by performing EM on several of the segments and confirmed they indeed formed fibrils in vitro. I also performed EM and kinetic assays on full-length mutant and wild-type human IAPP, providing evidence that IAPP is capable of forming two distinct fibril polymorphs originating from two different steric zipper spines. These results that illustrate the molecular basis for fibril polymorphism of IAPP suggests a mechanism of protein-only encoded information transfer of different prion strains.

To further understand the polymorphic nature of amyloid proteins, I then focused on structural characterization of Abeta. To elucidate Abeta polymorphism in atomic detail, my colleagues Jacques-Philippe Colletier, Arthur Laganowsky, Meytal Landau and I determined eight new micro-crystal structures of fibril-forming segments of Abeta. These structures, all of various forms of steric zippers, reveal a variety of modes of self-association of Abeta. Combining these atomic structures with previous nuclear magnetic resonance and electron tomography studies, we propose several fiber models, offering molecular models that further illustrate the polydispersity of Abeta assemblies. These structures and molecular models contribute fundamental information for understanding Abeta polymorphic nature and pathogenesis. We furthermore suggest that steric zipper
interactions are also the core of protofilaments binding together, explaining the immense heterogeneity in fibril morphologies as visualized under EM and various other characterization methods.

Structural characterization of fibril formation was carried to a third protein, tumor suppressor p53. It had recently been suggested that amyloid aggregation of mutant p53 may account for its gain of toxic function in cancer cells. Working with Alice Soragni, we elucidated the atomic details of the spine of p53 fibrils by identifying the aggregation-prone region and crystallizing two overlapping segments within the region. I also characterized a third segment that appears to exhibit a different type of steric zipper packing than other two segments. Results show that this short region within p53 displays the amyloid fibril polymorphism exhibited by Abeta and IAPP. In addition, these structures provide the basis for structure-based design of inhibitors of p53 aggregation as a potential cancer therapeutic.

A recent structure of a toxic amyloid oligomer, termed cylindrin, led me to also focus on a preliminary analysis of the mechanism of toxicity of this segment from alpha-B-crystallin. This was work done in collaboration with Arthur Laganowsky. I performed liposome disruption assays on the peptide, which suggests that the mechanism of toxicity of cylindrin may not be through membrane disruption. In addition, in collaboration with Professor Alex Van der Bliek, I attempted to transgenically express the peptide in *C. elegans*, as an in vivo model to examine toxicity. It appears cylindrin expression in *C. elegans* may induce slight toxicity, as it induces autophagosome accumulation and a slightly longer lifespan and larger brood size in the worms.
Finally, motivated by the extreme difficulty in crystallizing segments of amyloid proteins longer than eight residues, I helped in developing a methodology that has the potential to improve the chances of crystallizing proteins whose structure has remained elusive. In collaboration with Arthur Laganowsky, Minglei Zhao and Professor Todd Yeates, we developed a new crystallization approach, termed metal-mediated synthetic symmetrization, that introduces pairs of histidine or cysteine mutations onto the surface of target proteins, and, upon coordination with metal, generates novel crystal lattice contacts or oligomeric assemblies, thus producing a variety of new crystal forms, and increasing the chances of growing diffraction-quality crystals. We examined the method on two model fusion proteins, T4 lysozyme (T4L) and maltose-binding protein (MBP), and the approach resulted in 16 new crystal structures displaying a variety of oligomeric assemblies and packing modes, representing new and distinct crystal forms for these proteins. The results suggest this method has potential utility for crystallizing target proteins of unknown structure through either direct mutations on the target protein or fusion of the target protein to metal-site mutants of T4L or MBP, which could serve as crystallization chaperones.

Current work involves exploring non-typical steric zipper interactions. I have recently solved 3 more crystal structures of various segments of IAPP, one of which forms an out-of-register steric zipper. I have also solved 2 more out-of-register zipper structures of segments within p63 and p73, both paralogs of p53 and suggested to co-aggregate with mutant p53. Analysis of these out-of-register structures show that there is no weak interface among the hydrogen bonding interactions, unlike other structures that
have displayed out-of-register packing. Interestingly, cell viability assays showed that these peptides are not very toxic, suggesting the importance of these weak interfaces in amyloid toxicity. This work further confirms the polymorphic nature of amyloids.

The results embodied in this dissertation have assisted in advancing our understanding of molecular basis for amyloid fibril polymorphism and provides a preliminary characterization of the potential toxic amyloid oligomer cylinadrin. In addition, the new crystallization methodology described in this work has the potential to improve the chances of crystallizing longer amyloid segments and additional proteins of unknown structure. Greater comprehension of the structural details of amyloid proteins not only can shed light into amyloid-aggregation mechanisms, but can also offer insight into the mechanisms of toxicity and aid in the development of therapeutics that target amyloid fibrillization and block aggregation.
The dissertation of Angela Binamira Soriaga is approved.

____________________________________
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____________________________________
David Eisenberg, Committee Chair

University of California, Los Angeles
2013
DEDICATION

This work is dedicated to my parents, my brother and sister, and my fiancé, Robert.
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Throughout my time in the Eisenberg lab, I have been fortunate to be surrounded by very talented and generous scientists, doctoral students, and post-doctoral fellows. I would especially like to thank Duilio Cascio and Mike Sawaya for providing me with extensive training in X-ray crystallography. Their advice and help in structure determination have been critical in my graduate work, and I have enjoyed many synchrotron trips in their company. I also owe a special thanks to Arthur Laganowsky, who I considered a mentor in the lab and who helped me immensely in my first few years of graduate school with advice on crystallography, various experimental techniques, and basic research. In the past two years I have had the fortune of working with Alice Soragni, and I thank her for all her help in experiments, discussions, and writing. I am also grateful towards Jacques-Phillipe Colletier, Meytal Landau, Minglei Zhao, and Jed Wiltzius, who were also wonderful scientists to learn from and work with. In addition, I thank Dan Anderson for his help in the lab and for all his work in maintaining a safe and productive lab environment.

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Also, I thank Gary Fujii for the summer internship opportunity at his company. I thank support from the UCLA-NIH Biotechnology training grant and Dissertation Year Fellowship.

Chapter 1 is a published manuscript titled “Molecular mechanisms for protein-encoded inheritance” (Nat Struct Mol Biol. 2009 Sep;16(9):973-8). I thank Jed Wiltzius for allowing me to be a part of this project and for his help in experiments with IAPP. Thanks to Meytal Landau, Rebecca Nelson, and Marcin Apostol who helped write the manuscript. Thanks to the people at the UCLA Electron Imaging Center for
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Chapter 2 is a published manuscript on my work with Jacques-Phillipe Colletier and co-workers titled “Molecular Basis for Amyloid-β Polymorphism” (*Proc Natl Acad Sci. 2011 Oct 11;108(41)). I thank Jacques-Phillipe Colletier and Arthur Laganowsky for conceiving the project and conducting a lot of the crystallization work. Thanks to Meytal Landau also for her crystallization work, Lukasz Goldschmidt for performing the computational predictions, Minglei Zhao for his TEM work, and Duilio Cascio, David Flot and Michael Sawaya for helping X-ray data collection and processing. Thanks to Jacques-Phillipe Colletier for building the fiber models, Meytal Landau for drafting the manuscript, and David Eisenberg for revising the manuscript.

Chapter 3 is a draft manuscript on my p53 work titled “The atomic details of the steric zipper spine of p53 fibrils”. I thank Alice Soragni, who I worked with on this project and who was essential in our obtaining diffraction-quality crystals of the p53 segments. Thanks to JungReem Woo and Stuart Sievers for their help with crystal screening, crystal mounting, and data collection. Many thanks to the members of NE-CAT beamline ID-24-E at APS, especially K. Rajashanka, Jonathan Schuermann, Igor Kuorinov, and Malcolm Capel, for their help while at the synchrotron and giving us ample time to shoot out peptide crystals. I also thank Duilio Cascio, Michael Sawaya for helping in X-ray data collection and processing, and Jason Navarro and Michael Collazo for helping set up crystallization screens.
Chapter 4 is a draft manuscript on my cylindrin work titled “A preliminary analysis of cylindrin toxicity through membrane leakage experiments and transgenic expression in C. elegans”. I thank Arthur Laganowsky who I worked with on this project, who generated all the cylindrin constructs and did all the cloning work for generation of C. elegans cylindrin strains. I thank Gary Fuji for his summer internship opportunity at his company and Sam On Ho and Runjuhn Srivastava for help in liposome formulations and experiments. Regarding the C. elegans work, I thank Alexander van der Bliek for his collaboration and allowing me to work on C. elegans experiments in his lab and providing me with ample amount of resources to perform the experiments. Many thanks to Brian Head and Qinfang Shen also for their in C. elegans experiments.

Chapter 5 is a published manuscript on my work with Arthur Laganowsky and Minglei Zhao titled “An approach to crystallizing proteins by metal-mediated synthetic symmetrization” (Protein Sci. 2011 Nov;20(11):1876-90). I thank Arthur Laganowsky and Duilio Cascio for conceiving the project. Arthur Laganowsky worked with me on crystallizing and solving the T4 lysozyme constructs. I thank Minglei Zhao for providing all the work with MBP. Thanks to Duilio Cascio and Michael Sawaya for help in X-ray data collection and processing. Many thanks to Todd Yeates for his crystallography advice and revising the manuscript. Thanks to Malcolm Capel, K. Rajashankar, Frank Murphy, Jonathan Schuermann, and Igor Kourinov at NE-CAT beamline 24-ID-C at APS for their help in X-ray data collection. Thanks to Jason Navarro for setting up the crystallization screens.
Chapter 6 is a draft manuscript of the current work I am conducting in the Eisenberg laboratory. I thank Michael Sawaya and Duilio Cascio for their help in X-ray data collection and processing, Cong Liu for performing the cell-viability experiments, and Ramsay MacDonald for help with screening and mounting crystals. Thanks to Michael Collazo and Jason Navarro for setting up crystallization screens. Thanks to Malcolm Capel, K. Rajashankar, Frank Murphy, Jonathan Schuermann, and Igor Kourinov at NE-CAT beamline 24-ID-C at APS for their help in X-ray data collection.

Finally, I would like to acknowledge the many individuals who have helped me get to where I am today. I want to thank my college professors Jon Kull and Russell Hughes, who helped peak my interest in biochemistry and structural biology. I thank professors Jeffery Kelly and Andrew Dillin for introducing me into amyloid research, and Andrew Dillin for giving me the opportunity to work in his lab before graduate school. I am grateful to Robert Savinelli for his constant love and support, for helping me through my dissertation, and for moving to Los Angeles to wait for me to finish graduate school. I thank my brother Joseph for being a wonderful role model while I was growing up, and my sister Christine for her constant support and encouragement. Last, I would not be where I am today without the continual love, support, and guidance from my Mom and Dad, and I thank them for all they have given me.
VITA

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PUBLICATIONS AND PRESENTATIONS


Introduction

Amyloid and amyloid-associated diseases have constituted a vigorous area of research in the past two decades. Amyloids, a class of proteins that undergo changes in conformation that result in fibril formation, are implicated in a number of presently incurable illnesses including Prion diseases, Alzheimer’s disease, and Type II Diabetes. In more recent work, researchers have also associated amyloid aggregation with the progression of cancer. Despite intensive research, the study of amyloid structure, mechanism, and assembly has been hindered by their ability to form an extensive variety of oligomers and fibrils of different shapes and sizes, referred to as amyloid polymorphism.

The main structural component of amyloid, the fiber, exhibits certain attributes: (i) Congo red and thioflavin T binding, (ii) apple-green birefringence upon binding to Congo Red, and (iii) cross-beta diffraction pattern, beta sheet rich CD and FTIR spectra. Other common characteristics exhibited in most amyloids are a straight, unbranched 10-15 nanometer diameter morphology under electron microscopy, and an ability to exhibit nucleation-dependent kinetics upon Congo Red or Thioflavin T binding. X-ray diffraction analysis has shown that amyloids contain an ordered secondary structure that consists of a set of beta sheets parallel to the fibrillar axis, with their strands perpendicular to the axis. The fibril itself consists of several protofilaments, which are beta sheets that twist down the axis. Studies have found a structural motif common to short microcrystalline segments derived from a variety of amyloid-generating proteins. This motif consists of two beta-sheets with interdigitated
side chains, and is referred to as a steric zipper\textsuperscript{13,14}; it serves to significantly enhance the stability of the fiber and the rate of its nucleation.

While it was initially thought that fibrils comprised the toxic species of amyloid\textsuperscript{1,2,15,16}, increasing evidence suggests that the low molecular weight, soluble oligomers in some amyloids are more toxic\textsuperscript{17–23}. These oligomers share several characteristics: they are beta-rich and capable of also forming fibers; they exhibit greater cytotoxicity than either the monomer or fibrils formed from the same protein\textsuperscript{3,23}; and they are recognized by the conformational antibody A11 that binds oligomers but not fibrils\textsuperscript{24}. In contrast to the numerous structural studies on fibrils, the structural information of amyloid oligomers has been severely limited due to their transient, heterogeneous nature. Only very recently have some atomic-resolution details of amyloid oligomers been elucidated\textsuperscript{25,26}. These studies, particularly of an 11-residue segment from alpha-B-crystallin termed cylindrin\textsuperscript{25}, have revealed a novel packing motif consisting of out-of-register beta-sheets that has not been observed previously in amyloid species. Segments that formed these out-of-register motifs appear more cytotoxic than segments that form classic in-register fibrils\textsuperscript{25,26}; however, their mechanism of toxicity remains unknown.

While significant advances have been made to decipher the structure and mechanism of amyloid proteins, much remains to be learned for a more complete comprehension of the structural and mechanistic properties of the amyloid proteins and the roles they adopt in pathogenesis and normal function. My thesis work has assisted in advancing our understanding of the molecular mechanisms of amyloid aggregation, particularly of the fibril polymorphism of Islet Amyloid Polypeptide (IAPP), Amyloid-
beta (Aβ), and tumor suppressor proteins p53, p63, and p73. I have also attempted to examine the mechanism of toxicity of the cylindrin oligomer and aided in developing a new crystallization approach that has the potential to improve the chances of crystallizing longer amyloid segments and additional proteins of unknown structure.

**Dissertation Layout**

Chapter 1 is a published manuscript from *Nature Structural and Molecular Biology* titled “Molecular mechanisms for protein-encoded inheritance.” The paper proposes that the dry interface between self-complementing pairs of β-sheets of amyloid segments, termed steric zippers, is what provides the stability that allows for the protein-only encoded information transfer of different prion strains. Jed Wiltzius started this work by crystallizing several hexameric segments from IAPP, whose deposits are implicated in Type II diabetes. Each of the segments forms in-register steric zippers, suggesting IAPP displays fibril polymorphism, in which the same sequence gives rise to multiple packings and distinct steric zipper interfaces. Also contributing to this work were Meytal Landau and Marcin Apostol, who solved different polymorphs of other amyloid segments, and Rebecca Nelson and Michael Sawaya, who helped write the manuscript. My contribution to this project involved studies of full-length IAPP and short peptides using kinetics assays and electron microscopy. I show how various segments of IAPP that form steric zippers are capable of forming fibers in vitro, and performed EM that showed full-length wild-type and mutant IAPP forming distinct fiber morphologies. Furthermore, I performed a kinetics-based pH screen that provides evidence that full-length IAPP is
capable of two distinct fiber morphologies from two distinct spines.

Chapter 2 is a manuscript published in *Proceedings of the National Academy of Sciences* titled “Molecular basis for amyloid-β polymorphism” that expands the work from Chapter 1; here we illustrate the molecular basis for fibril polymorphism of Aβ, the protein whose deposits are implicated in Alzheimer’s disease28. Jacques-Phillipe Colletier and Arthur Laganowsky started the work by screening and crystallizing peptide segments derived from Aβ. Meytal Landau and I contributed by crystallizing several more segments. In particular, I crystallized segments 27-32 and 29-34 of Aβ. A total of eight new peptide structures are presented in the manuscript. Minglei Zhao performed TEM on the peptides and binary mixtures of peptides, showing that the morphologies of the fibrils formed by various mixtures of short segments are distinct from fibrils formed by a single segment. Combining these atomic structures with previous nuclear magnetic resonance and electron tomography studies, we propose several fiber models, offering molecular models that further illustrate the polydispersity of Aβ assemblies. These structures and molecular models contribute fundamental information for understanding Aβ polymorphic nature and pathogenesis.

Chapter 3 is a draft manuscript of my work involving structural studies of the aggregating segment of tumor suppressor protein p53. Working with Alice Soragni, we determined two crystal structures within the region of p53 suggested to be implicated in mutant p53’s gain of function in cancer cells6. Particularly residues 251-257 of p53 and 252-256 are capable of forming parallel steric zippers in which the isoleucine and leucine’s contribute in forming a dry interface along the fiber axis. Furthermore, we
identify a third segment suggested to form an anti-parallel steric zipper, providing evidence of the fibril polymorphism that can be exhibited by the protein. This work has aided in studies by Alice Soragni, who has designed peptide inhibitors that delay mutant p53 aggregation as a potential cancer therapeutic.

Chapter 4 is a draft manuscript describing a preliminary analysis of toxicity of the amyloidogenic segment from alpha-B-crystallin termed cylindrin. This was done in collaboration with Arthur Laganowsky, who had structurally characterized the segment and shown it was toxic to cultured cells\textsuperscript{25}. Our intent was to determine cylindrin’s mechanism of toxicity and characterize it \textit{in vivo}. I performed liposome assays while I was interning at Molecular Express as part of my UCLA-NIH Biotechnology training grant. Then, I attempted to test the peptide \textit{in-vivo} using transgenic \textit{C. elegans} strains that express cylindrin. The \textit{C. elegans} studies were done collaboration with Professor Alex Van der Bliek. Art generated all the clones for the strains, and I created all transgenic lines and performed all the \textit{C. elegans} work. Results suggest mechanism of toxicity of cylindrin is not through membrane disruption, which was described in supplemental information of the paper\textsuperscript{25}. In addition, results from \textit{C. elegans} work provide some evidence that cylindrin oligomer formation is off-pathway to fiber formation.

Chapter 5 contains a published manuscript from \textit{Protein Science} titled “An approach to crystallizing proteins by metal-mediated synthetic symmetrization. Here, we describe a new crystallization approach that takes advantage of protein engineering and metal-coordinated oligomeric assemblies. Arthur Laganowsky and I initiated the project
after solving two crystal structures using phasing from metals present in the structure. We introduced double or quadruple histidine mutations on the surface of T4 lysozyme (T4L) and were able to co-crystallize T4L with nickel, copper or zinc in new space groups. Minglei Zhao then applied this method to MBP, introducing similar histidine mutations and subsequently crystallizing new space groups of this protein upon incubation with metals. Metal-mediated symmetrization greatly expands the packing modes of a protein by generating new crystal contacts or oligomeric assemblies and it provides several distinct advantages over previous genetic engineering approaches. We propose two ways this method can be used to solve a protein of unknown structure.

Chapter 6 is a draft manuscript representing the current work and future directions as I conclude my graduate studies in the Eisenberg laboratory. The crystal structures determined in this chapter are results from co-crystallization attempts to capture the atomic details of a hetero-steric zipper, in which the dry interface of the steric zipper is formed from two different amyloidogenic segments. Previous studies had suggested the occurrence of amyloid hetero-association, though this type of association has not yet been seen in a crystal structure. Ultimately I determined the structures of only single segments; interestingly three of these structures, from IAPP and tumor suppressor proteins p63 and p73, reveal novel out-of-register fibril packing, a characteristic that has been associated with toxic oligomers and fibrils. I analyzed these structures in comparison to previously solved out-of-register structures observed some noticeable differences in the interfaces that suggests that the segments I studied may not be as toxic. Cell viability assays confirmed that the fibrils formed from these segments
exhibit only slight toxicity. Ramsay MacDonald aided in the crystallization studies of the IAPP segment NFGAILS, and Cong Liu performed all the toxicity assays.
References


Chapter 1: Molecular mechanisms for protein-encoded inheritance

Abstract
In prion inheritance and transmission, strains are phenotypic variants encoded by protein 'conformations'. However, it is unclear how a protein conformation can be stable enough to endure transmission between cells or organisms. Here we describe new polymorphic crystal structures of segments of prion and other amyloid proteins, which offer two structural mechanisms for the encoding of prion strains. In packing polymorphism, prion strains are encoded by alternative packing arrangements (polymorphs) of β-sheets formed by the same segment of a protein; in segmental polymorphism, prion strains are encoded by distinct β-sheets built from different segments of a protein. Both forms of polymorphism can produce enduring conformations capable of encoding strains. These molecular mechanisms for transfer of protein-encoded information into prion strains share features with the familiar mechanism for transfer of nucleic acid–encoded information into microbial strains, including sequence specificity and recognition by noncovalent bonds.
Introduction

Prions are infectious proteins that, in mammals, give rise to transmissible neurodegenerative diseases\(^1\) and, in fungi, produce heritable and sometimes beneficial phenotypes\(^2,3\). Although distinctly different in sequence and cellular role, the mammalian and most fungal prion proteins share similarities in their mechanisms of prion formation and propagation\(^2,4\). Prion formation involves a structural conversion, in which the protein changes from its normal, soluble structure to an aggregated, amyloid-like structure rich in \(\beta\)-sheets. This conversion involves breaking intramolecular noncovalent bonds and forming intermolecular hydrogen bonds and other noncovalent bonds. The resulting prion aggregates then seed the conversion of identical soluble protein molecules to the aggregated state. Mammalian and fungal prions also share the phenomenon of strains, in which structural conversions of the same protein give rise to different disease characteristics or phenotypes\(^2\). While mammalian disease strains show a correlation with differences in prion conformation\(^5-7\), the causal link has been proven in yeast: distinct conformations of aggregated Sup35 give rise to distinct strains of [\(PSI^+\)]\(^8,9\).

Amyloid fibrils, though not generally infectious, share with prion aggregates their cross-\(\beta\) spine structure and a propensity for conformational variation, or polymorphism. Amyloid conformers give rise to fibrils with distinct properties, such as NMR spectra\(^10\) and morphology\(^10, 11\) – for example, twisted versus flat fibrils, or fibrils of different widths. Similar morphologies have been observed for in vitro-formed mammalian prion fibrils\(^12\), suggesting commonalities between the conformational differences that produce amyloid fibril polymorphism and those that give rise to prion strains. The observation
that an amyloid disease is transmissible in mice\textsuperscript{13} further blurs the distinction between amyloid and prion.

Despite pioneering studies\textsuperscript{14-19}, little is known at the atomic level about the nature of the conformational differences that give rise to polymorphic amyloid fibrils and prion strains. In our previous work, we determined 13 fibril-like structures of segments from proteins known to fibrillize; these structures consisted of pairs of tightly packed, highly complementary $\beta$-sheets\textsuperscript{20, 21}, which we termed ‘steric zippers’. Each steric zipper is formed from identical short segments of protein molecules, stacked into $\beta$-sheets that run the entire length of the amyloid-like fibrils, and of the closely-related needle-shaped microcrystals\textsuperscript{21, 22} used to determine the atomic structures of the steric zippers. We noted that these steric zippers included three polymorphic pairs (alternative packing arrangements) that might be connected to the phenomenon of prion strains. Since then, in the course of determining new fibril-like structures, we have found that polymorphic structures are common among steric zippers. Here we present nine new structures that include three distinct types of steric zippers, and we present the structural and biochemical arguments that indicate the conformational differences of prion strains may be attributable to polymorphic steric-zippers. We also discuss the similarities in information transfer between protein-encoded prion inheritance and the more familiar nucleic acid-encoded inheritance.
Results

Packing polymorphism of steric zippers

In determining the atomic structures of steric zippers by X-ray microcrystallography, including nine new structures reported here (Tables 1-1, 1-2, and Supplementary Table 1-S1), we have found that some segments of amyloid and prion proteins form two packing types, or polymorphs. Four such pairs are shown in Figure 1. The fibril-forming segment\textsuperscript{22} SSTNVG is derived from islet amyloid polypeptide (IAPP), a 37-residue hormone that forms fibrillar amyloid deposits among the pancreatic β-islet cells of nearly all type II diabetics\textsuperscript{23}. Microcrystals of SSTNVG grown from different solutions revealed two structures (polymorphs), one featuring a pair of serine residues at the center (Fig. 1-1a, left)\textsuperscript{22} and the other, with a shifted registration of the two β-sheets, featuring a pair of asparagine residues at the center (Fig. 1-1a, right). In both polymorphs, a pair of tightly interdigitated β-sheets, with no water molecules in the interface, forms the basic dry steric zipper structure. A second pair of polymorphic steric zippers is formed by the segment VQIVYK (Fig. 1b), from the fibril-forming protein tau, associated with Alzheimer’s disease\textsuperscript{24}. Again, microcrystals grown under different conditions (Ref 5, and see Methods) produced different structures related by a shift in registration of the two sheets. These two pairs of polymorphs, of segments SSTNVG and VQIVYK, show that a given fibril-forming sequence can form distinct steric zippers by adopting distinct registrations of the two β-sheets. We term this ‘registration polymorphism’. 
Two additional pairs of steric zippers (Fig. 1-1c, d), one from the fibril-forming segment NNQQ of the yeast prion Sup35\(^{21}\), and the other from the segment NNQNTF of elk prion protein associated with strains\(^{25}\), reveal what we term ‘facial polymorphism’. In the steric zippers on the right, the two sheets are packed face-to-face, as in the SSTNVG and VQIVYK. The steric zippers on the left are packed face-to-back (NNQQ), or back-to-back (NNQNTF).

Each of the eight steric zippers of Figure 1-1 appears stable and is likely to be separated from its alternative polymorph by a high energy barrier. Each β-sheet of a zipper is stabilized by main chain hydrogen bonds between layers. The two sheets of a zipper are held together by van der Waals bonds between the highly complementary, interdigitated side chains, and in a few cases by intersheet hydrogen bonds, as in the left polymorph of SSTNVG (Fig. 1-1a). To transform one polymorph into another, it is necessary to break the inter-sheet contacts, reposition the two sheets, and interdigitate the side chains to form the second zipper. The high energy barrier presented by bond breaking means that the two polymorphs are distinct, stable, long-lived structures.

**Segmental polymorphism in steric zippers of IAPP**

The amyloid-forming protein IAPP displays a rich variety of fibril morphologies\(^{11,18}\). IAPP also shows a rich variety of steric zippers: six different segments of the IAPP sequence form distinct amyloid-like fibrils and microcrystals (Fig. 1-2). Rather than different packings of the same segment, described above, each of these
polymorphic steric zippers is formed from a different segment of the IAPP sequence (Supplementary Figs. 1-S1–S3).

The six IAPP segments of Figure 1-2 form fibrils and needle-shaped microcrystals suitable for X-ray diffraction studies. Each of the six microcrystals reveals a distinctive steric zipper structure. We expected fibrils and microcrystals formed by the same segment have similar structures, because fibrils and microcrystals often grow under the same condition (see the micrograph of NNFGAIL fibrils, Fig. 1-2); in both, the extended segment is perpendicular to the long axis, with the β-sheets parallel to the axis; both give characteristic amyloid diffraction peaks at ~10 and 4.7 Å (ref. 26), and occasionally we find fibrils that appear to emanate from the tips of microcrystals (see the micrograph of HSSNNF fibrils, Fig. 1-2). In short, six segments of one protein, IAPP, form six fibril-like structures, each differing from all the others at the atomic level, a striking segmental polymorphism.

**Full-length IAPP forms distinct steric zippers**

Our biochemical studies of human and mouse IAPP show that the spines of full-length IAPP fibrils can be built from steric zippers of at least two different segments. Human IAPP forms fibrils of several morphologies\(^\text{11, 18}\), whereas mouse IAPP does not form fibrils\(^\text{27}\) (Fig. 1-3a). The established involvement of the C-terminal region (residues 21-37) in human IAPP fibrillization\(^\text{28}\) is reinforced by differences in the human and mouse sequences (Fig. 1-2a and Supplementary Fig. 1-S1); five of the six residue differences are in the C-terminal region.
We find that the N-terminal region of IAPP (residues 1-20) is also sufficient to drive fibrillization. Replacement of Arg18 in mouse IAPP with histidine (R18H), as in human IAPP, imparts fibril-forming ability to mouse IAPP, although these fibrils form more slowly than those of human IAPP\(^9\) (Fig. 1-3a). Also, the pH profile of mouse IAPP R18H fibril formation shows an inflection at pH 6 (Supplementary Fig. 1-S5), roughly the pKa of the substituted histidine, further implicating His18 in fibrillization (Fig. 1-3b). Clearly the C-terminal region of mIAPP R18H is not responsible for fibrillation because of the mouse substitutions that prevent fibrillation (Fig. 1-2a). Moreover, the fibrils of human IAPP and mouse IAPP R18H differ in morphology (Fig. 1-3c). A further experiment suggests that a segment within the C-terminus preferentially forms the spine within full-length human IAPP. In this experiment (Fig. 1-3a), substitution of the mouse residue into human IAPP at position 18 (H18R) did not affect the kinetics of fibril formation (Fig. 1-3a). It does, however, lower the fluorescence maximum, probably due to lower ThT binding, perhaps from alternative packing of protofibrils into fibrils.

Thus, the human IAPP sequence contains at least two fragments capable of forming amyloid cross-β spines: the C-terminal region, with a high propensity to form a zipper spine, and a kinetically less favorable segment within the N-terminal region, containing His18 (Fig. 1-3a). The formation of a steric zipper within either region would preclude the formation of a steric zipper within the other region of the same molecule. Considering this result in light of the structures in Figure 1-2, we conclude that full-length IAPP can form polymorphs based on segmental polymorphism at the atomic level.
Discussion

Peptide polymorphs, prion strains, and amyloid polymorphs

Our determination of molecular structures for polymorphic short segments falls far short of revealing the molecular structures for the entire polymorphic fibrils themselves. Solid-state NMR, hydrogen-deuterium exchange, cryo-electron microscopy, and other methods have elucidated some fibrous molecular structures, but not yet atomic structures associated with strains. The highest resolution view to date of a prion fibril is that of HET-s, in which the sidechains protruding from pairs of β-strands interdigitate, much like the sidechains within steric zippers. That the structures of full proteins in fibrils are more complicated than the structures of short segments is certain: amyloid proteins generally have more than a single segment that forms a steric zipper, and thus protein fibrils probably contain more than a single type of β-sheet. In fact, one strain might differ from another in part by the order in which the various steric zippers are nucleated. The variety of steric zippers formed by IAPP (Fig. 1-2) gives a glimpse of the complexity of fibrous states that even a small protein can show. However, even before the complexity of prion strains is fully understood, the models for polymorphism reported here offer one basis for thinking about conformations of prion strains at the molecular level.

A similarity between the polymorphic segment structures reported here, and actual prion strains and amyloid polymorphs of full proteins, is that different conformations arise from different environmental conditions. For example different strains of Sup35-NM can be produced by incubation at different temperatures.
Similarly, distinct polymorphs of Alzheimer's β-amyloid fibrils can be produced by either agitating the dissolved precursor or not agitating\textsuperscript{10}. In our case, the polymorphs are produced under different solution conditions. In cells, different environmental conditions might arise from differential hydration, oxidative, or xenobiotic stress.

Lastly, the disruptive effect of proline scanning to fibrillation propensity is similar between peptide fibrils and full-length amyloid and prions. When proline residues from the non-fibrillizing mouse IAPP sequence are substituted into these fibrillizing segments from human IAPP, the segments no longer form fibrils. Three segments with proline replacements were tested: PPTNVG, PPTNVGSNTY, and PVLPPPT. None of these segments formed fibrils nor microcrystals over a period of a year. These observations strengthen the relevance of the short segments whose structures are presented here to the behavior of fibrils of the full protein.

**Information transfer in biology**

The results presented here suggest that the protein-encoded information transfer associated with prion strains shows similarities to nucleic acid-encoded information transfer of chromosomal inheritance (Table 1-3). The molecular basis for microbial strains is inherent in the central dogma of molecular biology: changes in gene sequence are translated into changes in protein structure; altered catalysis or interactions account for changed phenotypes of the mutant strains; and the phenotypes are heritable because the altered genes are inherited. In contrast, the prion hypothesis\textsuperscript{1} – that a protein conformation can define a transmissible or heritable phenotype – was slow to be
accepted, because the notion that multiple protein conformations can be heritable or transmissible fell outside common scientific experience. Proteins such as hemoglobin having alternative conformations have long been known. But the energy barriers between the conformations are low, and there was no known way that a conformation could be stabilized during the transmission to progeny cells. To achieve a high energy barrier between conformations there must be a strong non-covalent force that holds the protein in each distinctive conformation during the process of transmission.

The recognition that prion proteins can enter an amyloid-like, fibrillar state offers a molecular mechanism for a stabilized, enduring conformation: the fibrillar state is maintained by a high density of hydrogen bonds. More recently, we found that amyloid and prion-derived fibrils have a steric-zipper spine maintained also by strong van der Waals forces between the sheets and strong electrostatic polarization. The strong van der Waals forces bond self-complementary protein sequences, so that the interaction is sequence specific. Prion and amyloid-like fibrils are stable under physiological conditions, so that fibrils can be transmitted to progeny cells. Also, because a protein fibril introduced into a solution of the same protein can seed the dissolved molecules into fibrils, there is an evident molecular mechanism for prion conversion based on fibril properties.

Amyloid fibrils have the capacity for carrying the information of prion strains because they are polymorphic; the same protein can form a variety of distinct fibril types. Yet the molecular mechanism of fibril polymorphism has remained obscure. One proposal, for the specific case of Aβ(1-40) fibrils, is that one polymorph is
dimeric and the other trimeric\textsuperscript{16}. But because prion strains and fibril morphologies are so varied\textsuperscript{6, 39}, there must be molecular mechanisms that are capable of encoding not just two polymorphs, but many.

The results present here suggest that the molecular basis of fibril polymorphism may be based on the large variety of steric-zipper amyloid spines that can form from a single protein. These mechanisms are summarized in Figure 1-4. Considering the possible variety of packing, segmental, and combined structures for steric zippers, it is clear that a substantial variety of prion strains associated with a single protein can be encoded by steric zippers. Although combinatorial polymorphism has not yet been observed on the atomic level, biochemical evidence suggests it may operate in Sup35 fibril polymorphism\textsuperscript{14, 15, 17}.

The molecular aspects of the transfer of genetic information by the familiar mechanism of nucleic acid inheritance show similarities to the less familiar protein-based mechanism suggested here for prion strains. In both cases, transfer is by non-covalent bonding. In nucleic acid inheritance, information transfer is achieved by base-pairing, involving complementary hydrogen bonding between bases. In the mechanisms proposed here for prion strains, information transfer is achieved largely by the steric fit (i.e. van der Waals bonding) of short, self-complementary amino acid sequences, with hydrogen bonding maintaining the zipper spine. Both mechanisms are sequence specific, based on nucleic acid sequences or on self-complementary protein sequences. In the case of prions and amyloid proteins, hydrogen bonds maintain the integrity of the spine, whereas in nucleic acids, covalent bonds maintain the integrity of the backbone. The
greater integrity of the covalent backbone of nucleic acids provides a more robust mechanism for ensuring the continuity of life. Though the variation possible in protein-encoded inheritance is small in comparison to that in genomic DNA, the number of possible steric zippers is enormous. Though less structurally robust and more restricted in information content, protein-based inheritance could allow more rapid response to environmental changes than Mendelian mechanisms. In short, the steric zipper presents an alternative model of information transfer that to have been adopted by a few microbial and mammalian proteins and perhaps many others, yet to be discovered.

Methods

Amyloid propensity prediction. We predicted fibril-formation propensities for human and mouse IAPP sequences using the 3D Profile method. This algorithm uses the amyloid-like crystal structure of the NNQQNY segment as a structural template. Each six-residue proline-free segment sequence is threaded onto the NNQQNY backbone structure, and the energetic fit is evaluated using the RosettaDesign program. Based on energies of experimentally determined amyloid-like segments, we chose an energy threshold for fibril-formation propensity of –23 kcal mol\(^{-1}\). That is, segments with computed energies equal to or below this threshold are deemed to have high propensity to form fibrils. The energies of all hexameric segments in IAPP were plotted, assigning the computed energy to the first residue of the hexameric segment (Fig. 1-2a).

Crystallization and Structure Determination. All peptide segments (custom synthesis, CS Bio) were crystallized using the hanging drop/vapor diffusion method. Details of
crystallization, structure determination and refinement for each of the novel structures are provided in the Supplementary Information.

*Fibril Formation.* Lyophilized segments were dissolved to 1 mM in 100% hexafluoroisopropanol (HFIP), then diluted to 10 uM in 20 uM sodium acetate pH 6.5 (1% (v/v) HFIP final). Fibril formation was monitored by thioflavin T fluorescence at 450 nm excitation and 482 nm emission wavelengths. Data were collected in triplicate; error bars show the standard deviation between samples. For the experiments performed as a function of pH, samples were allowed to incubate at least one day and the final ThT fluorescence signal averaged for at least three samples is shown for comparison. The quiescent samples were incubated with 10uM ThT at 37°C in various buffer conditions for appropriate pH. The measured presence or absence of fibrils was confirmed by negative stain electron microscopy (data not shown).
Table 1-1. Data collection and refinement statistics for structures in Figure 1-1

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*One crystal was used for each data set. Values in parentheses are for highest-resolution shell.
Table 1-2  Data collection and refinement statistics for structures in Figure 1-2

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<td>3.1 (3.1)</td>
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**Refinement**

|                  |        |         |        |        |                |                |
| Resolution (Å)   | 19.29–1.85 | 25.97–1.84 (1.89–1.84) | 23.45–1.50 (1.68–1.68) | 43.31–1.40 (1.44–1.44) | 20.99–1.50 (1.67–1.67) | 28.51–1.60 (1.78–1.78) |
| No. reflections  | 379 (26) | 825 | 509 | 4723 | 662 | 984 |
| $R_{work}$ / $R_{free}$ | 19.2/23.2 | 23.8/28.2 | 17.3/20.7 | 22.2/26.5 | 14.8/15.8 | 12.4/15.9 |
| No. atoms        | 51      | 124     | 50     | 328    | 53     | 318     |
| Protein          |        |         |        |        |                |                |
| Ligand/ion       | 1       | 5       | 0      | 0      | 0     | 0       |
| Water            | 1       | 7       | 4      | 67     | 5     | 0       |
| $B$-factors      |        |         |        |        |                |                |
| Protein          | 13.5    | 6.7     | 4.2    | 11.8   | 3.9    | 2.5     |
| Ligand/ion       | 69.9    | 33.1    | —      | —      | —     | —       |
| Water            | 32.7    | 17.2    | 20.4   | 24.3   | 7.1    | —       |
| R.m.s. deviations|        |         |        |        |                |                |
| Bond lengths (Å) | 0.005   | 0.016   | 0.004  | 0.003  | 0.006  | 0.009  |
| Bond angles (°)  | 0.61    | 1.56    | 0.70   | 0.79   | 1.12   | 1.66   |

*Four crystals were used for NVGSNTY form 1. Two crystals were used for NFLVHSS. One crystal was used for each of the remaining data sets. Values in parentheses are for highest-resolution shell.
### Table 1-3. Comparison of molecular mechanisms of inheritance

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<td>High-density hydrogen bonding</td>
</tr>
<tr>
<td>Information content</td>
<td>Virtually unlimited</td>
<td>Potentially large</td>
</tr>
<tr>
<td>Adaptive advantages</td>
<td>High stability and information content</td>
<td>Rapid adaptation to environmental changes</td>
</tr>
</tbody>
</table>
Figure 1-1. Packing polymorphism of steric zippers, determined by X-ray microcrystallography. A steric zipper is a pair of interdigitated β-sheets, generally with a dry interface between them. The views here look down the fibril axes, showing three layers of the zipper. In actual fibrils and microcrystals, there are tens of thousands of layers. Each strand forms backbone hydrogen bonds to strands above and below it. Water molecules are shown as aqua spheres. 

a. Registration polymorphism of SSTNVG from islet amyloid polypeptide (IAPP). The left steric zipper (PDB code 3DG1) can be transformed to the right steric zipper by moving the top sheet to the left, and flipping side chains S2 and N4.

b. Registration polymorphism of VQIVYK from tau protein. The left zipper (PDB code 2ON9) can be transformed to the right zipper by moving the top sheet to the right.

c. Facial polymorphism of NNQQ from yeast prion Sup35. The left NNQQ steric (PDB codes 2ONX) zipper displays ‘face-to-back’ packing with N1 and Q3 amino acid side chains (yellow) of the top sheet interdigitated with Q4 and N2 (white) of the bottom sheet. In contrast, the right NNQQ steric zipper (PDB codes 2OLX) displays ‘face-to-face’ packing, with N1 and Q3 side chains (yellow) of both sheets forming the interdigitated interface.

d. Facial polymorphism of NNQNTF from elk prion protein. Both NNQNTF steric zippers are found in the same crystal structure, one face-to-face (right), with N1, Q3 and T5 (yellow) of both sheets forming the interdigitated interface; the other back-to-back, with sidechains N2, N4, and F6 interdigitated (white).
Figure 1-2. Segmental polymorphism in islet amyloid polypeptide (IAPP). a. IAPP sequences and segment propensities for fibril formation. The sequence of human IAPP (hIAPP) is shown at bottom, with residue replacements in mouse IAPP (mIAPP) below. The histogram at top shows the estimated energies of steric zippers formed by six-residue segments (starting at the listed residue) of IAPP. Segments having energies of -23 kcal/mol or lower are predicted to form fibrils. b. The six IAPP segments (highlighted with horizontal bars in a) were synthesized and found to form fibrils, as shown in the electron micrographs, top (scale bars are 100 nm). Each of the segments also forms microcrystals, shown in the light micrographs, upper middle (scale bars 50 μm). The structures of the six segments were determined, lower middle, and each revealed a steric zipper. Resolutions and R-factors are given at the bottom; details are described in Supplementary Information. The electron micrographs of segments HSSNNF and NNFGAIL seem to show both microcrystals and fibrils.
Figure 1-3. Evidence for at least two steric zipper polymorphs in full length IAPP. 

**a.** Mouse IAPP (mIAPP) does not form fibrils, in contrast to human IAPP (hIAPP) which rapidly forms fibrils. The mutation of R18 to H in mIAPP now permits mIAPP R18H to form fibrils. 

**b.** The pH dependence of mIAPP R18H fibrillization supports the involvement of His18. The error bars are the standard deviation of six replicates. 

**c.** hIAPP fibrils are commonly twisted and ~8 nm in width; mIAPP R18H fibrils are uniformly wider (~9-10 nm) and untwisted (scale bars are 50 nm), suggesting a different underlying structure.
Figure 1-4. Schematic summary of steric-zipper mechanisms for amyloid and prion polymorphism. On the left, an amyloid-forming protein is depicted with two segments (blue and yellow) each capable of forming a self-complementary steric zipper. Below the linear sequence is shown a steric zipper formed by the yellow segment with two $\beta$-sheets face-to-face. 

a. Packing polymorphism, in which the yellow segment has a sequence capable of forming a second steric zipper with the two $\beta$-sheets packing face-to-back as well as face-to-face.

b. Segmental polymorphism, in which both the yellow and blue segments have sequences capable of forming self-complementary steric zippers.

c. Combinatorial polymorphism, in which the blue and yellow segments have sequences capable of engaging in a steric zipper.

d. Single-chain registration polymorphism, in which two segments of the same chain form two steric zippers with different registrations of their sidechains. Compare this to Figures 1a and 1b where the registration polymorphs are formed from identical segments of different chains. Neither combinatorial nor singlechain-registration polymorphisms have not yet been observed at atomic resolution.
Supplementary Information

Supplementary Methods

In this paper, we report 9 new crystals structures of: NNQNTF, NFLVHS, NFLVHSS, HSSNNF, AILSST, two crystals forms of NVGSNTY, SSTNVG Form 2, and VQIVYK Form 2. The crystallization conditions are described below. Note that NNQNTF displays two unique steric zippers (Fig. 1 of the main text), thus our data present an overall of 10 new and unique steric zippers. The two polymorphic forms of NNQQ\textsuperscript{21}, as well as SSTNVG Form 1 (ref. 22), and VQIVYK Form 1 (ref. 21), were previously reported. Additional structures of SSTNVG Form 2 and VQIVYK Form 2 were determined out of various crystallization conditions. Some of these structures show different number of hetero atoms. The details are provided below.

**Crystallizing conditions:**

**NNQNTF:** Hanging-drop vapor diffusion; drop was a mixture of segment solution (30 mg ml\(^{-1}\) NNQNTF in 20mM BisTris) and reservoir solution (0.2M Ammonium Sulfate, 0.1M Tris pH 8.5, 25% PEG 3350). The structure was solved to 1.45Å resolution and contained one segment and two water molecules in the asymmetric unit.

**NFLVHS:** Hanging-drop vapor diffusion; drop was a mixture of segment solution (20 mg ml\(^{-1}\) segment in water) and reservoir solution (0.18M MgCl\(_2\), 0.09M HEPES pH 7.5, 27% isopropanol, and 10% glycerol). The structure was determined to 1.85Å resolution and contained one segment, one water molecule and one chloride ion in the asymmetric unit.

**NFLVHSS:** Hanging-drop vapor diffusion; drop was a mixture of segment solution (20
mg ml^{-1} segment in water) and reservoir solution (1.5 M ammonium sulfate, 0.1 M sodium acetate trihydrate pH 4.6). The structure was determined to 1.8 Å resolution and contained two segments, six water molecules and one sulfate ion in the asymmetric unit.

**HSSNNF**: Hanging-drop vapor diffusion; drop was a mixture of segment solution (20 mg ml^{-1} segment in water) and reservoir solution (0.1M Tris HCl pH 8.5, 8% PEG 8000). The structure was determined to 1.5Å resolution and contained one segment, and one water molecule in the asymmetric unit.

**AILSST**: Hanging-drop vapor diffusion; drop was a mixture of segment solution (40 mg ml^{-1} segment in water) and reservoir solution (0.2M MgNO₃ pH 5.9, 20% PEG 3350). The structure was determined to 1.4Å resolution and contained eight segments, and 67 water molecules in the asymmetric unit.

**NVGSNTY**: Hanging-drop vapor diffusion; drop was a mixture of segment solution (40 mg ml^{-1} segment in water) and reservoir solution (0.1M HEPES pH 7.5, 25% PEG 3350). Two crystals of NVGSNTY were grown in the above identical conditions but prepared for data collection by either flash freezing the crystals in liquid nitrogen or mounting dry crystals on glass capillaries. The crystal structure obtained from flash frozen sample was determined to 1.5Å resolution and contained one segment and five water molecules in the asymmetric unit (Form 1). The structure obtained from mounting dry crystals on glass capillaries was determined to 1.6Å resolution and contained two segments and three water molecules in the asymmetric unit (Form 2).

**SSTNVG (Form 1 space group C2)**: described previously¹ (PDB code 3DG1). Hanging-drop vapor diffusion; drop was a mixture of segment solution (2 mg ml^{-1}
SSTNVG in water) and reservoir solution (30% v/v MPD). The structure was solved to 1.65Å resolution and contained one segment and two water molecules in the asymmetric unit.

**SSTNVG (Form 2 space group P212121):** Hanging-drop vapor diffusion; drop was a mixture of segment solution (20 mM SSTNVG in water in the presence of 2 mM rifamycin SV sodium salt and 0.5 mM ascorbic acid) and reservoir solution (20% w/v PEG-3000, 0.1M HEPES pH 7.5, 0.2M NaCl). The structure was solved to 1.6Å resolution and contained one segment and three water molecules in the asymmetric unit.

Note: The same crystal packing was also obtained using the following crystallization conditions:

Hanging-drop vapor diffusion; drop was a mixture of 20 mM SSTNVG in 19% ethanol in the presence of 2mM curcumin, and reservoir solution (0.1M HEPES pH 7, 30% Jeffamine M- 600 pH 7). The structure was determined to 1.85Å resolution and contained one segment and three water molecules in the asymmetric unit.

**VQIVYK (Form 1 space group P21):** Described previously (PDB code 2ON9). Hanging-drop vapor diffusion; drop was a 1:1 mixture of segment solution (30 mg ml⁻¹ VQIVYK in water) and reservoir solution (0.2 M ammonium acetate, 0.1M Na HEPES pH 7.5, 45% v/v MPD). The structure was solved to 1.5Å resolution and contained two segments and seven water molecules in the asymmetric unit.

**VQIVYK (Form 2 space group C2):** Hanging-drop vapor diffusion; drop was a 1:2-2:1 mixture of segment solution (10mM SSTNVG in 80% DMSO in the presence of 1 mM perphenazine) and reservoir solution (14% iso-propanol, 0.07M HEPES-Na pH 7.5,
0.14M Sodium Citrate, 30% Glycerol). The structure was solved to 1.5Å resolution and contained one segment and one water molecule in the asymmetric unit.

**Note** – the same crystal packing was also obtained using the following crystallization conditions; all contained one segment in the asymmetric unit:

1. Hanging-drop vapor diffusion; drop was a 1:2-2:1 mixture of segment solution (10mM VQIVYK in 60% DMSO with and without 1 mM or 2 mM curcumin) and reservoir solution (0.1M Tris HCl pH 8.5, 70% v/v MPD). The structure of the crystals grown in the presence of 1mM Curcumin was determined to 1.7Å resolution and contained one water molecule in the asymmetric unit. The structure of the crystals grown in the presence of 2 mM curcumin was determined to 1.7Å resolution and contained no water molecules. The structure of the crystals soaked with 1mM Curcumin was solved to 1.5Å resolution and contained one water molecule in the asymmetric unit. The structure of the crystals grown without Curcumin was determined to 1.7Å resolution and contained one water molecule in the asymmetric unit.

2. Hanging-drop vapor diffusion; drop was a 1:2-2:1 mixture of segment solution (10mM VQIVYK in water in the presence of 1mM 2-Methoxy-4-methylphenol (Creosol)) and reservoir solution (1M succinic acid pH 7, 0.1M HEPES pH 7, 1% w/v Polyethylene glycol monomethyl ether 2,000). The structure was determined to 1.7 Å resolution and contained one water molecule in the asymmetric unit.

3. Hanging-drop vapor diffusion; drop was a 1:2-2:1 mixture of segment solution (10 mM VQIVYK in 40% DMSO in the presence of 1 mM juglone) and reservoir solution (0.1M Tris.HCl pH 8.5, 70% v/v MPD). The structure was determined to 1.8Å resolution
and contained one water molecule in the asymmetric unit.

4. Hanging-drop vapor diffusion; drop was a 1:2-2:1 mixture of segment solution (10 mM VQIVYK in 10% DMSO in the presence of 1 mM neocuproine) and reservoir solution (0.1M Tris pH 8, 20% v/v MPD). The structure was determined to 1.8Å resolution and contained no water molecules in the asymmetric unit.

5. Hanging-drop vapor diffusion; drop was a 1:2-2:1 mixture of segment solution (10mM VQIVYK in water in the presence of 1 mM thioflavin S) and reservoir solution (1M succinic acid pH 7.0, 0.1 M HEPES pH 7, 1% w/v Polyethylene glycol monomethyl ether 2,000). The structure was determined to 1.5Å resolution and contained one glycerol molecule in the asymmetric unit.

6. Hanging-drop vapor diffusion; drop was a 1:2-2:1 mixture of segment solution (10 mM VQIVYK in 25% ethanol in the presence of 2 mM curcumin) and reservoir solution (0.1M MMT buffer pH 8, 30% PEG 1500). The diffraction data from this condition was merged with data from additional crystal, also prepared using hanging-drop vapor diffusion: drop was a 1:2-2:1 mixture of segment solution (10mM VQIVYK in 25% ethanol in the presence of 2mM Curcumin) and reservoir solution (1M succinic acid pH 7.5, 0.1M HEPES pH 7.5, 1% w/v Polyethylene glycol monomethyl ether 2,000). The structure was determined to 2.1Å resolution and contained one water molecule in the asymmetric unit.

**Structure determination and refinement**

X-ray diffraction data were collected at beamline 24-ID-E of the Advanced Photon
Source, Argonne National Laboratory; wavelength of data collection was 0.9792 Å. NNQNTF diffraction data were collected at European Synchrotron Radiation Facility, beamline ID-13; wavelength of data collection was 0.9465 Å. Data were collected at 100 K. Molecular replacement solutions for all segments were obtained using the program Phaser. The search models consisted of available structures of different crystal forms of the same segment, or geometrically idealized β-strands with side chain conformations modeled as the most frequently observed rotamer defined in the graphics program O. Crystallographic refinements were performed with the program Refmac. Model building was performed with Coot and illustrated with PyMOL. There were no residues that fell in the disallowed region of the Ramachandran plot.
Figure 1-S1. The microcrystal structures of steric zippers formed by segments NFLVHS, NFLVHSS, and HSSNNF of IAPP. Water molecules are shown as aqua spheres. Both NFLVHS and the NFLVHSS segments are antiparallel with respect to β-strands within a β-sheet. A chloride ion (shown as a green sphere) is coordinated between histidine residues of every other β-strand within each β-sheet of NFLVHS. A sulfate ion lies within the NFLVHSS structure. The structure of HSSNNF reveals parallel β-strands within β-sheets and dry, identical interfaces between each pair of sheets. The β-strands between adjacent β-sheets are in the same plane for NFLVHS and NFLVHSS, whereas they are staggered in HSSNNF.
The microcrystal structures of steric zippers formed by segments from residues 21–33 from IAPP. The NNFGAIL and SSTNVG structures as published previously. The structure of the AILSST segment reveals antiparallel β-strands within adjacent β-sheets. The β-strands between adjacent β-sheets are in the same plane. The structure of the second packing of SSTNVG shows a shift in the registry between adjacent β-sheets. The center of the interface is now Asn31, rather than Ser29.
Figure 1-S3. The preparation of microcrystals derived from amyloid-like fibrils influences the hydration state of the wet interface. The structures of NVGSNTY determined in identical conditions (see Supplemental Methods) were solved by either flash freezing the crystals in liquid nitrogen (top left) or mounting dry crystals on glass capillaries (top right). The flash frozen sample shows more water molecules at the wet interface. Importantly, the dry interface at the center of the structure remains identical. This dry interface reveals an intricate hydrogen-bonding network, rather than closely interdigitated sidechains.
Figure 1-S4. The 3D profile method provides a computational explanation for the aggregation of mIAPP R18H. 

a, The scan of computed energies versus sequence for segments of human IAPP reveals several aggregation-prone hexameric segments spanning from residue 13 all the way through the final hexamer at position 32. 

b, Mouse IAPP scores poorly due to the Arg residue at position 18 and the Pro residues at positions 25, 28, and 29. The final hexamer at position 32 still scores well because these residues are the same in the mouse and human constructs. The upstream proline residues may prevent this segment from forming a cross-β, steric-zipper spine in the full-length mouse construct. 

c, The mIAPP R18H protein can form fibrils because these segments contain His18, permitting the formation of a steric zipper spine incorporating this residue.
Figure 1-S5. Fibrils of the mIAPP R18H construct contain a cross-β spine outside the C-terminal putative amyloidogenic domain. **a,** The primary structure diagram above shows color-coded segments that form both fibrils and microcrystals within hIAPP. The Pro residues within mIAPP R18H at positions 28 and 29 prevent this region from forming a cross-β spine. A segment composed of PPTNVS is incapable of forming fibrils or microcrystals (data not shown). **b,** Fiber formation of hIAPP as a function of pH shows that hIAPP forms fibrils regardless of pH. The fiber formation capability of mIAPP R18H has an inflection point near the pKa of His, suggesting a direct involvement of this position in the fiber formation of this construct.
Table 1-S1. Features of the steric zipper (dry interface) of microcrystals derived from fibril-forming proteins.

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<th></th>
<th>Strand Orientation</th>
<th>Area buried (Å²)</th>
<th>Shape complementarity</th>
<th>Inter sheet distance (Å)</th>
<th>Rosetta score (kcal/mol)</th>
<th>PDB code</th>
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<td>-25.9</td>
<td>3FTL</td>
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a. The Area buried was calculated using areaimol[^44,45] with a probe radius of 1.4Å. The difference between the accessible surface areas of one β-strand alone and within the steric zipper structure constitutes half of the reported area buried.

b. Lawrence and Colman’s shape complementarity index[^46]

c. Sheet-to-sheet distances were calculated as the shortest distance between two least squares lines fitted to the main chain atoms of two opposing strands in the dry interface. The lines were projected onto a plane normal to the “fibril axis”.

d. The Rosetta score was calculated as described in the Methods (main text).

e. NNQNTF structure contains two different polymorphs. The numbers of the left side correspond to the steric zipper of the polymorph shown on the right panel of Figure 1d.

f. The steric zipper of AILSST contains water molecules, thus is not a completely dry interface. The calculations for the area buried, shape complementarily and inter sheet distance were performed using the deposited PDB structures, while the Rosetta score is calculated for a minimized structure (repacking of the side chains) with added hydrogens.
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Chapter 2: Molecular Basis for Amyloid-β Polymorphism

Abstract

Amyloid-beta (Aβ) aggregates are the main constituent of senile plaques, the histological hallmark of Alzheimer’s disease. Aβ molecules form β-sheet containing structures that assemble into a variety of polymorphic oligomers, protofibers and fibers that exhibit a range of lifetimes and cellular toxicities. This polymorphic nature of Aβ has frustrated its biophysical characterization, its structural determination, and our understanding of its pathological mechanism. To elucidate Aβ polymorphism in atomic detail, we determined eight new micro-crystal structures of fiber-forming segments of Aβ. These structures, all of short, self-complementing pairs of β-sheets termed steric zippers, reveal a variety of modes of self-association of Aβ. Combining these atomic structures with previous NMR studies allows us to propose several fiber models, offering molecular models for some of the repertoire of polydisperse structures accessible to Aβ. These structures and molecular models contribute fundamental information for understanding Aβ polymorphic nature and pathogenesis.
**Introduction**

The amyloid hypothesis (1, 2) was based on the observation that amyloid-beta (Aβ), a 39–43 amino-acid peptide that forms fibrillar, β-sheet rich structures, is the main constituent of proteinaceous deposits observed in the brains of Alzheimer’s patients (3, 4). Evidence implicating Aβ in the pathogenesis of Alzheimer’s disease includes the appearance of Alzheimer’s symptoms in animal models that express the Aβ peptide (5) and the early onset of the disease coupled with massive depositions of Aβ in patients with the rare Aβ mutation, Asp23-to-Asn (Iowa mutant) (6). *In vitro*, the Aβ Iowa mutant forms fibers considerably faster than wild type (7). This finding that accelerated fiber formation is correlated to pathology points to fibers as the etiologic agent. In contrast, recent studies point to short fibers and soluble oligomeric forms of Aβ as the more toxic species (8-10). Thus it appears that different assemblies of Aβ are toxic and they may share common structural features (11-13). Also supporting structural similarity of fibers and oligomers is the observation that many compounds, including analogs of the common amyloid ligands congo-red and thioflavin T, bind to both Aβ oligomers and fibers (14-17).

Despite decades of research, we lack full understanding of the molecular mechanisms of toxicity in Alzheimer’s and other aggregation diseases. Part of the problem is the lack of structural information on the proteins mainly involved in the etiology, Aβ and Tau (3, 18). Atomic structures of oligomers of Aβ have been especially elusive due to their metastability and their heterogeneity in size and shape. More structural information is
available for amyloid fibers, such as those associated with Alzheimer’s disease and other aggregation diseases (19-35).

Different amyloid fibers display similar biophysical characteristics (36), most notably their common ‘cross-β structure’ indicated by their x-ray fiber-diffraction patterns, displaying orthogonal reflections at about 4.8 Å and 10 Å spacings (37-39). The atomic features of the cross-β structure have been clarified by X-ray-derived atomic models of amyloid-like structures, revealing a motif consisting of a pair of tightly mated β-sheets, called a “steric zipper” (20). Steric zippers are formed from short self-complementary sequences, and account for amyloid aggregation (20, 40, 41). These short peptide segments form well-ordered fibers (42) and have the biophysical characteristics of the fibers of their parent proteins (43). The structures of microcrystals of over 80 of these amyloid-like segments from different disease-associated proteins have been determined ((44-49) and unpublished results). These structures help to define “cross-β structure”, suggesting that stacks of identical short segments form the ‘cross-β spine’ of the protofilament, the basic unit of the mature fiber, while the rest of the protein adopts either native-like or unfolded conformation peripheral to the spine (20, 50). Here, we hypothesize that steric zippers not only serve as the spine of the protofilament, but also can mediate the interactions between protofilaments that associate to form mature fibers.

Amyloid-forming proteins, and Aβ in particular, can display a bewildering variety of oligomeric and fiber forms, or polymorphs (27, 29, 31, 51-53). For example, Aβ1-40 was suggested to form five amyloid structures with distinct β-sheet contents and fiber stabilities (28). Experiment-based models of Aβ1-40 and Aβ1-42 described several fiber
polymorphs. Solid-state nuclear magnetic-resonance (ss-NMR) provided models for Aβ1-40 (21, 29, 31, 34, 35). A model for Aβ1-42 was derived using hydrogen-bonding constraints from quenched hydrogen/deuterium-exchange NMR, together with information from mutagenesis and previous ss-NMR studies (19). The models suggest that in these particular polymorphs, the Aβ molecule adopts a U-shaped protofilament structure, which hydrogen-bonds with identical molecules to form a pair of in-register, parallel β-sheets. However, the models differ in the precise location of the U-turn in the sequence, as well as in the specific interactions between distal regions, demonstrating that polymorphism is present at the protofilament level. Interestingly, the protofilament structure of Aβ1-40 fibers seeded from brain plaques was reported to differ from the earlier synthetic Aβ1-40 structures, with the C-terminal β-sheet flipped in relation to its interface with the N-terminal β-sheet (21, 34, 35). Tycko and coworkers further expanded the range of structures available to Aβ by proposing an anti-parallel protofilament-structure for Asp23-to-Asn Aβ1-40 (Iowa-mutant) fibers; that is, successive stacked β-strands in each β-sheet run in opposite directions (7). Because all models reported so far were obtained from Aβ material that had been prepared by serial rounds of seeding, they presumably represent only a fraction of all conformations available to Aβ protofilaments. Higher level of polymorphism is manifested in varied modes of association of protofilaments into fibers (31, 34). For example, ss-NMR studies reported models for Aβ1-40 fibers containing either two or three protofilaments (29). Lastly, electron-density maps of Aβ fibers produced by cryo-electron microscopy displayed a variety of fiber forms (32, 51, 54, 55). It is interesting to note that in prion protein fibers, structural
motifs other than tightly mated pairs of β-sheets were proposed. For example, an NMR-based model of HET-s(218–289) prion suggests a fiber formed via a β-solenoid triangular hydrophobic core (33).

The variety of polymorphs suggests that multiple interaction sites exist within each Aβ molecule, giving rise to differences in fiber morphologies and physicochemical properties on the surface of the fibers that may be correlated with varying levels of cellular toxicity (29, 31, 35, 56). This variety may provide an explanation for the poor correlation between the extent of amyloid deposition and the severity of neurological symptoms (4, 31, 57). Therefore, to better understand the nature of Aβ polymorphism in atomic detail, we report eight new crystal structures of Aβ segments that, together with three previously determined structures (45), span the sequence range Aβ<sub>16-42</sub>. The eleven structures, all of which are steric zippers, reveal multiple modes of homotypic interactions (between identical segments), giving rise to a large variety of possible assemblies of Aβ molecules via different steric zipper spines. Combining our crystal structures of homotypic steric zippers with previous experiment-based models of Aβ, which suggest heterotypic interactions between distal segments in pairs of β-sheets (19, 21, 29, 34, 35), allows us to generate models of Aβ protofilament associations that exemplify the range of possible polymorphs.

**Results**

*Identifying fiber-forming segments in Aβ using the 3D-profile method*

We identified fiber-forming segments of Aβ predicted to form the spines of Aβ fibers (Fig. 2-1A). For this we used the 3D-profile method that scores six-residue sequence
segments for their propensity to form steric zippers, based on the structural profile of a
canonical steric zipper with a parallel, face-to-face, β-sheet orientation (for nomenclature
see (45)). Generally, the strands are allowed to translate in respect to each other but the
orientation of the strands (parallel vs. anti-parallel) remains fixed (41, 59). Several
segments within the regions of Aβ_{11-25} and Aβ_{27-42} were predicted either to self-associate
into homotypic steric zippers (Fig. 2-1A), or to form heterotypic steric zippers in which
one of the two β-sheets is composed of one segment and the complementary β-sheet is
composed of a second segment (Fig. 2-1C). These predicted heterotypic interactions
correlate with ss-NMR studies of Aβ_{1-40} (21, 29) and an experiment-based model of Aβ_{1-42}
(19). In addition, the predicted heterotypic interactions within the Aβ_{27-42} region
correlate with a conformation of the Aβ_{28-42} segment when fused to the C-terminal region
of RNase (60). In this crystal structure, Aβ_{28-42} forms a small anti-parallel β-sheet with a
bend formed by Gly37, yielding heterotypic interaction between residues 30-36 and 38-42, similar to the predictions of the 3D-profile method (Fig. 2-1C).

Crystal structures of 6-8 residue segments of Aβ

The 3D-profile method predicted several six-residue segments to be amyloidogenic
(Fig. 2-1A). We examined these segments, as well as longer segments, for their ability to
form fibers and crystals. Five segments (Aβ_{16-21}, Aβ_{27-32}, Aβ_{29-34}, Aβ_{30-35} and Aβ_{35-42})
formed micro-crystals, with some forming more than a single crystal form (Fig. 2-2).
Other segments suffered from low solubility or fast fibrillation, limiting their structural
characterization to fiber diffraction and electron microscopy, as described below.
Previously, we described eight classes of steric zipper symmetries (45). Together, the steric zippers of Aβ presented here (Table 2-S1) and in a previous publication (45) occupy six of these eight classes, suggesting the variety of possible arrangements of Aβ associations via different spine packings. We find both parallel and anti-parallel packing of β-strands within β-sheets, as detailed in the following.

**Crystal structures of the Aβ_{16}^{16}KLVFFA_{21}^{21} segment.** The Aβ_{16-21} segment crystallized in three crystal forms (Fig. 2-2 A-C), all displaying an anti-parallel β-strand stacking of the type “face=back” (45). That is, the β-sheets are equifacial, with identical side chains at the face and back of the β-sheet, a consequence of an internal two-fold screw symmetry element. Aβ_{16-21} forms I and III (Fig. 2-2A and 2-2C) display similar interfaces with different conformations of Lys16, and a slight registration slip of the steric zipper interface. Aβ_{16-21} form II displays two steric zipper interfaces that differ in rotamer conformation of Phe20 (Fig. 2-2B). In all zippers, the pairs of β-sheets are packed together via hydrophobic side chains, forming a dry interface. All four steric zippers belong to symmetry class 7 (45).

Corresponding to our structures, ss-NMR characterization of a one-residue longer peptide, Aβ_{16-22} (26), as well as a one residue shorter peptide, Aβ_{17-21} (30), showed an anti-parallel organization of β-sheets in the fibers. Fibers of longer segments, Aβ_{11-25} (30) and Aβ_{34-42} (61), also display an anti-parallel β-strand orientation. The anti-parallel orientation might be associated with pathology seeing that it was observed for a subset of fibers of the “Iowa” Aβ mutant that is related to a familial, early onset, Alzheimer’s disease (6, 7). In addition, Aβ oligomers were also suggested to form anti-parallel β-sheet
structures (62-65). In contrast, the wild-type, full-length Aβ fibers display a parallel orientation (25).

**Crystal structure of the Aβ^{27}NKGAII^{32} segment.** The Aβ_{27-32} segment forms a parallel β-sheet stacking with two different steric zipper interfaces. Both interfaces show β-sheets packed together via interdigitating hydrophobic side chains, typical of symmetry class 1 (45). One interface shows a “face-to-face” orientation and the other “back-to-back” (Fig. 2-2D).

**Crystal structure of the Aβ^{29}GAIIGL^{34} segment.** The Aβ_{29-34} segment forms an anti-parallel β-sheet with a dry steric zipper interface displaying a “face-to-back” orientation (Fig. 2-2E). The two non-equifacial anti-parallel β-sheets are related to each other by a simple translation vector, corresponding to symmetry class 6 (45). The registration between neighboring anti-parallel strands is such that the last two residues in each strand fall outside the hydrogen bonding pattern of the β-sheet. Specifically, Gly33 deviates from β-sheet geometry, placing Leu34 outside the β-sheet. The conformation of Gly33 and Leu34 are different in the two anti-parallel strands in the asymmetric unit, which correspond to neighboring strands within the β-sheet.

**Crystal structure of the Aβ^{30}AIIGLM^{35} segment.** The Aβ_{30-35} segment forms a parallel β-sheet with a dry steric zipper interface of the type “face-to-back”, symmetry class 2 (Fig. 2-2F). This steric zipper interface resembles a “knobs-into-holes” type of packing (66); i.e., Ile32 and Leu34 from one β-sheet form the “knob” that enters the “hole” between Ile31 and Met35 of the mating β-sheet, created by the presence of Gly33 (lacking a side chain).
Crystal structures of the Aβ<sup>35</sup>MVGGVVI<sup>42</sup> segment. The Aβ<sub>35-42</sub> segment crystallized in two crystal forms displaying both parallel (“face-to-back” orientation, symmetry class 2) (Fig. 2-2G) and anti-parallel (“face=back” orientation, symmetry class 7) (Fig. 2-2H) β-sheet stacking. Interestingly, the two steric zippers display a similar interface with minor conformation differences of side chains, and a “knobs-into-holes” type of packing similar to that described for Aβ<sub>30-35</sub>. The “knob” is formed by Val39 and Ile41 that accommodates the “hole” formed by the presence of glycine residues.

Previously described structures of Aβ<sub>35-40</sub> and Aβ<sub>37-42</sub> segments (45) are shown in Figure 2-2 I-K. Aβ<sub>35-40</sub> crystallized in two forms, both displaying anti-parallel β-sheets with a “face=back” orientation, symmetry class 8 (Fig. 2-2 I-J). Aβ<sub>37-42</sub> forms parallel β-sheets with a “face-to-back” orientation, symmetry class 4 (Fig. 2-2K).

Quasi-crystalline fibers of long (11-20 residue) Aβ segments

Our attempts to crystallize longer segments of Aβ: Aβ<sub>11-25</sub>, Aβ<sub>16-35</sub>, Aβ<sub>22-35</sub>, and Aβ<sub>30-42</sub>, resulted in highly disordered micro-crystals. The x-ray diffraction patterns (Fig. 2-S1) show a mix of crystalline and fiber diffraction, termed quasi-crystalline fiber diffraction (67). These diffraction patterns display a distinguishable feature at reciprocal spacing of 4.8Å, which is consistent with parallel, in-register, β-sheet structures. A feature at a spacing of 9.6Å, expected for either anti-parallel β-sheets or out-of-register parallel β-sheets, is not present.

Fiber formation of Aβ segments analyzed by electron microscopy
We examined the fiber-forming propensities of Aβ segments, including 6-8 residues segments that form micro-crystals (Aβ16-21, Aβ30-35, Aβ35-40, Aβ35-42 and Aβ37-42) (Fig. 2-S2A), as well as 11-20 residue segments (Aβ11-25, Aβ16-35, Aβ30-40 and Aβ30-42) (Fig. 2-S3A). All of the Aβ segments formed fibers. It is noteworthy that Aβ30-35 forms small micro-crystals even under fibrillation conditions, and fibers can grow from the tip of micro-crystals (Fig. 2-S2A), suggesting common structural features for fibers and micro-crystals (48).

Can distal Aβ segments associate to form the spine structures of amyloid fibers?

Identification of fiber-forming segments in Aβ using the 3D-profile method predicted the association of distal segments to form heterotypic steric zippers (Fig. 2-1C). Based on these predictions, we carried out co-crystallization screens of 1:1 mixtures of distal peptide segments (Aβ16-21+Aβ30-35, Aβ16-21+Aβ35-40, Aβ16-21+Aβ35-42, Aβ16-21+Aβ37-42, Aβ11-25+Aβ30-40, Aβ15-25+Aβ30-40, Aβ11-25+Aβ30-42 and Aβ15-25+Aβ30-42), but failed to produce diffracting crystals containing two differing peptide segments. Nonetheless, electron micrographs of fibers grown from certain mixtures display a morphology that is distinct from the morphologies of the individual segments (Figs. 2-S2B and 2-S3B).

Discussion

Aβ, as well as several of its peptide segments, readily forms fibers (Figs. 2-S2 and 2-S3) (4). Eleven short segments (6-8 residues) also form micro-crystals permitting us to determine their structures in atomic detail (Fig. 2-2). These structures represent 13
diverse steric zipper interfaces, each of which can serve as the spine for fiber formation (20). In previous work, we termed this phenomenon segmental polymorphism (48).

Types of Amyloid Polymorphism

Four steric zipper structures (Aβ16-21, Aβ27-32, Aβ35-40 and Aβ35-42) show a second type of amyloid polymorphism, termed packing polymorphism, in which the same sequence can form distinct steric zipper structures by virtue of different packing in the spine (48) (Fig. 2-2). Aβ35-42 shows a new mode of packing polymorphism, with β-sheets stacking via both parallel and anti-parallel β-strands (Fig. 2-2 G-H). Of particular importance, this new type of polymorphism may be related to Aβ toxicity. The Aβ “Iowa” mutation (Asp23-to-Asn) (6) is the determinant for familial, early onset, Alzheimer’s disease. The majority of fibers formed from the mutant Aβ1-40 suggest an anti-parallel orientation (7) and deposit massively compared to wild-type Aβ fibers, which exclusively exhibit a parallel orientation (25). Of interest, the two types of polymorphs (parallel vs. anti-parallel) were observed within the same sample of the “Iowa” mutant Aβ1-40 (7). This observation can be explained structurally by our crystal structures. Two different polymorphs of Aβ35-42 showing parallel and anti-parallel β-sheet orientation nevertheless show similar interfaces of the two steric zippers with only slightly dissimilar side chain conformations (Fig. 2-2 G-H). Also, anti-parallel β-sheet structures have been reported for Aβ oligomers (62-65), which recent studies point as more toxic than fibers (8-10).

The steric zipper structures of Aβ segments (Fig. 2-2), as well as of segments from other disease-related amyloid proteins (44-49), all show homotypic interactions, with the pair of β-sheets formed from the same segment of the protein. Heterotypic interactions,
between β-sheets formed from different Aβ segments, were proposed based on NMR studies (19, 21) and the interpretation of cryo-electron microscopy maps (32, 51, 54, 55). Our predictions, based on the 3D-profile method, suggest the association of distal segments to form heterotypic steric zippers (Fig. 2-1C). Our observations of fiber formation of the different Aβ segments are compatible with this notion, as fibers formed from mixed pairs of Aβ segments display different morphologies compared to fibers formed from individual segments (Figs. 2-S2 and 2-S3). The heterotypic interactions suggest a fourth mode of amyloid polymorphism, *heterotypic polymorphism*, which is an example of *combinatorial polymorphism* suggested in previous work (48). With the numerous modes of segmental, packing, and heterotypic polymorphism available for fiber formation, a given Aβ fiber may contain more than a single type of protofilament, each displaying a different kind of polymorphism, as discussed in the following.

*Pseudo-atomic Aβ fiber models*

Using our atomic structures of steric zippers of Aβ segments, combined with models of the protofilament structure (19, 34), we constructed several atomic models of Aβ fibers that exemplify the numerous possibilities for fiber morphology (Figs. 2-3 and 2-S4-6). The protofilament models, namely pairs of tightly mating β-sheets, one of Aβ1-40 derived from ss-NMR studies (34), and another of experiment-based model of Aβ1-42 (19), show a U-shaped structure. In Aβ1-40, residues 23-29 form a bend in the backbone to bring two β-sheets, composed of residues 10-22 and 30-40, to form a heterotypic interface (34). In Aβ1-42, the heterotypic interactions are between β-sheets formed by residues 18-26 and 31-42 (19). Few examples of Aβ fiber models are constructed from the steric zipper
structures of Aβ35-42, Aβ16-21 or Aβ27-32 mediating the interactions between the two different types of protofilaments (Figs. 2-3 and 2-S4). The inter-protofilament interface suggested by the model of figure 2-S4A differs from those of figures 2-3 and 2-S4B, and involves the pairing of the N-terminal β-sheets for the former and the pairing of the C-terminal β-sheets for the latter. A quaternary model that includes the association of the C-terminal β-sheets was previously suggested by ss-NMR studies (34). The inter-protofilament interface in this NMR model covers residues 30-40, which is longer than the interfaces of figures 2-3 and 2-S4B which cover residues 35-42 or 27-32, respectively, and thus might represent a more stable polymorph.

Overall, our models (Figs. 2-3 and 2-S4) incorporate different segments as the spines, exemplifying *segmental polymorphism*. They display diverse interfaces within the fiber, incorporating variation within the protofilament structure, as suggested by experiments (19, 34), as well as variation in the interactions between protofilaments composing the mature fiber. Additional fiber models displaying the association of multiple protofilaments via several different core regions illustrate a higher level of *segmental polymorphism* (Fig. 2-S5).

Our predictions of fiber-forming segments show a cluster of predicted interactions within residues 30-42 of Aβ (Fig. 2-1C). Structures of segments within this region, of Aβ30-35 and Aβ35-42, show steric zippers forming a “knobs-into-holes” type of packing (66) (Fig. 2-2 F-H). Correspondingly, we modeled a steric zipper that is longer than those determined by the crystal structures and spans residues 31-42, displaying a similar kind of “knobs-into-holes” packing between two protofilaments (Fig. 2-S6A). In this longer
model, residues Val39 and Ile41 protrude into the void created by Gly33, and Met35 protrudes into the void created by Gly37-Gly38, similar to the structure of Aβ35-40 (Fig. 2-2J). The NMR-based quaternary model of Aβ1-40 (34) displays a similar, but slightly shifted, “knobs-into-holes” interface, with Ile31 forming the “knob” that protrude into the void created by Gly37-Gly38, and Met35 forming the “knob” that protrude into the void created by Gly33. Lastly, in order to demonstrate polymorphism that is associated with the disease-related “Iowa” Aβ mutant (6, 7), we constructed a fiber model based on the crystal structure of Aβ16-21 displaying an anti-parallel orientation (Fig. 2-S6B).

Our results offer a molecular basis for amyloid polymorphism. Thirteen different steric zipper interfaces display a variety of polymorphic arrangements (Fig. 2-2). By combining our crystal structures with previous NMR studies, we offer fiber models that illustrate the structural variety of Aβ assemblies. Polymorphism produces a variety of structures with a variety of cellular toxicities, and a molecular view into the different structures may advance our understanding of the mechanisms of amyloid toxicity.

**Materials and Methods**

*Materials*

Peptide segments (custom synthesis, minimal purity of 98%) were purchased from CS Bio. Chemicals were purchased from Thermo-Fisher and Sigma-Aldrich.

*Crystallization conditions*
All crystals were grown at 18 °C via hanging-drop vapor diffusion. Details of
crystallization, structure determination and refinement are provided in the Supporting
Information.

*Modeling of full-length Aβ fibers*

Models of Aβ fibers were contracted based on experiment-based models of Aβ1-40 and
Aβ1-42 protofilaments (19, 34) aligned with the steric zipper interfaces and refined as
described in the Supporting Information.

*Fiber formation assessed by electron microscopy*

Samples were prepared as described in the Supporting Information.
Figure 2-1. Amyloidogenic propensity of Aβ homotypic and heterotypic interactions predicted by the 3D-profile method. (A) The 3D-profile method calculates the RosettaDesign energy (58) for the self-association (homotypic interactions) of six amino-acid peptide segments (41, 59). The histogram of peptide segments is colored in rainbow from blue to red for segments with low-to-high predicted amyloid propensity. The Aβ amino acid sequence and residue numbering are shown at the top. (B) Six-residue Aβ segments whose crystal-structures have been determined are shown as arrows; blue and purple code for structures first presented here or in a previous publication (45), respectively. (C) The 3D-profile method prediction for the association of hetero- and homo- Aβ segments is presented on a 2-dimensional interaction heat-map colored as in panel A. Each element represents the interaction energy of the hypothetical steric zipper of six residues that starts at the residues at the corresponding positions on the axes. Three main cross-peaks predicting high fiber-formation propensity are boxed.
Figure 2-2. Crystal structures of Aβ segments, shown in projection down the fiber axes. The Aβ segments are packed as pairs of interdigitated β-sheets, generally with a dry interface between them, termed steric zippers, forming the basic unit of the fiber (44, 45). The view here looks down the fiber axis, showing only four layers of β-strands in each β-sheet; actual fibers can contain more than 100,000 layers. Each panel is labeled with the amino acid sequence of each segment and the starting and ending residue numbers. Molecules are shown as sticks with non-carbon atoms colored by atom type. In structures with β-sheets composed of parallel strands (Panels D, F, G and K), the carbons are in white. Anti-parallel strands forming β-sheet structures (Panel A-C, E, H-J) are alternately colored with carbons colored white and blue. Closest partners across the dry interface share the same color. Some of the panels are split in two halves; each half represents a different dry interface within the same crystal structure.
Figure 2-3. Models of protofilament associations. The crystal structure of Aβ35-42 Form II was used to model interactions between two protofilaments. The protofilament structure is derived from experiment-based models of Aβ1-40 (residues 1-9 are disordered in the fiber) (34) (upper model) or Aβ1-42 (residues 1-17 are disordered in the fiber) (19) (lower model).
**Supplementary Methods**

*Crystallization conditions*

All crystals were grown at 18 °C via hanging-drop vapor diffusion. All crystal appeared within one week, except Aβ_{35-42} Form I and II that were found in a 1.5 year-old crystallization tray.

**Aβ_{16-21} (16-KLVFFA-21) Form I:** The segment was dissolved in water at 5 mg/ml and mixed with 0.2 M ammonium acetate, 0.1 M Bis-Tris pH 5.5, and 45 % v/v 2-Methyl-2,4-pentanediol (MPD).

**Aβ_{16-21} (16-KLVFFA-21) Form II:** The segment was dissolved in 20% dimethyl sulfoxide (DMSO) at 4.5 mg/ml in the presence of 1 mM 2-(1-(6-[[2-fluoroethyl](methyl)amino]-2-naphthyl)ethylidene)malononitrile (FDDNP) (68) and mixed with 30% (v/v) Jeffamine M-600, 0.1 M Mes pH 6.5 and 0.05 M CsCl.

**Aβ_{16-21} (16-KLVFFA-21) Form III:** The segment was dissolved in water at 5 mg/ml and mixed with 0.2 M ammonium acetate, 0.1 M Tris buffer pH 8.5 and 30% isopropanol.

**Aβ_{27-32} (27-NKGAII-32):** The segment was dissolved at 25 mg/ml in water and mixed with 2.4 M Sodium Malonate.

**Aβ_{29-34} (29-GAIIGL-34):** The segment was dissolved at 7 mg/ml in water and mixed with 14.4% PEG 8000, 0.08 M Na Cacodylate pH 6.5, 0.16 M Calcium Acetate, and 20% Glycerol.

**Aβ_{30-35} (30-AIIGLM-35):** The segment was dissolved in water at 1 mg/ml and mixed with 2 M sodium chloride.
**Aβ35-42 (35-MVGGVVIA-42) Form I**: The segment was dissolved in water at 0.5 mg/ml and mixed with 1.26 M Na phosphate monobasic monohydrate, and 0.14 M K phosphate dibasic pH 5.6.

**Aβ35-42 (35-MVGGVVIA-42) Form II**: The segment was dissolved in water at 0.5 mg/ml and mixed with 0.1 M Hepes pH 7.5 and 0.5 M Mg formate.

**Aβ16-35 (16-KLVFFAEDVGSNKGAIIGLM-35)**: The segment was dissolved in 2% DMSO at 1 mg/ml and mixed with i/ 0.1 M Na citrate pH 5.6, and 35% (w/v) tert-Butanol; ii/ 0.2 M Na/K tartrate; and iii/ 0.2 M Mg acetate, 0.1 M Na cacodylate pH 6.5, and 30% (w/v) MPD.

**Aβ22-35 (22-EDVGSNKGAIIGLM-35)**: The segment was dissolved in water at 2 mg/ml and mixed with 50 mM NaCl, 0.1 M Tris pH 8.5, and 12% (w/v) isopropanol.

**Aβ11-25 (11-EVHHQKLVFFAEDVG-25)**: The segment was dissolved in 50 mM ammonium hydroxide pH 11.1, 0.2 M sodium chloride, and 10% DMSO at 1.5 mg/ml and mixed with 4% (v/v) tacsimate pH 4.0, and 12% (w/v) PEG 3350.

**Aβ30-42 (30-AIIGLMVGGVVIA-42)**: The segment was dissolved in 50 mM ammonium hydroxide and 10% DMSO at 1 mg/ml and mixed with 0.1 M Bicine pH 9, and 10% (w/v) PEG 6000.

**Data collection and structure refinement**

All structures were obtained by mounting dry crystals on sharpened glass capillaries in the presence of 15-20% glycerol, except for the structure of Aβ29-34, which was obtained from a flash frozen crystal. Data were collected at 100 K using a microfocus beam (5x5
μm²) at beamline ID23-EH2 of the European Synchrotron Radiation Facility (ESRF), and beamline 24-ID-E of the Advanced Photon Source (APS), Argonne National Laboratory. Data indexation, integration and scaling were performed using XDS/XSCALE (69) and DENZO (70). Molecular replacement solutions for all segments were obtained using the program PHASER (71). The search models consisted of geometrically idealized β-strands, or previously determined polymorphs of the same segment. Crystallographic refinements were performed with REFMAC5 (72), PHENIX (73), and BUSTER (74). Model building was performed with COOT (75) and illustrated with PYMOL (76). Crystals of Aβ30-35 displayed pseudo-translational symmetry, and those of Aβ35-42 displayed translational symmetry and twinning.

We note that the charged termini of the peptide segments in the crystal are satisfied by interaction with neighboring steric zippers, water molecules and sometimes counter-ions (e.g. acetate in Aβ16KLVFFA21 Form I). In the in-register parallel β-sheets structures with a face-to-face orientation (Aβ27NKGAII32), the β-sheets are staggered (Table 2-S1), which allows the positively charged N-termini to stack in between rows of negatively charged C-termini. In the second interface of the Aβ27NKGAII32 (Table 2-S1), the termini interact with polar side chains (Fig. 2-2D).

**Modeling of full-length Aβ fibers**

NMR structures of Aβ1-40 and Aβ1-42 protofilaments were used as starting models (19, 34). The protofilament structures were aligned with the steric zipper interfaces, and the models were minimized using Crystallography and NMR system (CNS) with a dielectric
constant of 80 kT and non-bonded interactions cutoff of 13 Å (77, 78). Pseudo-crystal symmetry was used to maintain the distances between strands equal along the β-sheets, and psi/phi angles values from the original Aβ protofilaments structures were used as restraints. After minimization, a slow-cooling simulated-annealing was performed, with a starting temperature of 5000 K and a cooling rate of 10 K. Integrated time steps at each temperature were 0.25 psec.

Fiber formation assessed by electron microscopy

Lyophilized peptides were solubilized in DMSO at 5 mM and diluted in 150 mM HEPES pH 7.4 and 150 mM NaCl to a final concentration of 0.5 mM, and incubated for 24 hr. Aβ_{16-21} N-acetyl-KLVFFA-NH2 was dissolved in phosphate buffered saline (PBS) at 200 μM and incubated for 5 days. Turbidity at 450 nm was monitored as a function of time on a Varioskan plate reader (Thermo Scientific) (data not shown).

The samples were fixed on carbon-coated parlodion support films mounted on copper grids, which were charged by high-voltage, alternating current, glow-discharge immediately before use. 5 uL samples were applied directly onto grids and allowed to adhere for 3 minutes. Grids were rinsed with 2 drops of distilled water and negatively stained with 1% uranyl acetate for 1 minute. Specimens were examined either in a Hitachi H-7000 electron microscope at an accelerating voltage of 75 kV or a FEI CM120 electron microscope at an accelerating voltage of 120 kV. Images from Hitachi H-7000 were recorded on Kodak electron microscope film 4489 and later scanned into digital
Images from FEI CM120 were recorded digitally by TIETZ F 224HD CCD camera (2K x 2K, pixel size 24μM).
Supporting Figures

Figure 2-S1

Figure 2-S1. Eleven-to-twenty residue Aβ segments yield quasi-crystalline fibers. X-ray diffraction pattern of 11-20 residue Aβ segments point to quasi-crystalline fibrils with parallel β-sheet stacking arrangements, indicated by the brag peak spacing of 4.8Å (marked in red).
Figure 2-S2. Fiber-formation of 6-8 residue Aβ segments and their binary mixtures. Electron micrographs of negatively stained fibers formed by short Aβ segments (A) and their binary mixtures (B). Aβ30-35 forms micro-crystals and fibers, which can grow out from the tip of the crystals. Aβ35-40 forms sheet-like structures. The binary mixtures show different morphology from the component segments. The scale bars in the micrographs correspond to 100 nm.
Figure 2-S3. Fiber-formation of 11-20 residue Aβ segments and their binary mixtures. Electron micrographs of negatively stained fibers formed by 11-20 residue Aβ segments (A) and their binary mixtures (B). The morphology of fibers forming from the mixtures differs from that of individual segments. Scale bars in all micrographs correspond to 100 nm.
Figure 2-S4. Models of protofilament associations via different steric-zipper interfaces displaying segmental polymorphism. The interface between two β-strands within the crystal structures of Aβ_{16-21} Form II (A) or Aβ_{27-32} (B) was used to model interactions between two protofilaments. The protofilament structure is derived from ss-NMR studies of Aβ₁₋₄₀ (residues 1-9 are disordered in the fiber) (34) (A₁ and B₁), or an experiment-based model of Aβ₁₋₄₂ (residues 1-17 are disordered in the fiber) (19) (A₂). Notably, the Aβ_{27-32} steric zipper can only associate Aβ₁₋₄₀ protofilaments, since this region is part of the turn in the Aβ₁₋₄₂ protofilament model (19).
Figure 2-S5. Models of protofilament association via two different steric-zipper interfaces exemplify higher level of segmental polymorphism. The interactions between four protofilaments were modeled using two main interfaces that are based on the crystal structure of Aβ segments. Here, six examples illustrate the large variety of possible assemblies (polymorphs). The protofilament structure is derived from the studies of Aβ1-42 (19) or Aβ1-40 (34).
Figure 2-S6

Figure 2-S6. Models of unique types of Aβ polymorphism. (A) Association of two protofilaments (derived from the ss-NMR studies of Aβ1-40 (34)) via a long steric zipper interface. Two segments within the Aβ30-42 region that were predicted to interact by the 3D-profile method (Fig. 1C) form the β-sheets that associated into a steric zipper interface. Notably, an interaction between the Aβ30-35 and Aβ35-42 segments was implied by the electron micrographs of the binary mixtures of these segments that showed different fiber morphology comparing to the individual segments (Fig. S2). The interface resembles a “knobs-into-holes” type of packing (66), similar to the steric zipper interface of Aβ30-35 and Aβ35-42 (Fig. 2); Val39 and Ile41 form the “knob” that accommodates into the void left by Gly33, and Met35 protrudes into the void created by Gly37-Gly38. (B) A model of the D23N “Iowa” Aβ mutant, which displays an anti-parallel orientation of the β-strands according to ss-NMR measurements (7). The two anti-parallel protofilaments were associated via the steric zipper interface of Aβ16-21 Form II. For clarity, only residues 16-23 are shown in sticks. This model, comparing to models of parallel β-sheet stacking (Figs 3 and S4), display a unique mode of packing polymorphism (48), namely parallel/anti-parallel polymorphism.
Supporting Tables

Table 2-S1. Features of the dry steric zipper interface of microcrystals derived from fibril-forming Aβ segments.

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<th>Aβ segment</th>
<th>Strand orientation</th>
<th>Steric zipper type</th>
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<th>Shape complementarily$^b$</th>
<th>PDB code</th>
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<sup>a</sup>Area buried was calculated using AREAIMOL (79, 80) with a probe radius of 1.4Å. The summation of the difference between the accessible surface areas of a) one β-strand alone and in contact with the opposite β-sheet, and of b) the β-sheet alone and in contact with the opposite β-strand, constitutes the reported area buried. In structures with anti-parallel β-strand orientation, as well as in parallel β-strand orientations with different conformations, the average area buried per β-strand is reported.

<sup>b</sup>Lawrence and Colman’s shape complementarity index (81).

<sup>c</sup>The structures of 16KLVFFA<sup>21</sup> Form II and 27NKGAII<sup>32</sup> show two different steric zipper interfaces. The upper values correspond to the left interface of Fig. 2, and the lower values to the right interface.

<sup>d</sup>The structures of 30AIIGLM<sup>35</sup> and 35MVGGVVIA<sup>42</sup> Form I contain two similar steric zippers in the asymmetric unit that differ in side-chain orientations. Values for area buried and shape complementarily are reported for the two steric zippers. The structures depicted in Fig. 2 correspond to the values reported here on the left.
Table 2-S2. Data collection and refinement statistics for the crystal structures of Aβ segments.

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| α, β, γ (°) | 99.8, 95.6, 104.7 | 89.9, 90.0, 89.9 | 90.2, 89.8, 103.6 | 90, 103.57, 90 |
| Resolution range (Å) (*) | 9.06 – 1.3 (1.35-1.3) | 45.23 – 2.00 (2.12 – 2.00) | 47.69 – 1.90 (2.01 – 1.90) | 23.80 – 2.1 (2.23 – 2.10) |
| Completeness (%) (**) | 93.4 (98.9) | 97.5 (88.5) | 82.4 (76.8) | 90.9 (89.6) |
| Rmerge(Linear) (%) (***) | 11.8 (32.4) | 10.5 (18.8) | 6.5 (33.0) | 14.3 (42.2) |
| I/σI (*) | 15.8 (4.65) | 4.91 (2.11) | 4.60 (1.94) | 2.54 (1.59) |

| Unique Reflections | 1658 | 1679 | 2132 | 896 |
| Observations/parameters ratio | 4.87 | 1.24 | 1.31 | 1.09 |
| Rwork (%) (b) | 14.3 | 22.2 | 21.4 | 20.2 |
| Rfree (%) (c) | 19.6 | 26.7 | 23.1 | 24.6 |
| r.m.s.d. bond length (Å) | 0.01 | 0.02 | 0.02 | 0.02 |
| r.m.s.d. bond angles (°) | 1.18 | 2.71 | 2.33 | 2.89 |
| Number of atoms in asymm. unit | 85 | 338 | 408 | 204 |
| Average B-factor | 9.82 | 23.11 | 23.38 | 11.90 |

(*) Values in brackets are for the highest resolution shells.
(a) $R_{merge(\text{linear})} = \sum (|I - \langle I \rangle|) / \sum I$, where I is the observed intensity of the reflection HKL and the sum is taken over all reflections HKL.
(b) $R_{work} = \sum |F_o| - |F_c| / \sum |F_o|$.
(c) $R_{free}$ as defined by (82).
References


Chapter 3: The atomic details of the steric zipper spine of p53 fibrils

Abstract

It has been shown previously that fragments of p53 can undergo amyloidogenic aggregation \textit{in vitro}. Recent studies suggest that this aggregation may account for mutant p53’s gain-of-function activity in cancer cells, illustrating a possible mechanism by which p53 aggregation leads to cancer formation. In this work, we elucidated the atomic details of the aggregation-prone region of p53 (residues 251-258). We determined the crystal structures of two overlapping peptide segments, which form parallel, in-register steric zippers. Structural analysis of a third segment suggests the formation of an anti-parallel steric zipper, providing an example of amyloid fibril polymorphism. These results provide the basis for structure-based design of aggregation inhibitors of p53 as a novel cancer therapeautic.
Introduction

The tumor suppressor p53 is a transcription factor responsible for a number of essential cell functions including regulating the cell cycle, activating DNA repair proteins, and initiating apoptosis. It is one of the most highly mutated proteins in cancer, as mutations of p53 are implicated in half of all reported human tumor cases\textsuperscript{1,2}. These mutations result in the inactivation of p53 wild-type function and the acquisition of dominant-negative activity that promotes tumor progression\textsuperscript{3,4}. While the structures of the various domains of wild-type p53 are known and the mutations implicated in cancer have been widely studied, the mechanisms behind its toxic gain of function remain largely unknown\textsuperscript{5–9}.

Wild-type p53 assembles natively as a homotetramer with DNA-binding properties\textsuperscript{10}. Each monomer consists of several domains (Fig. 3-1a): a transactivation domain (residues 1-44), proline-rich region (residues 58-101), DNA-binding or core domain (residues 102-292), and tetramerization domain (residues 325-356)\textsuperscript{9}. 95\% of the missense mutations in malignant tumor cases occur in the DNA-binding domain, with six “hot spot” residues, R175, G245, R248, R249, R273, and R283 being the most frequently mutated\textsuperscript{8,11}. The mutations can be divided into two categories: (i) DNA contact mutants that directly affect p53 DNA-binding ability while not affecting the native structure and (ii) conformational mutants that thermodynamically destabilize the native structure. Conformational mutations account for 30\% of reported clinical cancer cases\textsuperscript{9}.

Recently, it was suggested that these conformational mutations of p53 induce an amyloid-like aggregation that may account for their toxic gain of function in cancer
Xu et al. showed that conformational mutations of p53 form cytoplasmic punctate aggregates that can bind thioflavin T, an amyloid-binding dye. Analysis of the sequence using the TANGO aggregation algorithm identified residues 251-257 in the DNA-binding region as having a high aggregation propensity. While this sequence is buried in the native structure (Fig. 1a), the authors argued that destabilization of the native structure could expose this region and subsequently induce aggregation. The authors showed that conformational mutants of p53 co-aggregate with wild-type p53, resulting in loss of wild-type function. Furthermore, mutant p53 can recruit its paralogs p63 and p73, resulting in their loss of transcriptional activity. Interestingly, both p63 and p73 share a similar aggregation-prone sequence in their DNA-binding domains.

These results, along with additional studies that show that fragments of p53 can undergo amyloidogenic aggregation in vitro, suggest a mechanism by which p53 aggregation could lead to cancer formation. In this context, cancer can be considered a protein aggregation disease, and the concept of preventing the aggregation of p53 to deter its oncogenic gain of function provides a novel strategy for cancer drug discovery. Here, we elucidated the atomic details of the aggregating region of the DNA-binding domain of p53. We synthesized several overlapping peptide segments of p53 and crystallized them. We determined the structures of two segments, which formed in-register, parallel steric zippers. We obtained data for a third segment, which we suggest forms an anti-parallel steric zipper. From these results, it appears that the aggregating region of p53 is capable of forming amyloid zipper structures, displaying polymorphism exhibited by other well-studied amyloid proteins. This structural characterization of p53
aggregation provides the basis for structure based design of aggregation inhibitors of p53\textsuperscript{27}.

**Results**

*Identification of the aggregation-prone region of p53 using the 3D-profile method*

We scanned the p53 sequence through the 3D-profile algorithm that identifies six-residue segments capable of forming steric zippers\textsuperscript{28,29}. Indeed, region 251-258 of p53 appears to be amyloidogenic (Fig. 3-1b). We examined various hexameric and heptameric segments within the region and tested their ability to form fibers and crystals. Three segments formed microcrystals; however one of the segments, p53\textsubscript{251-256} (ILTIIIT), exhibited low solubility and fast radiation decay, limiting its structural characterization.

*Parallel steric zipper structures from p53*

We identified two overlapping segments of p53 predicted to form steric zippers, a 7-residue peptide p53\textsubscript{252-258} (LTIITLE) and 6-residue peptide p53\textsubscript{253-258} (TIITLE). Both formed fiber-like aggregates as visualized under EM (Fig. 3-2a). LTIITLE appears to form molecular ribbons similar to those shown in a previous study of aggregated p53\textsuperscript{30}. TIITLE forms thinner, more typical amyloid-like fibrils. Microcrystals of both peptides formed within one week (Fig. 3-2b). We successfully determined the crystal structure of each peptide using diffraction data collected from these crystals. Data collection and refinement statistics can be found in Table 3-1, and steric zipper statistics in Table 3-2.

*Crystal structure of the p53\textsubscript{252-258} LTIITLE segment.* The peptide LTIITLE forms beta strands that are arranged as parallel, in-register beta sheets, with a dry steric zipper interface displaying a face-to-back orientation, indicative of a class 2 steric zipper\textsuperscript{24}. In
this structure, the fibril core is composed of interactions between mainly leucine and isoleucine, with L252, I254, and the methyl from T256 of one strand interdigitating with L257 and I255, and the methyl from T253 on the other strand (Fig. 3-3a). An extended network of hydrogen bonds between strands runs along the fiber axis (Fig. 3-3b). In addition, both the strands and the sheets pack in a parallel orientation (Fig. 3-3c).

**Crystal structure of the p53<sub>253-258</sub> TITITLE segment.** The hexameric segment TITITLE also forms a similar parallel, in-register steric zipper. In this structure, the peptide forms a pair of parallel beta sheets in a face-to-face orientation (class 1), with I255, L257, and the methyl group from T253 from each sheet constituting the dry steric zipper interface (Fig. 3-4a). The wet interface involves T256 interacting with a water molecule that brings together another sheet (Fig. 3-4d). In addition, while strands are stacking in a parallel orientation, the sheets pack anti-parallel to each other (Fig. 3-4c).

*p53 amyloidogenic segments may also form anti-parallel steric zippers*

We were able to obtain fibrils and microcrystals of a third segment, p53<sub>251-256</sub> (ILTIIT) (Fig. 3-4a and b). However, fast radiation decay and polymorphism among crystals grown under the same crystallization conditions prevented its structure determination. Diffraction patterns from all single crystals of ILTIIT did show Bragg-spacings of 9.6Å (Fig. 3-4c), indicating that the repeating units along the fibril axis are 9.6Å apart (Fig. 3-4d). This corresponds to the stacking of the beta strands having an antiparallel orientation; thus, we can assume that ILTIIT forms an antiparallel steric zipper.
Discussion

In this study, we show that the aggregation-prone region of p53 exhibits various types of fibril polymorphism. Three peptide segments form distinct steric zipper interfaces that can each serve as the spine for fibril formation\textsuperscript{23}. This was previously termed segmental polymorphism\textsuperscript{26}. That the sequences from these segments overlap significantly suggests a second type of amyloid polymorphism displayed, termed packing polymorphism, in which the same sequence forms distinct steric zipper structures due to different packing in the spine\textsuperscript{26}. Crystals of segment ILTIIT also displayed this polymorphism, as the unit cell dimensions among different crystals were not consistent, ultimately preventing its structure determination. Both segmental and packing polymorphism have been exhibited by other well-studied amyloid proteins such as Amyloid-beta and Islet Amyloid Polypeptide\textsuperscript{25,26}.

The atomic-level details elucidated here provide the basis for fibril formation of conformational mutants of p53. Destabilization of the native structure of p53 exposes a normally buried but highly aggregation-prone segment, residues 251-258 (Fig. 3-1a), which serves as a template for amyloid formation via steric zipper interactions. The structures presented here suggest the spine of these fibrils consists of hydrophobic interactions among the leucine, isoleucine, and threonine residues, particularly of I255, L257, and T253 (Fig. 3-3a and 3-4b). Interestingly, both p63 and p73 contain a highly homologous sequence in their DNA-binding domains\textsuperscript{12}. We hypothesize that is through heterotypic steric zipper interactions between these sequences that drive p53 aggregates to recruit wild-type p63 and p73.
Future work involves using the information gained from this study to develop an inhibitor specific to p53 fibrils, designed to cap the fibril spine and block its aggregation\textsuperscript{27}. Considering that region 251-258 displays polymorphism, finding a single inhibitor may prove challenging. However, given the previous success in such structure-based design for cases in which the formation of fibers, and not oligomers, constitutes the toxic entity\textsuperscript{27,31}, it is promising that such an inhibitor could be developed as a novel cancer therapeautic to fight p53 aggregation and restore wild-type activity.

**Materials and Methods**

*Sample preparation and crystallization.* Peptides were synthesized at greater than 97% purity from CS. Bio (Menlo Park, CA) and Celtek Bioscience (Nashville, TN) and dissolved in 10 mM lithium hydroxide (LiOH). Crystals were grown at 18 °C via hanging-drop vapor diffusion. Peptide 252-LTIITLE-258 was dissolved at 2 mg/ml and crystallized in the presence of 0.1 M Tris buffer pH 8.5 and 20% ethanol. Peptide 253-TIITLE-258 was dissolved at 6 mg/ml and crystallized in the presence of 0.01 M zinc chloride, 0.1 M MES buffer pH 6, and 20% PEG 6000. Peptide 251-ILTIIT-256 was dissolved at 3 mg/ml and crystallized in 1.0 M ammonium phosphate and 0.1 M acetate pH 4.5.

*Data collection and structure refinement.* Crystals of 253-TIITLE-258 and 251-ILTIIT-256 were mounted on the ends of pulled glass capillaries in the presence of 20% glycerol. Crystals of 252-LTIITLE-258 were mounted on 20-50 μm Mitegen LD (Ithaca, NY) loops in the presence of 20% glycerol and flash cooled in liquid nitrogen. Data was
collected at 100 K using a microfocus beam (5x5 μm²) at beamline 24-ID-E of the Advanced Photon Source (APS) at Argonne National Laboratory. Data indexing, integration and scaling were performed using XDS/XSCALE\textsuperscript{32} and DENZO/SCALEPACK\textsuperscript{33}. The merged scaled data was imported into the CCP4 format with programs from the CCP4 program suite organized under the “CCP4i” interface\textsuperscript{34}. Molecular replacement solutions for the segments were obtained using the program PHASER\textsuperscript{35}, using a polyalanine beta-strand as the search model. Crystallographic refinements were performed with REFMAC\textsuperscript{56}, and PHENIX\textsuperscript{37}. Model building was performed with COOT\textsuperscript{38} and illustrated with PYMOL\textsuperscript{39}.

Transmission electron microscopy. Lyophilized peptides were dissolved in 10mM LiOH at their respective crystallization concentrations, and incubated either at 37 degrees for 24 hours or at room temperature for one week. The samples were fixed on carbon-coated EM grids (Ted Pella), glow-discharged immediately before use. 5 uL samples were applied directly onto grids and allowed to adhere for 4 minutes. Grids were rinsed twice with 5 uL distilled water and negatively stained with 1% (wt/vol) uranyl acetate for 2 minutes. Specimens were examined in a FEI T12 electron microscope at an accelerating voltage of 120 kV. Images were recorded digitally by a Gatan 2K x 2K CCD.
Figure 3-1. Domain architecture of p53 and prediction of fiber-forming segments by the 3D profile method. (a) Domain architecture of p53 and structure of the DNA-binding domain. Starting at the N-terminus, full-length p53 contains a transactivation domain (TA), proline-rich region (PR), DNA-binding domain (DBD), and tetramerization domain (TD). Mutations of p53 that tend to destabilize the native structure and induce aggregation are located in the DBD. The structure of the DBD (pdb 1TSUR) is shown with the aggregating region (residues 251-258) predicted by Xu et al highlighted in red. (b) Prediction of fiber-forming segments of the aggregating region of p53 by the 3D profile method. The Rosetta energy of steric zipper-like self-association was calculated by scanning through the sequence with a six-residue window. The profile for residues 251-258 is shown. Energies below -23 kcal/mol, indicated by a horizontal gray line, are predicted to form amyloid-like fibrils containing a steric zipper interaction\textsuperscript{28,29}. The calculated energies of segments are colored in rainbow from green to red for low to high predicted propensity for fiber formation. The segments studied in this chapter are highlighted in the arrows.
Figure 3-2. Fibrils and microcrystals of 252-LTIITLE-258 and 253-TIITLE-258. (a) Electron micrographs of LTIITLE and TIITLE after incubation at 37 degrees for 24 hours. LTIITLE appears to form molecular ribbons similar to a previous study of aggregated p53\(^{30}\). TIITLE forms thinner, more typical amyloid-like fibrils under EM. (b) Microcrystals of LTIITLE and TIITLE observed by light microscopy. These crystals were used for diffraction studies. The width of these crystals is about 5 um.
Figure 3-3. 252-LTIITLE-258 forms a parallel class 2 steric zipper. (a) View down the fiber axis reveals a steric-zipper interface between the isoleucines and leucines of the peptide. LTIITLE forms a pair of parallel beta sheets in a face-to-back (class 2) orientation. (b) View perpendicular to the fiber axis reveals the hydrogen bonding network between each strand and its neighboring strands within a beta sheet. (c) Cartoon representation looking perpendicular to the fiber axis, revealing the interaction between sheets. The strands are in a parallel orientation and the sheets pack parallel to each other. (d) View parallel to the fiber axis reveals the packing of the beta sheets, showing the dry and wet interfaces between the sheets.
Figure 3-4. 253-TIITLE-258 forms a parallel class 1 steric zipper. (a) View down the fiber axis reveals a steric-zipper interface between the isoleucines and leucines of the peptide. In this structure however, the peptide forms a pair of parallel beta sheets in a face-to-face (class 1) orientation, revealing polymorphism of this aggregating region. (b) View perpendicular to the fiber axis showing the hydrogen bonding network. (c) Cartoon representation looking perpendicular to fiber axis, revealing the interaction between sheets. The strands are stacking in a parallel orientation, however the sheets pack anti-parallel to each other. (d) View parallel to the fiber axis reveals the packing of the beta sheets, showing the dry and wet interfaces between the sheets.
Figure 3-5. 251-ILTIIT-256 appears to form an antiparallel steric zipper. (a) Electron micrograph of ILTIIT fibrils after incubation at room temperature for 1 week. The peptide appears to form fibrils growing out of microcrystals. (b) Microcrystals of ILTIIT observed by light microscopy. Data was collected on these crystals; however, they decayed quickly and due to polymorphism of the crystals its structure could not be determined. (c) Diffraction pattern from a single crystal of ILTIIT with a 5 degree oscillation showed Bragg-spacings of 9.6Å, indicating that the stacking of the beta strands of each sheet is antiparallel. These spacings were seen in all crystals of ILTIIT analyzed, regardless of their polymorphism. (d) Cartoon representation of a model of an antiparallel ILTIIT sheet. Each color represents the strands going in one direction. That ILTIIT forms an antiparallel zipper suggests that the aggregating region of p53 is capable of displaying packing polymorphism.
Table 3-1. Statistics of X-ray data collection and refinement for p53 peptide structures

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a. Values in parentheses correspond to the highest resolution shell.

b. $R_{\text{merge}} = \sum |I - <I>| / \sum I$.

c. $R_{\text{work}} = \sum |F_o - F_c| / \sum F_o$.

d. $R_{\text{free}} = \sum |F_o - F_c| / \sum F_o$, calculated using a random set containing 10% reflections that were not included throughout structure refinement.
Table 3-2. Features of the dry steric zipper interface of microcrystals derived from fibril-forming p53 segments.

<table>
<thead>
<tr>
<th>p53 segment</th>
<th>Strand orientation</th>
<th>Steric zipper type</th>
<th>Area buried (Å²)</th>
<th>Shape complementarily&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>252-LTIITLE-258</td>
<td>Parallel</td>
<td>Face-to-back In register β-sheets</td>
<td>176</td>
<td>0.87</td>
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<tr>
<td></td>
<td></td>
<td>Symmetry class 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>253-TIITLE-258</td>
<td>Parallel</td>
<td>Face-to-face In register β-sheets</td>
<td>184</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Symmetry class 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Area buried was calculated using AREAIMOL<sup>40</sup> with a probe radius of 1.4Å. The summation of the difference between the accessible surface areas of a) one β-strand alone and in contact with the opposite β-sheet, and of b) the β-sheet alone and in contact with the opposite β-strand, constitutes the reported area buried. In structures with anti-parallel β-strand orientation, as well as in parallel β-strand orientations with different conformations, the average area buried per β-strand is reported.

<sup>b</sup> Lawrence and Colman’s shape complementarity index<sup>41</sup>. 

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References


Chapter 4: A preliminary analysis of cylindrin toxicity through membrane leakage experiments and transgenic expression in *C. elegans*

Abstract
The recent characterization of an 11-residue peptide from alpha-B-crystallin by X-ray crystallography elucidates the first atomic details of a toxic amyloidogenic peptide segment in its oligomeric state. While this peptide, termed cylindrin, was shown to be toxic in cultured cells, its mechanism of toxicity, as well as the extent of toxicity in relation to other amyloid proteins, remains to be determined. The work in this chapter provides a preliminary analysis of cylindrin toxicity. First, through liposome disruption assays, we suggest that the mechanism of toxicity of cylindrin is not through membrane disruption, as cylindrin failed to induce significant dye release of calcein-enclosed liposomes. Second, we attempt to examine cylindrin toxicity *in vivo*, through transgenic expression of the peptide in *C. elegans* through extrachromosomal arrays. We confirm expression of cylindrin and perform initial experiments suggesting slight toxicity induced by the peptide. However, these strains do not exhibit the same paralysis phenotype exhibited in previous studies of amyloid transgenics. We suggest further experiments that could be performed to expand on preliminary analysis provided here.
**Introduction**

Of the variety of intermediate stages proposed to progress during the conversion of soluble amyloid proteins into fibrils, increasing evidence suggests it is the transient, soluble, lower molecular weight oligomers that comprise the toxic agents in amyloid-related diseases\(^1\)–\(^7\). These oligomers share several characteristics: they are beta-rich and capable of also forming fibers; they exhibit greater cytotoxicity than either the monomer or fibrils formed from the same protein\(^1,\)\(^8\); and they are recognized by a “conformational” antibody (A11) that binds oligomers but not fibrils\(^9\). Despite intensive research, the transient polymorphic nature of small amyloid oligomers has prevented the elucidation of their assembly and pathology mechanisms. While atomic-resolution models have been experimentally determined for amyloid fibrils\(^10\), the structural details for oligomers remain largely unknown.

A recent study of an 11-residue segment from alpha-B crystallin by X-ray crystallography sheds some light onto these complex mechanisms, as the authors determined the first atomic-resolution structure of a toxic amyloidogenic peptide segment in its oligomeric state\(^11\). This segment, termed cylindrin, forms six identical antiparallel \(\beta\)-strands in the shape of a barrel, with a dry interior of the barrel resembling the dry interior of a steric zipper interface\(^10\)–\(^12\). In contrast to in-register \(\beta\)-strands typically observed in amyloid fibril structures, the \(\beta\)-strands in cylindrin are out-of-register. Notably, cylindrin exhibits properties shared by amyloid oligomers, such as \(\beta\)-sheet–rich structure, cytotoxicity, and recognition by A11 conformational antibody, suggesting that cylindrin may represent a common state of all amyloid oligomers\(^11\). Furthermore,
molecular dynamics (MD) studies suggest that the cylindrin oligomer may be involved in a distinct aggregation pathway separate from that of an in-register fibril\textsuperscript{11}.

The structure and characterization of cylindrin raises many important questions. It has yet to be determined whether cylindrin is a common oligomeric state of well-studied toxic amyloid proteins, such as Amyloid-beta (Abeta) and Islet Amyloid Polypeptide (IAPP), or if it shares similar toxic mechanisms. In addition, while cylindrin was shown to be toxic in cultured cells, it remains unclear whether this toxicity can be observed \textit{in-vivo}. The work in this chapter thus aims to address these queries, in a preliminary investigation of the mechanism of cylindrin toxicity. First, through liposome disruption assays, we suggest that the mechanism of toxicity of cylindrin is not through membrane disruption, as cylindrin constructs failed to induce significant dye release of calcein-enclosed liposomes. Second, we attempt to examine toxicity \textit{in vivo}, through transgenic expression of the peptide in \textit{Caenorhabditis elegans} through extrachromosomal arrays. Initial characterization of these strains confirms expression of cylindrin. However, these worms do not exhibit the same paralysis phenotype shown in previous studies of amyloid transgenics\textsuperscript{13–16}. We suggest further experiments that could be performed to expand on analysis provided here.

\textbf{Results}

\textit{Construction of cylindrin variants}

In the crystal structure of cylindrin, the dry interior of the cylinder, is closed by three valines, particularly V2, V4, and V8\textsuperscript{11}. We thus targeted various cylindrin mutants
that would be predicted to strengthen or disrupt this packing within the structure (see Table 4-1 for complete list of sequences). We synthesized wild-type K11V-TR and K11V^{V2L}-TR, which were studied previously\textsuperscript{11}. K11V^{V2L} is a sequence variant in which the valine at position 2 is replaced by a leucine; K11V^{V2L}-TR was subsequently crystallized and used in the cell-culture experiments that demonstrated cylindrin’s cytotoxicity\textsuperscript{11}. A third peptide, K11V^{PG}-TR, is the V2L variant that also contains a mutation in the linker region of a glycine to a proline, resulting in a pro-gly linker instead of gly-gly linker. This linker would be predicted to reinforce the beta-hairpin turn between the repeating strands within the cylindrin structure.

For \textit{in vivo} analysis, we focused on three variants that would be predicted to disrupt cylindrin oligomer formation through steric clash of core, buried residues (Table 4-1). K11V^{V4W}-TR is the V2L variant that contains a V4W substitution in both repeats, which would thus place a large aromatic residue in buried core. Interestingly, this peptide was not toxic in cell culture\textsuperscript{11}. In K11V^{6P}-TR, the core valines are replaced by the beta-sheet breaker proline. Finally, K11V^{R2D2}-TR contains the longer, charged arginine and aspartate residues in place of two core valines.

\textit{Cylindrin constructs induce minimal membrane leakage compared to IAPP}

It was previously shown that cylindrin was toxic to cultured cells\textsuperscript{11}. To examine whether this cytotoxicity could be due to membrane disruption, such as in the case for human islet polypeptide (hIAPP)\textsuperscript{17,18}, we performed liposome dye-release experiments. K11V-TR, K11V^{V2L}-TR, K11V^{PG}-TR, and hIAPP\textsubscript{8-37} (residues 8-37) were incubated in
the presence of calcein-containing liposomes, and fluorescence was measured over time. In addition, a 23-residue peptide, IAV-M2eA1, that binds membranes but does not induce leakage, was used as a negative control \(^{19-21}\) (see materials and methods). In two independent experiments, the hIAPP\(_{8-37}\) peptide diminished liposome integrity leading to dye release, but the K11V-TR constructs did not (Fig. 4-1 and 4-2). While the cylindrin constructs induced slightly more leakage than the control (Fig. 4-1b), leakage was minimal despite the peptides being present at a 10-fold higher concentration compared to hIAPP\(_{8-37}\). This suggests that the mechanism of toxicity of cylindrin is not membrane disruption.

**Generation of transgenic strains of C. elegans that express cylindrin**

To further study the mechanism of cylindrin toxicity, we turned to an *in vivo* model, using *Caenorhabditis elegans* as the organism of study. Previous studies had shown that transgenic expression of amyloids in worms induced noticeable phenotypes \(^{13-16}\); thus, our intent was to examine if cylindrin expression caused similar effects. To assemble cylindrin for transgenic expression, the sequence coding for the cylindrin tandem repeat peptide was fused to a secretion signal sequence \(^{15}\) and incorporated into two expression vectors (Fig. 4-3a and b). The first vector, pPD96.52, contains a *myo-3* promoter that induces expression in all muscle cells except for those in the pharynx \(^{22}\). The second vector is a chimera constructed by extracting an *hsp-16.41* promoter region from pPD49.83 into pPD96.52, resulting in expression via a heat shock response in almost all tissues, including neurons, muscle, intestine and pharynx but not the germ.
Using this strategy, we successfully cloned K11V^{V2L}-TR (hereafter mentioned as K11V) and the destabilization mutants K11V^{V4W}-TR (V4W), K11V^{6P}-TR (6P), K11V^{R2D2}-TR (R2D2) under both promoters.

Transgenic animals were then generated by gonad microinjection\(^25,26\), using coinjection of each cylindrin plasmid with three markers (Fig. 4-3c). Plasmid pRF4, contains a mutant C. elegans collagen gene \([rol-6(sul1006)]\) that produces a dominant, distinct "Roller" phenotype, which allows for identification of animals that maintain the injected transgenes\(^27\). The other two markers, driven under the \(myo-3\) promoter for muscle cell expression, were injected for fluorescent-tagging experiments. The CFP::LGG-1 dominant marker contains cyan fluorescent protein (CFP) fused to LGG-1, an autophagosomal marker protein\(^28\). This marker thus stains autophagosomes, which are known to accumulate as a result of toxic amyloid expression\(^29\). The second marker, TOM-70::YFP, contains yellow fluorescent protein (YFP) fused to the first 30-residues of a protein TOM-70 known to anchor proteins to the outer mitochondrial membrane; expression of this marker thus stains the outer mitochondrial membrane\(^30,31\). We predicted cylindrin toxicity could induce morphological changes in the mitochondria, which could then be visualized through confocal fluorescence microscopy. In addition, the use of these GFP variants would allow for simultaneous imaging of both TOM-70 and LGG-1\(^30\).

Using this microinjection procedure, and selecting for the transgenic worms that exhibited the roller phenotype, we established multiple independent transmitting (extrachromosomal) lines for \(myo-3/K11V\) (DE101 and DE105), hsp16-41/K11V (DE201), and the respective mutants V4W (DE102, DE106, and DE202), 6P (DE103 and
DE203), and R2D2 (DE104 and DE204). We also generated a strain by co-injection of only the markers, which we used as negative control (DEXX-1). The entire list of strains is shown in Table 4-2. The transmitting lines established in our study had a meiotic stability of 30-60%. The worms expressing wild-type or mutant K11V showed no difference in appearance from the control strain (data not shown).

Expression of myo-3/cylindrin appears to induce autophagosome accumulation

We examined the expression of cylindrin proteins under the control of the myo-3 promoters in the generated C. elegans strains. Lysates of day 1 K11V and V4W transgenic strains and the control strain were isolated and subjected to SDS–PAGE. Cylindrin expression was examined by immunoblotting with a rabbit polyclonal cylindrin antibody 5521, which was generated against the K11V-TR peptide and shown to bind specifically to synthetic wild-type K11V-TR, K11V$^{V2L}$, and K11V$^{V4W}$ (Arthur Laganowsky, unpublished results). Two specific ~29 kDa and ~39 kDa protein bands were detected in the lysates of two strains expressing K11V and V4W (Fig. 4-3d and e, lanes 1 and 2). Interestingly, this does not correspond to the electrophoretic mobility of synthetic K11V$^{V2L}$-TR (Fig. 4-3d, lane 8). However, the control strain in which only the markers were injected did not show any specific bands (Fig. 4-3d and e, lane 3). Also, two strains in which K11V and V4W were injected at lower concentrations (see Table 4-2) showed very faint bands (Fig. 4-3d, lanes 4 and 5).

To examine any autophagosome and mitochondria effects induced by cylindrin expression, we examined CFP and YFP localization on control (DEXX-1) and myo-
3/K11V (DE105) worms. There were no noticeable differences in the morphology of the mitochondrial outer membrane, indicated by imaging the YFP-stained outer mitochondrial membrane (Fig. 4-4a and c). However, this was not the case regarding LGG-1 accumulation. While the DEXX-1 worms displayed a predominantly diffuse CFP::LGG-1 localization (Fig. 4-4b), DE105 worms displayed various CFP-puncta (Fig. 4-4d), suggesting autophagosome accumulation in *C. elegans* muscle induced by cylindrin expression. Interestingly, Abeta expression has been shown to induce autophagosome aggregation in transgenic *C. elegans*.[29]

*Expression of cylindrin does not induce a paralysis phenotype*

In previous studies, transgenic amyloid expression of Abeta, polyglutamate, or superoxide dismutase in *C. elegans* induced significant amyloid aggregation, which resulted in paralysis throughout the body except for the pharynx[13–16]. We examined whether our cylindrin strains exhibited this same phenotype; however, neither myo-3/K11V nor hsp16-41/K11V worms become paralyzed (data not shown). We also attempted to assess toxicity through lifespan and brood analysis on the hsp16-41/cylindrin strains. We examined the brood size of various adult K11V and control worms that were heat-shocked at 30° for four hours at L4 larval stage to induce cylindrin expression. Results show that K11V has a noticeably higher brood size than the control strain (Fig 4-5a). This was not expected as we predicted that toxicity would negatively affect brood size. In addition, we performed a lifespan analysis on K11V and control worms. Worms at day 1 of adulthood were heat-shocked at 30° to induce cylindrin
expression, and kept at the same temperature until death. While K11V worms initially
die off faster than control worms, the survivors have a slightly longer lifespan than the
control worms (Fig. 4-5b).

Discussion

The preliminary results presented here support the notion from previous studies of
a distinct aggregation pathway of cylindrin that is separate from the in-register pathway
of fibrils\textsuperscript{11}. First, the mechanism of toxicity of cylindrin appears to differ from amyloids
such as Abeta or IAPP, whose cytotoxicity has been associated with membrane
disruption\textsuperscript{17,18,32,33}. Our liposome dye-release experiments show that cylindrin fails to
induce significant disruption of calcein-enclosed liposomes, suggesting a mechanism of
toxicity more complicated than membrane disruption. In addition, previous studies of
transgenic \textit{C. elegans} have shown that amyloid expression in worm models induces a
paralysis phenotype, due to amyloid fibrils accumulating in muscle cells\textsuperscript{14–16}. We
constructed and characterized various strains of transgenic \textit{C. elegans} that express
cylindrin; however, they do not exhibit the same phenotype, suggesting lower
accumulation of amyloid fibrils in these worms. That cylindrin expression does not
induce significant fibrillization is consistent with molecular dynamics studies suggesting
the cylindrin oligomer is off-pathway to fibers\textsuperscript{11}.

Cylindrin expression does appear to induce autophagosome accumulation, a
characteristic that is shared in Abeta-expressed worm strains\textsuperscript{29}. Autophagy, a ubiquitous
cellular process involving the lysosomal degradation of unnecessary or dysfunctional
cytoplasmic material, is often upregulated in the presence of various stressors or toxins\textsuperscript{28,29,34}. Thus, it is possible that cylindrin expression in worms might be inducing slight toxicity that results in autophagosome accumulation, indicated by LGG-1::CFP puncta in K11V strains. Furthermore, it has been shown that low-level stressors at 20-25\% of the minimum toxic dose often enhance future stress resistance and increase longevity, through a process called stress-induced hormesis\textsuperscript{35–37}. This could explain the stress-resistant effect of K11V strains versus control in response to their higher brood size and slightly longer lifespan. In western blot experiments on lysates using a cylindrin-specific antibody, we confirm expression of the peptide in strains generated for muscle expression, though the peptide appears to be forming higher molecular weight species \textit{in vivo}, in comparison to that of the synthetic peptide (Fig. 4-3, lanes 1, 2, and 8). It remains to be determined whether this band is an SDS-induced artifact or an SDS-resistant oligomer\textsuperscript{38,39}.

Ultimately, additional experiments are required to fully understand the mechanism of the toxicity of cylindrin, both \textit{in vitro} and \textit{in vivo}. Liposome assays offer at best a crude assessment of a segment’s membrane disruption capability; thus, additional cell-based assays will need to be performed to confirm these results. In addition, it is possible that transgenic cylindrin expression can induce significant aggregation in worms that results in their paralysis; however, the amount of cylindrin DNA we injected (Table 4-2) in the worms may not have been sufficient to induce such a phenotype. In addition, while strains were generated for mutants of K11V, they have not yet been studied. Future work involves a deeper examination of K11V toxicity in \textit{C.}
**elegans**, including comparison of mutant K11V transgenic expression versus wild-type, confocal microscopy experiments showing additional autophagic and mitochondrial effects from K11V expression, and examination of cylindrin localization in the worm by use of cylindrin antibody. Also, it will be important to determine whether conformational antibodies such as A11 and OC⁹ or histological dyes such a thioflavin-T and congo-red are able to bind cylindrin aggregates in these transgenic worms.

**Materials and Methods**

**Preparation of Large Unilamellar Vesicles (LUVs).** Calcein-containing LUVs were prepared as described previously¹⁸, with minor modifications. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-glycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Mixtures of POPC and POPG in a 7:3 molar ratio were dissolved in 1:1 chloroform:methanol. The solvent was evaporated under dry nitrogen gas to yield a lipid film that was further dried under vacuum for at least 24 hours to remove any residual organic solvent. The film was then hydrated in 70 mM calcein (Sigma Aldrich, St. Louis, MO) and 10mM Tris-HCl (pH 7.4) at a lipid concentration of 5mM. The suspensions were subjected to 10 freeze-thaw cycles of temperatures of -80 and 50 °C followed by extrusion through two 0.2 µm pore size filters (Whatman, Florham Park, NJ). Non-encapsulated calcein was separated from calcein-filled LUVs by size exclusion using a Sephadex G-75 (GE Healthcare, Piscataway, NJ) equilibrated in buffer (10 mM Tris-HCl pH 7.4, 100 mM sodium chloride). The calcein-containing LUVs were concentrated to 3-5 mM and stored at 4 °C.
Phospholipid content and concentrations of the LUVs were determined by RP-HPLC using 100% Methanol and 100 mM TEAC (tetraethylammonium chloride) pH 7.8 as the solvent and a C18 column. Diameter of LUVs was determined using a Microtrac UPA 150 (York, PA). LUVs preparations displayed diameters between 170-200 nm for at least 10 days. LUVs were used for experiments within 5 days of preparation.

Membrane Leakage Experiments. K11V-TR constructs were cloned and recombinantly synthesized as described previously. These peptides and synthetic peptide IAV-M2eA1 (a gift from Gary Fujii; sequence: SLLTEVETPIRNEWCRCNDSSD) were solubilized in water. Three different stock solutions were made at concentrations of 0.2 mM, 0.8 mM, and 2 mM. 5 uL of each peptide stock solution was added to a well containing 195uL of 7:3 calcein-containing POPC:POPG LUVs in a buffer (10mM Tris-HCL pH 7.4, 100mM sodium chloride) with or without 2.5% DMSO. Dye leakage was measured using a SpectraMax5 (Molecular Devices, Sunnyvale, CA) at 485 nm excitation and a 535 nm emission. Measurements were taken every 10 minutes for 30 hours. Each well contains a final LUV concentration of 100 uM with final peptide concentrations of 5 uM, 20 uM, and 50 uM. Synthetic human Islet Amyloid Polypeptide (hIAPP), residues 8-37, was purchased from CS-Bio (Menlo Park, CA). Lyophilized hIAPP was dissolved in 100% hexafluoro-2-propanol (HFIP) and put under vacuum to evaporate the HFIP. hIAPP was then reconstituted in water at 0.2 mM, filtered using a 0.2 uM filter, and added to 6 wells containing 100 mM Calcein-containing LUVs to a final concentration of 5 uM hIAPP. Fluorescence at a given time point was normalized as described.
previously\textsuperscript{17} \cite{23}, using the equation: \[ F_{\text{normalized}} = \frac{(F_{\text{max}} - F_{\text{min}})}{(F_t - F_{\text{min}})}. \] \( F_t \) is the measured fluorescence intensity, \( F_{\text{min}} \) the fluorescence of 100 mM calcein-containing LUV’s alone, and \( F_{\text{max}} \) is the maximum fluorescence determined by incubation of 100mM LUVs in the presence of 0.5\% Triton X-100.

\textit{Molecular cloning.} A minigene encoding the beta-cylin-repeat peptide \( (K11V^{V2L-TR}) \) attached to a 16-residue secretion signal\textsuperscript{15,40} was assembled in two rounds of PCR. In the first round, the \( K11V^{V2L-TR} \) coding sequence was amplified from a cDNA clone\textsuperscript{11} using a forward primer that contained the last 8 residues of the secretion signal, followed by a KpnI site and the first 9 residues of \( K11V^{V2L-TR} \). The C-terminal sequence contained a stop codon followed by a Xho I and Sac I restriction. The second round of PCR constructed the entire signal sequence attached to \( K11V^{V2L-TR} \), using the same C-terminal sequence from round 1, and a forward sequence that included an Nhe I site, start codon, and all residues from the signal peptide. This PCR product was then cleaved with Nhe I and Sac I restriction endonucleases, and inserted between the unique Nhe I and Sac I sites of vector pPD96.52 that contains a myo-3 promoter for muscle cell expression. To generate cylindrin expression via the heat shock response, a chimera was constructed by extracting the \( hsp-16.41 \) promoter region from pPD49.83 using Hind III and Bam HI, and ligating it into a Hind III/Bam HI digested pPD96.52.

\textit{Cylindrin mutant plasmid construction.} The \( K11V^{6P}, K11V^{V4W}, \) and \( K11V^{R2D2} \) mutations on the DNA sequence were performed on both \( K11V^{V2L-TR} \) plasmids as described
previously\textsuperscript{11}, using a Site-Directed Mutagenesis kit (QuickChange XL, Stratagene, La Jolla, CA) with site-directed primers designed using manufacturers online ‘QuickChange Primer Design Program’ (Stratagene, La Jolla, CA). Thus, each construct was generated under both the \textit{myo-3} promoter and \textit{hsp16.41} promoter. The open reading frame of each mutant was confirmed by sequencing.

\textit{Generation of transgenic C. elegans.} Following standard techniques\textsuperscript{26}, extrachromosomal transgenic lines were established by injecting each cylindrin construct into Bristol N2 \textit{C. elegans} syncytial gonads together with several markers: (i) the \textit{rol-6} (pRF4) (rol-6 REF) dominant marker\textsuperscript{27}, (ii) a \textit{cfp:llg-1} dominant marker that targets augophagosomes\textsuperscript{28}, and (iii) a \textit{mito:yfp} marker that stains the outer mitochondrial membrane\textsuperscript{30} (gifts from Alexander van der Bliek). DNA concentrations were 50 ng/µl for pRF4, 10 ng/µl for the fluorescent markers, and 10-30 ng/µl (Table 4-2) for the cylindrin plasmids. Transmitting lines were established and maintained by selection for the Roller marker phenotype. At least three independent lines for each construct were generated and used for analysis. General handling techniques of \textit{C. elegans}, such as strain maintenance and culture were carried out according to standard protocols\textsuperscript{41}. Nematodes were grown at 20°C on nematode growth medium (NGM) agar plates and live bacteria (\textit{Escherichia coli} strain OP50) were provided as nutrients. Strains were frozen in liquid nitrogen and stored indefinitely at -80°C; they were recovered when needed for assays\textsuperscript{41}. 

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Western blot analysis. Worms were age-synchronized by treatment with hypochlorite and grown to L4 larval stages on 100 x 15 mm NGM agar plates (Fisher Scientific, Pittsburgh PA). On day 1 of adulthood, 150 rollers from each strain, or a single line from a certain strain, were harvested and washed times with M9 buffer (22 mM KH2PO4, 42 mM NaH2PO4, 86 mM NaCl). Samples were diluted with 6X sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 5% glycerol, 1% NP-40 and 0.01% bromophenol blue), boiled for 10 minutes, then passed through a 25g syringe (BD; Sparks, MD) to shear the DNA. Samples were boiled again, then loaded onto 4-12% NuPAGE Bis-Tris gels (Invitrogen; Carlsbad, CA) and electrophoresed. Synthetic K11V-TR was also loaded onto the gel as a positive control. Proteins were transferred to nitrocellulose membranes and blocked with 10% fat free milk in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20). The membranes were then incubated with a polyclonal cylindrin antibody 5521 (1:250 dilution in 5% fat free milk, TBST buffer), which was generated against the cylindrin peptide and shown to bind specifically to wild-type K11V-TR, K11V V2L, and K11V V4W (Arthur Laganowsky, unpublished results). The membranes were washed three times in TBST buffer before incubating with anti-rabbit HRP-linked antibody (1:5000 dilution in 5% fat free milk, TBST buffer) (Invitrogen; Carlsbad, CA) at room temperature for 1 hour. After washing the membranes three times in TBST buffer, films were developed following the protocol in the Kit (Thermo Scientific Pierce ECL Western Blotting Substrate, #32209).
**Confocal Fluorescence Microscopy.** Fluorescence images were captured using a Zeiss Axiovert 200M equipped with 40×/NA 1.3 Plan-Neofluar, 63×/NA 1.4 Plan-Apochromat, or 100×/NA 1.45 α-Plan-Fluar objectives and an ORCA ER-CCD camera (Hamamatsu, Shizuoka, Japan). Young adult worms (picked 1 day after L4) were used for imaging of muscle cell mitochondria. YFP-tagged mitochondria or CFP-tagged LGG-1 in muscle cells were imaged with a Zeiss LSM 5 Pascal confocal microscope equipped with a 100×/NA 1.45 Plan-Fluar objective. CFP was excited with 458-nm and YFP with 514-nm.

**Brood Size Analysis.** DEXX-1, and hsp/K11V worms (3 independent lines) were grown at 20°C on 6-cm NGM plates seeded with OP50 bacteria. At L4 stage, worms from each line were shifted to 30°C for four hours. At this time, the strains under the hsp16.41 promoter would induce expression of K11V and mutants, while the control strain would not. After temperature Ten to fifteen L4 worms from each line of a certain strain were transferred to new plates and allowed to lay eggs for 5 days at 20°C. During these 5 days, worms were transferred to new plates after every 48 hours. The F1 progeny from each individual worm was counted every 24 hours, also on the same plate series.

**Lifespan Analysis.** Age-synchronized DEXX-1, and hsp/K11V worms (3 independent lines) were grown at 20°C on 6-cm NGM plates seeded with OP50 bacteria. At L4 stage, worms were shifted to 30° to induce K11V expression via heat-shock response and kept at that temperature until death. Worms were scored by gently prodding the nose and tail
with a platinum wire to test for live or dead animals. Worms were scored either dead or alive every hour until the last worm died. A total of 60 worms (10 worms per plate) were scored for each independent line.
Table 4-1. Cylindrin peptide abbreviations and amino acid sequences

<table>
<thead>
<tr>
<th>Peptide Abbreviation</th>
<th>Sequence</th>
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<tr>
<td>K11V-TR</td>
<td>GKVVKVLGDVIEVGGKVLGDVIEV</td>
</tr>
<tr>
<td>$K11V^{Y2L}$-TR*</td>
<td>GKLKVLGDVIEVGGKLKVLGDVIEV</td>
</tr>
<tr>
<td>K11V$^{PG}$-TR</td>
<td>GKLKVLGDVIEVPGLKLKVLGDVIEV</td>
</tr>
<tr>
<td>K11V$^{W4}$-TR</td>
<td>GKLKWLGDVIEVGGKGLKWLGVDVIEV</td>
</tr>
<tr>
<td>K11V$^{6P}$-TR</td>
<td>GKPKPLGDPIEVEGGKPKPLGDPIEV</td>
</tr>
<tr>
<td>K11V$^{R2D2}$-TR</td>
<td>GKLKRLGDDIEVGGKLKDLDGDRIEV</td>
</tr>
</tbody>
</table>

* This peptide was denoted as K11V-TR in the study by Laganowsky et. al\textsuperscript{11}
**Table 4-2. List of the cylindrin strains generated by gonad microinjection**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cylindrin construct&lt;sup&gt;a&lt;/sup&gt;</th>
<th>promoter&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ng/ul injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEXX-1</td>
<td>N/A (markers only)</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>DE101</td>
<td>K11V&lt;sup&gt;V2L&lt;/sup&gt;-TR</td>
<td>myo-3</td>
<td>10</td>
</tr>
<tr>
<td>DE102</td>
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<sup>a</sup> Construct is preceded by a secretion signal peptide sequence in the plasmid.

<sup>b</sup> Promoter that drives cylindrin expression.
Figure 4-1. Beta cylindrin tandem repeat peptides induce minimal membrane leakage. (a) Liposome dye-release experiments were performed with K11V-TR, K11V<sup>V2I</sup>-TR, K11V<sup>P</sup>-TR, hIAPP<sub>8-37</sub>, or IAVMe2-A1 peptides. The concentrations used in experiments are shown (inset). IAVMe2-A1, known to bind membranes but not induce leakage<sup>19–21</sup> (REF), was used as a negative control. hIAPP<sub>8-37</sub> was used as a positive control<sup>17,18</sup> (REF). Also present in all conditions was the presence of 2.5% DMSO, which aids in liposome stability<sup>42,43</sup>. Peptides were incubated with calcein-containing liposomes and calcein fluorescence was measured over time. The calcein fluorescence is normalized to detergent solubilized liposomes with 0.5% Triton-X. For hIAPP, leakage was observed up to 50%. (b) Zoom of boxed section in (a). IAVMe2-A1 failed to induce any leakage. The cylindrin peptides induced leakage of less than 10%. Of the three cylindrin constructs studied, K11V<sup>P</sup>-TR induces the most leakage.
Figure 4-2. In the presence of less stable membranes, beta cylindrin tandem repeat peptides still fail to induce substantial membrane leakage. Figure adapted from Laganowsky et al. Liposome dye-release experiments were performed with K11V-TR, K11V\textsuperscript{V2L}-TR, or hIAPP\textsubscript{8-37} (residues 8-37) peptides. In this experiment, no DMSO was present in the conditions. Concentrations used in experiments are shown (inset). Peptides were incubated with calcein-containing liposomes and calcein fluorescence was measured over time. Dye release from detergent solubilized liposomes treated with 0.5% Triton-X was used to normalize the fluorescence. For hIAPP\textsubscript{8-37}, leakage was observed up to 60%. The K11V-TR or K11V\textsuperscript{V2L}-TR peptides reached a maximum leakage of only 10%; again, its concentration was 10 times higher compared to the hIAPP\textsubscript{8-37} peptide. This suggests that membrane disruption by beta cylindrin is not the mechanism of toxicity.
Figure 4-3

Figure 4-3. Generation of transgenic *C. elegans* that express cylindrin. (a) For expression in muscle cells, the sequence coding for the cylindrin tandem repeat peptide was fused to a secretion signal sequence (ss) and incorporated into expression vector pPD 96.52 that contains a myo-3 promoter. (b) For expression through the heat shock response, an *hsp-16.41* promoter from pPD49.83 is extracted and incorporated into pPD96.52. (c) Transgenic animals were generated using coinjection of each cylindrin plasmid with three markers: in black, plasmid pRF4 [rol-6(sul1006)] that produces a dominant "Roller" phenotype; in red, LGG-1::CFP dominant marker that stains autophagosomes; and in green, TOM-70::YFP that stains the outer mitochondrial membrane. Strains generated are shown in Table 4-2. (c) Western blot using a polyclonal cylindrin antibody on lysates of myo-3/K11V strains and control, shows specific bands in the DE105 and DE106 strains (lanes 1 and 2), but not in the controls (lane 3) or in K11V strains with lower concentration of cylindrin plasmid injected (lanes 4 and 5; see Table 4-2). In lane 8, synthetic K11V is blotted as a positive control. (d) A second blot shows the same results, confirming cylindrin expression in DE105 (K11V) and DE106 (V4W) strains.

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**Table 4-2**

<table>
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<tr>
<td>V4W</td>
<td>Green</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Green</td>
</tr>
</tbody>
</table>

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**Figure 4-3**

(a) DE100 (DE100) and (b) DE200 (DE200) diagrams showing the expression vectors and promoters used for cylindrin expression.
Figure 4-4. Cylindrin expression induces autophagosome accumulation in transgenic *C. elegans*. Fluorescence images of outer mitochondrial membrane (a and c) and autophagosomes (b and d) on Day 1 control (DEXX-1) and myo-3/K11V (DE105) strains reveal no noticeable changes in morphology of muscle mitochondria in worms expressing K11V. However, these worms exhibit punctate LGG-1::CFP staining (d), indicative of autophagosome accumulation. Transgenic amyloid expression has previously been shown to induce this effect.29
Figure 4-5

(a) Brood size of K11V or control strains after heat-shock at 30° for four hours. Results show that each independent line of K11V has a higher brood size than the control strain.

(b) Lifespan analysis K11V and control worms. Worms at day 1 of adulthood were heat-shocked at 30° to induce cylindrin expression and kept at the same temperature until death, and worms were scored either dead or alive every hour until the last worm had died. While K11V worms initially die off faster than control worms, the survivors have a slightly longer lifespan than the control worms.
References


   doi:10.1073/pnas.0905127106


Chapter 5: An approach to crystallizing proteins by metal-mediated synthetic symmetrization

Abstract
Combining the concepts of synthetic symmetrization with the approach of engineering metal binding sites, we have developed a new crystallization methodology termed metal-mediated synthetic symmetrization. In this method, pairs of histidine or cysteine mutations are introduced on the surface of target proteins, generating crystal lattice contacts or oligomeric assemblies upon coordination with metal. Metal-mediated synthetic symmetrization greatly expands the packing and oligomeric assembly possibilities of target proteins, thereby increasing the chances of growing diffraction-quality crystals. To demonstrate this method, we designed various T4 lysozyme (T4L) and maltose-binding protein (MBP) mutants and co-crystallized them with one of three metal ions: copper (Cu\(^{2+}\)), nickel (Ni\(^{2+}\)) or zinc (Zn\(^{2+}\)). The approach resulted in 16 new crystal structures – 8 for T4L and 8 for MBP – displaying a variety of oligomeric assemblies and packing modes, representing in total 13 new and distinct crystal forms for these proteins. We discuss the potential utility of the method for crystallizing target proteins of unknown structure by engineering in pairs of histidine or cysteine residues. As an alternate strategy, we propose that the varied crystallization-prone forms of T4L or MBP engineered in this work could be used as crystallization chaperones, by fusing them genetically to target proteins of interest.
Introduction

A common bottleneck in protein X-ray crystallography is the ability to grow diffraction-quality crystals. Although high-throughput robotics now makes it possible to screen vast numbers of crystallization conditions, some proteins remain recalcitrant to crystallization. Many approaches have been developed to try to improve the success rate for crystallization, e.g. systematically truncating the target protein\textsuperscript{1,2}, methylating the lysine residues\textsuperscript{3,4}, removing post-translational modifications\textsuperscript{5-7}, screening homologues of the target protein for crystallization\textsuperscript{8,9}, fusing the target protein to a carrier protein\textsuperscript{10-13}, crystallizing racemic mixtures of the target protein\textsuperscript{14-20}, and co-crystallizing the target protein with antibodies or other binding proteins\textsuperscript{21-23}. A number of those methods have been reviewed\textsuperscript{24}.

Recently, rational mutagenesis of protein surface residues has been proposed to improve the crystallizability of target proteins, including methods referred to as 'surface entropy reduction' (SER)\textsuperscript{25} and 'synthetic symmetrization' (or 'crystal lattice engineering')\textsuperscript{26-28}. The SER method typically replaces solvent-exposed, flexible amino acids, such as Lys, Glu, and Gln, with less flexible residues, such as Ala. It has been successfully used for several protein targets\textsuperscript{29-31}, and a web-based server has been created to suggest suitable mutation sites in any given protein sequence\textsuperscript{30}. Synthetic symmetrization involves introducing into the surface of a protein specific motifs likely to drive symmetric self-association. Motivation for the approach derives in part from the observation that protein oligomers tend to crystallize in space groups that support the point group symmetry of the oligomer\textsuperscript{26}. In such cases, some of the contacts required to
establish a crystal lattice are essentially built in to the oligomer, meaning that fewer fortuitous contacts are required\textsuperscript{16}. The general strategy is also amenable to variation; different modes of engineered oligomerization lead to distinctly different opportunities for crystallization. One method already demonstrated for synthetically dimerizing a protein is to introduce a single cysteine on the surface and then form an intermolecular disulfide bond. That method was applied first to crystallize bacteriophage T4 lysozyme (T4L) in several new crystal forms\textsuperscript{26} and then to crystallize an enzyme of previously unknown structure\textsuperscript{27}. In a parallel strategy (alternately coined 'crystal lattice engineering'), a series of surface-exposed helix residues were mutated to leucine to promote dimerization by way of an intermolecular leucine zipper\textsuperscript{28}; a subsequent application of that approach produced crystals by way of heterotypic interactions between the engineered half-leucine zipper and a distinct surface region from another protein molecule, rather than by way of a symmetric self-association\textsuperscript{32}. Despite the successes demonstrated so far by the disulfide and leucine-zipper based approaches to synthetic symmetrization, they suffer from a limited type of symmetric association; both are designed to produce dimers.

It is known that metals play a very important role in oligomerization and crystallization of proteins\textsuperscript{33-35}. For example, in the high resolution crystal structure of truncated alphaA crystallin\textsuperscript{36}, a zinc binding motif is formed by three chains, and zinc promotes protein oligomerization and crystallization. The compound tetrathiomolybdate, used in the treatment of copper metabolism disorders, reacts with the copper binding metallochaperone, Atx1, forming a stable complex\textsuperscript{37}. The crystal structure of this
complex reveals a trimer of Atx1 molecules mediated through the bound tetrathiomolybdate molecule. Tezcan and co-workers have studied metal-directed protein self-assembly on the model protein, cytochrome b562, focusing primarily on the evolution of metal coordination in protein folds and complexes. Their work has shown that by introducing histidine mutations on the alpha helical surface of cytochrome b562, the protein can oligomerize to form dimers to tetramers, all of which are mediated through metal binding. Furthermore, the metals present within a protein crystal can provide the experimental phases needed for its structure determination. Whereas the incorporation of selenomethionine into proteins has become a standard method for exploiting anomalous scattering information, natural metal-binding sites containing copper, iron, nickel, cobalt, or zinc atoms have also been utilized for protein structure determination via anomalous dispersion (see refs. and references therein).

Here we propose a new crystallization methodology, termed metal-mediated synthetic symmetrization, which combines the idea of synthetic symmetrization with strategies – following work by Tezcan, et al. – for engineering metal binding sites at protein interfaces. This method has the potential to produce a variety of new crystal forms by introducing new contacts between protein molecules in the lattice through metal binding interactions, thereby increasing the chances of obtaining diffraction-quality crystals. We demonstrate the applicability of metal-mediated symmetrization using two proteins commonly used as fusion partners for crystallization trials: T4L and maltose binding protein (MBP). We discuss potential advantages of this method over current approaches, and ways to employ the method on proteins of unknown structure, through
either direct mutations on the target protein or fusion of the target protein to metal-site mutants of T4L or MBP, which could serve as crystallization chaperones.

Results

Rationale and design of mutations

Based on previous studies regarding natural or engineered metal binding sites, we rationalized that mutations of solvent-exposed residue pairs $i$ and $i+4$ of a helix$^{50-53}$ or $i$ and $i+3$ of a helix-loop$^{37}$ to a pair of either histidine or cysteine residues would induce homooligomeric assemblies upon incubation with metal. Specifically, we predicted the metal site within these assemblies to take on a tetra- or octahedral coordination, in which the mutated histidines or cysteines among the molecules participate in the metal coordination. Such coordination geometries are commonly found in proteins$^{33,54}$ and have been engineered previously$^{37-42}$. Based on the crystal structures of T4L and MBP, in each protein we chose three pairs of solvent-accessible residues that are located close to the ends of helices (Fig. 5-1). The introduction of metal binding sites near the ends of helices, compared to sites in the middle of helices, were expected to cause fewer steric clashes and allow greater coordination possibilities among molecules. In both T4L and MBP, the mutations were chosen to be distant from the C-terminus, to avoid potential interference with a fusion protein that might be attached. The determination of which residues are on the surface-exposed side of a helix was straightforward in our test experiments with T4L and MBP, whereas this represents a challenge when the structure is unknown. A recent application of disulfide-based synthetic symmetrization$^{27}$
illustrates the use of sequence alignments and bioinformatics tools to make reasonable choices for engineering residues on the sides of helices.

T4L and MBP mutants – most containing two mutations and one containing four mutations (summarized in Table 5-1) – were expressed, purified, and subjected to co-crystallization in the presence of either copper (Cu$^{2+}$), nickel (Ni$^{2+}$), or zinc (Zn$^{2+}$). The ratio of metal to protein was approximately 1.5 to 1. In some cases, the addition of metal induced slight precipitation of the protein-metal solution that could be reversed by the addition of EDTA. However, no additional purification step, besides filtration through a 0.22 µm membrane, was performed once the metal was added (see Methods for details). Crystallization was carried out using standard commercial crystallization screens (typically only three to four screens). For all of the mutant constructs, crystals formed in a variety of conditions within 1 week, and in some cases within 2 hours, of setting up crystal screens. All structures were solved from crystals mounted from preliminary screens without any further optimization (Supplementary Table 5-S3).

**T4L histidine mutants**

For T4L, we created two double-histidine mutants, T4L$_{76H/80H}$ and T4L$_{61H/65H}$, and one quadruple-histidine mutant, T4L$_{61H/65H/76H/80H}$, that emulates the engineering design by Tezcan, et al.$^{33}$ in which four histidine mutations are introduced as two pairs of proximal residues in a long helix. Crystallization of these mutants with the various metals resulted in eight crystal structures (Table 5-1), which represent six distinct crystal forms; in some mutants nickel and copper produced similar metal binding sites and
crystal packing arrangements. The crystal forms obtained were all different from those observed before for T4L. The molecular structures and crystal packing diagrams are shown in Figures 5-2 and 5-3, and crystallographic statistics are shown in Supplementary Table 5-S1.

Different combinations of mutations and metals led to various oligomeric forms of T4L. The protein can form either dimers (Fig. 5-2a-c, Fig. 5-2e) or trimers (Fig. 5-2d) through metal coordination, illustrating a greater range of oligomerization possibilities compared to other methods of symmetrization. Moreover, in one case a metal-mediated dimer further associated into a hexameric ring within the crystal asymmetric unit (Fig. 5-2e). With respect to metal site formation, the crystallization results were likewise variable and unpredictable. The same mutant was sometimes crystallized in different forms in the presence of different metals (Fig. 5-2a and Fig. 5-2e) or even in different forms with the same metal (Fig. 5-2b and d). Unexpectedly, in some structures one of the mutated histidines is not in coordination with the metal (Fig. 5-2b-d). Instead, either natural (wild type) surface residues or solvent molecules complete the coordination (Table 5-1). However, in all of the cases where this occurs, the neighboring free histidine is in a π stacking arrangement with the coordinating histidine (Fig. 5-2b-d), indicating it may be necessary for the coordination. Finally, not all the T4L molecules in the asymmetric unit are coordinated by the metals. In two cases, a non-coordinated molecule is present (Fig. 5-2c and d). The variations in metal site geometry and protein oligomerization enable the numerous new crystal forms observed here for T4L (Table 5-1 and Fig. 5-3).
MBP histidine mutants

To demonstrate that this methodology applies not only to T4L, we applied it to a second protein, MBP. We prepared three double-histidine mutants of MBP: MBP$_{216H/220H}$, MBP$_{26H/30H}$, and MBP$_{310H/314H}$ and crystallized them in the presence of metals (Table 5-1). The molecular structures and crystal packing diagrams of these mutants are shown in Figures 5-4 and 5-5, and crystallographic statistics are shown in Supplementary Table 5-S2. Similar to T4L histidine mutants, MBP mutants can either dimerize (Fig. 5-4a) or trimerize (Fig. 5-4e) upon addition of metal ions. In one case, a dinuclear zinc site is present (Fig. 5-4c). Notably for the MBP mutants, metal coordination can be involved in lattice contacts and drive the formation of metal-mediated polymers (Figs. 5-4b-d and 5-5). Depending on the crystallization conditions and type of metal, a single mutant construct MBP$_{216H/220H}$ can pack into three different forms (Fig. 5-4a-c). Interestingly, in all the structures, natural surface residues, usually Glu and Asp, participate in the coordination, and all molecules in the asymmetric unit are coordinated by the metals. The wild type MBP construct used as the starting point in this study crystallized exclusively in space group P6$_1$ in previous work. The structures of all the MBP mutants shown here are distinct from the P6$_1$ crystal form obtained with wild type MBP, and therefore represent new packing modes driven by metal coordination.
Considering that cysteine residues are also commonly found in coordination with metals – zinc finger proteins being a particularly well-studied case\textsuperscript{54,56} – we explored whether cysteine mutations could also be used for metal-mediated symmetrization. We generated two double-cysteine mutants of T4L: T4L\textsuperscript{76C/80C}, and T4L\textsuperscript{125C/128C} (Fig. 5-6 and Supplementary Table 5-S1) following the design of an $i$ and $i+3$ mutation at a helix-loop. The T4L\textsuperscript{125C/128C} mutant was inspired by the crystal structure of copper-binding Atx1 in complex with tetrathiomolybdate and copper, in which natural Cys15 and Cys18 of Atx1 participate in a complex metal binding network\textsuperscript{37}. Both mutants were purified in the presence of 1 mM dithiothreitol (DTT) to prevent cysteine oxidation, and crystallization was carried out in the presence of zinc. Interestingly, T4L\textsuperscript{125C/128C} crystallized as a trimer mediated by a novel multi-nuclear zinc sulfur cluster, which to our knowledge has not been reported previously (Fig. 5-6a and Supplementary Fig. 5-S1). Four zinc atoms form a tetrahedron with six sulfur atoms – two from each protein – coordinating from six edges, forming an adamantane-like cage. The zinc sulfur cluster is reminiscent of the copper (Cu$^{1+}$) sulfur cluster stabilized by Atx-1 and tetrathiomolybdate in anaerobic conditions\textsuperscript{37}. The second double cysteine mutant, T4L\textsuperscript{76C/80C}, crystallized in more than 60% of the crystallization conditions from an initial screen, and some crystals diffracted to ~1.5 Å without any further optimization. The structure of this mutant revealed a D$_2$ tetramer covalently linked by four disulfide bonds (Fig. 5-6b). The tetramer is constituted by two dimers that have similar structures to the copper-bound T4L\textsuperscript{76H/80H} (Fig. 5-2a). The disulfide bonds are all homotypic, with
the two Cys76-Cys76 bonds lying on one dyad axis of symmetry and the two Cys80-Cys80 bonds lying on one of the other dyad axes. Surprisingly, no metal ions were observed in the electron density, although zinc ions were added to the crystallization drops. For both double cysteine mutants of T4L, the crystal packing modes are novel (Fig. 5-7), although in the second case there was no mediation through zinc.

Discussion

In this study, we demonstrate a crystallization method that combines the concept of synthetic symmetrization with the approach of engineering metal binding sites. We generated several different double or quadruple histidine mutants of proteins T4L and MBP, which were chosen in part for their prior use as fusion proteins. Upon incubating these constructs with various metals (Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$), we obtained fourteen new crystal structures, representing eleven new and distinct crystal forms, showing various symmetries and packing modes mediated by the added metal ions (Table 5-1). Moreover, we crystallized two different double cysteine mutants of T4L with added zinc ions, and obtained two further distinct crystal forms exhibiting more complex packing arrangements, in one case based on direct disulfide bonding rather than metal-mediated interactions.

We obtained crystals with new symmetry and packing from all the mutation pairs we tested. Some mutants crystallized with great ease and variety. For example, MBP$^{216H/220H}$ and T4L$^{61H/65H/76H/80H}$ could be crystallized in many different forms, depending on the crystallization condition and the type of metal. Crystals having the
same unit cell dimensions and symmetry as the wild type protein (P321 for T4L and P61 for MBP) were also sometimes obtained, but at a relatively low rate (less than 1/3 of the time). Analysis of the crystallization conditions for all mutants (Supplementary Table 5-S3) shows that conditions that facilitated metal-mediated oligomeric assembly tended to either have high concentrations of salt or polyethylene glycol of various molecular weights.

The metal ions were incorporated into the overall crystal lattice in different ways in different crystals. In a number of structures, the metal fell on a crystallographic axis (Table 5-S1), suggesting that the metal-induced homooligomeric assembly helped drive lattice formation. This is especially apparent in the polymeric assemblies of MBP216H/220H and MBP26H/30H in the presence of zinc (Supplementary Fig. 5-S2), in which metal coordination generates helical arrangements of subunits compatible with crystal symmetry. Both types of situation support the notion that symmetrized oligomers may gain an advantage over asymmetric monomers in crystal formation. We note that in all the crystal structures obtained, despite the variety of protein oligomeric states induced, that generally the individual metal ions added were coordinated by two subunits. We also observed that in slightly more than half of the structures, only one of the two introduced histidines participated in the coordination. In those cases, natural surface residues, usually Glu or Asp, or solvent molecules were involved in metal binding instead. This suggests the possibility in future work of combining Glu and Asp with single or double His mutations for metal-mediated synthetic symmetrization.
The oligomeric protein arrangements observed by metal-mediated symmetrization were highly variable. It is likely that even greater diversity could have been realized through additional investigations. For several constructs, there were too many favorable leads from initial crystallization trials to permit a complete characterization of all the possible crystal forms. Furthermore, other experimental variables that might have produced additional crystal forms were not investigated. The relative concentration of metal ions was held fixed in our experiments, though this variable might be expected to influence the oligomeric modes of metal-mediated protein association.

Polymorphic assembly behavior was also observed under different crystallization conditions for some individual combinations of protein constructs and metals. This was evident in the multiple structures of T4L_{61H/65H/76H/80H} with copper, for example, in which slight variations in the crystallization conditions (Supplementary Table 5-S3) led to outcomes in which the geometry of the metal coordination by T4L molecules varied from square planar to tetrahedral (Table 5-1, Fig. 5-2b and 5-2d). In such cases, alternate metal-driven protein arrangements may be favored by slightly different solution conditions, or by different crystal packing arrangements. The likelihood that multiple distinct arrangements coexist in solution in some cases raises the possibility that heterogeneity could hinder crystallization. However, the multitude of our successful crystallization results, including multiple distinct forms from some individual combinations of protein construct and metal ion, argues otherwise. The reversibility of metal coordination probably has a positive effect in this regard, making it possible for
favorable crystal packing arrangements to drive otherwise heterogeneous mixtures into specific, well-ordered crystal forms. Furthermore, the coordination geometries of the metal sites were often slightly distorted (Table 5-1), suggesting that flexibility in metal coordination could also help enable the formation of well-ordered crystal packing arrangements.

Another potential advantage of metal-mediated synthetic symmetrization is the phasing power introduced by metals. The metal sites are well defined in most of our structures (Supplementary Fig. 5-S2) and anomalous signals were observed for most of them, despite that the data were not collected using an X-ray wavelength close to the absorption edge for the metals introduced (Supplementary Fig. 5-S1). For the T4L$^{125C/128C}$ mutant, we confirmed that experimental phases could be obtained using the anomalous scattering from the zinc atoms (Fig. 5-S1). By using synchrotron radiation tuned to the optimal wavelength, we anticipate that it will be generally possible to obtain diffraction phases from crystals grown by the metal-mediated symmetrization approach.

Based on our findings, we propose that one could utilize the concept of metal-mediated synthetic symmetrization to crystallize more difficult protein targets that have eluded crystallization using traditional methodologies. Figure 8 summarizes the “rational mutagenesis of surface residues” methods that have been developed to facilitate protein crystallization. In contrast to previously proposed methods, our approach allows the formation of oligomers in diverse arrangements and symmetries, giving rise to greatly expanded opportunities to grow diffraction-quality crystals. The flexibility of our
approach also allows it to be potentially combined with methods such as surface entropy reduction in which high-entropy surface residues can be mutated.

We propose two variations by which the approach could be used to promote the crystallization of new target proteins: (i) crystallizing fusion constructs in which a target protein is fused to the various engineered forms of T4L or MBP described here, or (ii) directly crystallizing a target protein after mutating pairs of surface residues to histidine or cysteine. The first approach is based on the observation that both T4L and MBP have been successfully used to crystallize otherwise difficult proteins, including membrane proteins and amyloid proteins\textsuperscript{10,57-61}. The mutation pairs characterized in this study could be readily used to increase the chance of getting crystals. The crystal packing arrangements of such fusion proteins would depend on surface properties of the target protein, and would therefore likely be different from the structures presented here. In the second, direct approach, positions for making mutation pairs in a target protein would be chosen based on predictions of secondary structure and surface exposure, or based on a homology model, when available. Double histidine or cysteine mutations are preferably introduced close to the ends of a helix (Fig. 5-1). For long helices, two histidine pairs can be introduced at both ends. The spacing between the two histidines are preferably $i$ and $i+4$, and for cysteines either $i$ and $i+3$ or $i$ and $i+4$. Theoretically, histidine mutations should have a broader application since they will not interfere with native cysteines. While additional purification of oligomeric species after metal addition was not necessary for success in our studies, this step might be useful in confirming metal-mediated assembly and optimizing the chances of success with more difficult protein targets.
Considering that it is easy to introduce double mutations, and all the crystals in this study were solved directly from robotic screens without any optimization, one could apply the approach relatively easily to generate and test several varied constructs for a given protein of interest. Such an approach could prove valuable for crystallizing asymmetric proteins or protein complexes that have eluded traditional crystallization methodologies.

**Methods**

*T4 Lysozyme Plasmid Construction.* Cysteine-less T4 Lysozyme (T4L) (a kind gift from Mark Fleissner and Wayne Hubbell at UCLA), residues 1-162, was PCR amplified with Platinum Taq Polymerase (Invitrogen, Carlsbad, CA). The N-terminal primers contained a six base pair overhang, NdeI restriction site, TEV protease cleavage site, and a short linker of residues GP to aid TEV protease cleavage. The C-terminal primer contained a stop codon, XhoI restriction site, and a three base pair overhang. The PCR product was agarose gel purified and extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR product and pET28b (Novagen, Gibbstown, NJ) were digested with NdeI and XhoI according to manufacturer protocol (New England Biolabs, Ipswich, MA). Digested pET28b and T4L products were gel purified and extracted (as described above). DNA concentrations were determined using BioPhotometer UV/VIS Photometer (Eppendorf, Westbury, NY). The vector pET28 and T4L were ligated using the Quick Ligation Kit (New England Biolabs, Ipswich, MA) according to manufacturer protocol, and transformed into *E. coli* cell line TOP10 (Invitrogen, Carlsbad, CA).
colony was grown overnight, and the pET28-TEV-T4L plasmid was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA).

_T4 Lysozyme and Maltose-Binding Protein Mutants._ All mutations in the DNA sequence were performed on the pET28-TEV-T4L or the pMal-a1 (a kind gift from Prof. Cynthia Wolberger at Johns Hopkins University) plasmids using a Site-Directed Mutagenesis kit (QuickChange II XL, Agilent, Santa Clara, CA) with site-directed primers designed using manufacturers QuickChange Primer Design Program available online (Agilent, Santa Clara, CA), according to the manufacturer’s protocol. The final constructs were sequenced prior to transformation into _E. coli_ expression cell line BL21 (DE3) gold cells (Novagen, Gibbstown, NJ).

_Protein Expression._ A single colony was inoculated into LB Miller Broth (Fisher BioReagents, Fisher Scientific, Pittsburgh, PA) supplemented with 30 µg/mL Kanamycin (Fisher Scientific, Pittsburgh, PA) (LB_Kan) or 100 µg/mL Ampicillin (Fisher Scientific, Pittsburgh, PA) for T4L and MBP mutants, respectively. One liter of LB_Kan or LB_Amp in a 2 L shaker flask was inoculated with 7 mL of overnight culture and grown at 37 °C until the culture reached an OD_{600} 0.6-0.8. For T4L mutants, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 0.5 mM, and grown for one and a half hours at 37 °C. For MBP mutants, IPTG was added to a final concentration of 1 mM, and grown for four hours at 37 °C. Cells were harvested by centrifugation at 5,000
x g for 10 minutes at 4 °C. The cell pellet was frozen and stored at -80 °C prior to purification.

**T4L Protein Purification.** The cell pellet was thawed and resuspended in buffer A (50 mM sodium phosphate, 0.3 M sodium chloride, 20 mM imidazole, pH 8.0) supplemented with Halt Protease Inhibitor Cocktail (Peirce, Thermo Fisher Scientific, Rockford, IL) and 1 mM DTT for cysteine containing mutants at 25 mL per 1 L of culture volume. The resuspended culture was sonicated and centrifuged at 12,000 x g for 25 minutes at 4 °C. The clarified lysate was filtered through a 0.45 μm syringe filtration device (HPF Millex-HV, catalog no. SLHVM25NS, Millipore, Billerica, MA) before loading onto a 5mL HisTrap-HP column (GE Healthcare, Piscataway, NJ). The HisTrap-HP column was washed with five column volumes of NHA buffer and protein eluted with linear gradient to 100% in four column volumes of NHB buffer (50 mM sodium phosphate, 0.3 M sodium chloride, 500 mM imidazole, pH 8.0). For cysteine containing mutants, buffer A and B were supplemented with 5 mM beta-mercaptoethanol (BME). Protein eluted around 40-60% buffer B and peak fractions were pooled. TEV protease, produced and purified as described\textsuperscript{36}, was added at a volume of 1/100\textsuperscript{th} the pooled volume, and ethylenediaminetetraacetic acid (EDTA) was added to final concentration of 1 mM. After 20-30 minutes, pooled protein was transferred to a Slide-A-Lyzer 10,000 MWCO dialysis cassette (Pierce, Thermo Fisher Scientific, Rockford, IL), and dialyzed against buffer C (20 mM TRIS pH 8.0, 200 mM sodium chloride, 20 mM imidazole, 1 mM DTT) at room temperature overnight. The dialyzed protein fraction was transferred
to a 50 mL conical falcon tube (Fisher Scientific, Pittsburgh, PA). After two days of TEV protease cleavage, cut protein was passed over a 5 mL HisTrap HP column pre-equilibrated in buffer A and the flow-through containing His-tag removed T4L was collected and concentrated prior to loading onto a Superdex Prep Grade 75 gel filtration column equilibrated in GF buffer (100 mM sodium chloride, 1 mM DTT, 20 mM TRIS pH 8.0). Peak fractions were pooled and concentrated. Protein concentration was determined by UV absorbance at 280 nm with extinction coefficient of 24750 M$^{-1}$ cm$^{-1}$ of protein.

*MBP Protein Purification.* Cells were resuspended in a lysis buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA. Phenylmethylsulfonyl fluoride (PMSF) was added to the cell resuspension to a final concentration of 1 mM. Clarified cell lysate, as described above, was loaded onto a self-packed amylose column (150 mL column volume, resin from New England Biolabs, Ipswich, MA). The column was first washed with Buffer A (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) for one column volume and then eluted with Buffer B (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM maltose). Peak fractions were pooled and concentrated using an Amicon Ultra-15 concentrator (30 kDa MW cutoff; Millipore, Billerica, MA) prior to loading onto a Superdex S-200 column (GE Healthcare, Piscataway, NJ) equilibrated in SEC buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl and 5 mM maltose). Peak fractions were pooled and concentrated. Protein concentration was determined by absorbance at 280 nm with the calculated extinction coefficient of 67800 M$^{-1}$cm$^{-1}$. 

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**Protein Crystallization.** Concentrated T4L and MBP protein mutants were diluted to ~1 mM and supplemented with 1.25-1.5 mM of metal: copper sulfate, nickel sulfate, or zinc acetate. Protein solutions containing metals were filtered through a 0.22 µm Ultrafree-MC centrifugal filter device (Amicon, Bedford, MA) prior to crystallization experiments in hanging drop plates. Crystallization experiments were carried out at the UCLA crystallization core facility (http://www.doe-mbi.ucla.edu/facilities/crystallization) and stored at 20 °C. The crystallization conditions are provided in Supplementary Table S5-3.

**Structure Determination.** All data were collected at 100 K at Advanced Photon Source (Chicago, IL) beam lines 24-ID-C and 24-ID-E, and in-house on a Rigaku Raxis-IV++ imaging plate detector using Cu Kα radiation from a Rigaku FRE+ rotating anode generator with confocal optics (Supplementary Table 5-S1). Single crystals were cryoprotected with glycerol and mounted with CrystalCap HT Cryoloops (Hampton Research, Aliso Viejo, CA). Crystals were flash-cooled in liquid nitrogen prior to data collection. All data were processed using DENZO/SCALEPACK\textsuperscript{62} or XDS/XSCALE\textsuperscript{63}. Initial phases of T4L and MBP mutants were calculated by molecular replacement using structures with PDB codes 3LZM and 1ANF, respectively, as search models using PHASER\textsuperscript{64}. Model building was done using COOT\textsuperscript{65}. All model refinement was done using REFMAC\textsuperscript{66}, PHENIX\textsuperscript{67} and BUSTER\textsuperscript{68}.
Figure 5-1

Figure 5-1. Surface residues of T4 lysozyme (T4L) and maltose-binding protein (MBP) selected for mutations. (a) T4L is shown as a cartoon representation (blue). Three pairs of residues are labeled (red, yellow and green), each corresponding to one double-residue mutant: D61/K65 to histidines, R125/E128 to cysteines, R76/R80 to either histidines or cysteines in two different constructs. A quadruple histidine mutant for D61/K65/R76/R80 was also made. (b) MBP is shown as a cartoon representation (orange). Three pairs of residues are labeled (green, blue and purple), each corresponding to one double histidine mutant. All selected pairs are three or four residues apart and are close to the ends of helices.
Figure 5-2. Crystal structures of metal-mediated symmetrization of T4 Lysozyme (T4L) histidine mutants. For each panel, a close-up view of the metal binding site is shown as an inset with coordinating residues labeled. Copper and zinc atoms are shown as bronze and grey spheres, respectively. Coordination to metal atoms is shown as dashed yellow lines. (a) The mutated histidine residues of T4L76H/80H form a 2-fold NCS (non-crystallographic symmetry) dimer mediated through the bound copper atom. T4L76H/80H has similar packing and interactions when co-crystallized with nickel (data not shown). (b) The quadruple histidine mutant, T4L61H/65H/76H/80H, forms a dimer through two copper atoms. Three mutated histidine residues, H61/H65/H76, a natural aspartate, D72, and two water molecules (red spheres) complete each copper binding site. (c) The double histidine mutant, T4L61H/65H, forms a dimer (purple and green) through two copper atoms. This dimer is accompanied by a non-copper bound molecule (blue) packed within one asymmetric unit. Each copper site is formed by one mutated histidine residue, H65, a natural glutamine, Q69, and completed by N-terminal glycine residues derived from the tobacco etch virus (TEV) protease cleavage site. (d) The four histidine mutant, T4L61H/65H/76H/80H, also forms a trimer. The copper binding site is formed by the mutated histidine, H61, located on a three-fold crystallographic axis. Three other protein molecules in the asymmetric unit, not bound to copper ions, are also shown (light yellow, light orange and light red). (e) The two histidine mutant, T4L76H/80H, also forms a hexameric ring structure mediated by three zinc atoms. The zinc binding sites are formed by two mutated histidine residues, H76/H80, from two neighboring molecules.
Figure 5-3. Crystal packing of T4L histidine mutants. (a-e) The crystal packings of five T4L histidine mutants are shown. The annotation below each image indicates the construct, the co-crystallizing metal and the space group. The metal binding sites are highlighted by red dots. Different chains in the asymmetric unit are colored differently. Molecules related by crystallographic symmetry are shown in the same color. The packing arrangements are shown projected along one of the unit cell edges as denoted by a coordinate system at the lower left corner of each image. A projection of the unit cell is also shown by a black quadrilateral in each image.
Figure 5-4. Crystal structures of metal-mediated symmetrization of maltose-binding protein (MBP) histidine mutants. The insets show close-up views of the metal binding sites. Metal atoms are colored the same as described in Fig. 5-1. Water and chloride ions are shown as blue and green spheres, respectively. (a-c) A double histidine mutant, MBP^{216H/220H}, forms a variety of metal mediated interactions. (a) A copper mediated dimer is formed through the mutated histidine, H216, the natural histidine, H40, and glutamate, E222, residues. (b) A polymeric assembly is created through crystal lattice contacts mediated by zinc atoms. The zinc binding site is formed by the mutated histidine, H216, and the natural glutamate residues, E39/E222. (c) Crystal lattice contacts are produced through zinc binding. Two zinc atoms are bound by the two mutated histidines, H216/H220, the two natural glutamates, E222/E310, and also by acetate ions (orange). The MBP^{216H/220H} mutant protein displays the versatility of metal binding through use of different metals. (d) Crystal lattice contacts are mediated through zinc atoms for the double histidine mutant, MBP^{26H/30H}. The two mutated histidines, H26/H30, and the natural aspartate, D165, form the zinc binding site. (e) The double histidine mutant, MBP^{310H/314H}, forms a trimer assembled through three zinc atoms. Two natural glutamates, E292/E289, and the mutated histidines, H310/H314, complete each zinc binding site.
Figure 5-5

(a-e) Crystal packings of five MBP histidine mutants are shown. The annotation below each image indicates the construct, the co-crystallizing metal and the space group. The metal binding sites are highlighted by red dots. Different chains in the asymmetric unit are colored differently in panels a, d and e. Molecules related by crystallographic symmetry are shown in the same color. The packing arrangements are shown projected along one of the unit cell edges as denoted by a coordinate system at lower left corner of each image. A projection of the unit cell is also shown by a black quadrilateral in each image.
Figure 5-6. Crystal structures of metal-mediated symmetrization of T4 Lysozyme (T4L) double cysteine mutants. (a) The double cysteine mutant, T4L$^{125C/128C}$, forms a trimeric complex assembled through a zinc cluster. Three pairs of cysteines from neighboring molecules coordinate four zinc atoms in an adamantane-like structure stabilized by four chloride ions (shown in the inset). (b) A covalent tetramer is formed by the double cysteine mutant, T4L$^{76C/80C}$, through disulfide bonds. Shown are four molecules related by a 4-fold crystallographic symmetry axis. Four disulfide bonds between the mutated cysteines, C76 and C80, covalently link all the molecules into a ring. The disulfide bonds are highlighted in the inset.
Figure 5-7. Crystal packing of T4L cysteine mutants.  

(a and b) Crystal packings of two T4L cysteine mutants are shown. The annotation below each image indicates the construct, the co-crystallizing metal and the space group. The metal binding sites are highlighted by red dots. Note that the packing of T4L$^{76C/80C}$ is mediated by disulfide bonds instead of metals. Different chains in the asymmetric unit are colored differently in panel a. Molecules related by crystallographic symmetry are shown in the same color. The packing arrangements are shown projected along one of the unit cell edges as denoted by a coordinate system at lower left corner of each image. A projection of the unit cell is also shown by a black quadrilateral in each image.
Figure 5-8

Figure 5-8. A scheme summarizing approaches for crystallizing protein targets by rational mutagenesis of surface residues. A protein (red box) can be mutated according to the idea of 'surface entropy reduction'\textsuperscript{30} (orange box), which typically involves replacing long flexible amino acid side chains by alanine. Alternatively, or in combination, surface residues can be modified in a way that specifically promotes symmetric oligomerization. This general idea is referred to as 'synthetic symmetrization'\textsuperscript{26}; a closely related idea has been called 'crystal lattice engineering'\textsuperscript{28}. Single cysteine mutations have been used successfully for dimerization\textsuperscript{26,27} (green box). A method based on inserting multiple leucine residues in a surface helix has been used successfully by others (blue box)\textsuperscript{28,32}. In this work we propose metal-mediated synthetic symmetrization (purple boxes), which involves introducing either double histidine mutations (lower right box) or double cysteine mutations (lower left box), followed by the addition of metal ions. The metal-mediated approach leads to a rich variety of oligomeric arrangements and crystal packing opportunities.
Table 5-1. Characteristics of metal interactions for various T4L and MBP mutants and their crystals.

<table>
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<tr>
<th>Type of Metal</th>
<th>Group</th>
<th>Space Group</th>
<th>Residues involved in oligomers</th>
<th>Corresponding Geometry of Metal</th>
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<tbody>
<tr>
<td>Cu</td>
<td>1+</td>
<td>R-3</td>
<td>Cu2+Cu2+Cu2+</td>
<td>Tetrahedral</td>
</tr>
<tr>
<td>Cu</td>
<td>1+</td>
<td>P63/mmc</td>
<td>Cu2+Cu2+Cu2+</td>
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<tr>
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<td>I432</td>
<td>Cu2+Cu2+Cu2+</td>
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<tr>
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<td>P213</td>
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</tr>
<tr>
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<td>Cu2+Cu2+Cu2+</td>
<td>Tetrahedral</td>
</tr>
</tbody>
</table>

Legend:
- For each mutant, the metal ion involved in either crystallographic or local contact.
- Highest symmetry element mediated by metal coordination.
- N/A = Not applicable, same structure in the asymmetric unit.
- A. Residues in pairs are natural amino acids that participate in metal coordination.
Figure 5-S1. Stereo views of the zinc-sulfur cluster in T4L$^{125C/128C}$. **(a)** A stereo view of the zinc-sulfur cluster. Three crystallographically related copies of T4L$^{125C/128C}$ are colored in green, blue and purple. Zinc and chloride atoms are shown as grey and green dots. A 2Fo-Fc $\sigma_A$ weighted electron density map is shown in blue, contoured at 1.3 $\sigma$. A difference anomalous map is shown in magenta at 8.0 $\sigma$. Note that the diffraction data was collected at 0.9795 Å, where the anomalous scattering contributions of zinc and sulfur atoms are about 2.5 and 0.2 electrons, respectively. **(b)** The same stereo view as in (a), showing a composite simulated annealing omit map (green), contoured at 1.3 $\sigma$ only around the zinc sulfur cluster. The map was calculated by omitting the entire zinc sulfur cluster (four zinc atoms, four chloride atoms and six sulfur atoms).
Figure 5-S2

Representative electron density of the metal binding sites. 2Fo-Fc \( \sigma_A \) weighted electron density maps are shown in blue at 1.3 \( \sigma \). Composite simulated annealing omit maps are shown in green at 1.3 \( \sigma \) only around the metal atoms. The omit maps were calculated by omitting the metals. (a) Electron density maps of T4L \( \text{His}^{61/65/76/80} \) crystallized in space group \( P2_12_12_1 \) with copper. A close-up view of the metal binding site is shown as a stereo view on the right. (b) Electron density maps of MBP \( \text{His}^{310/314} \) crystallized in space group \( P2_12_12_1 \) with zinc. A close-up view of the metal binding site is shown as a stereo view on the left.
Table 5-S1. Statistics of X-ray data collection and refinement for T4L mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>PDB accession code</th>
<th>Bond angle (°)</th>
<th>Bond length (Å)</th>
<th>Overall Factors</th>
<th>Non-protein atoms</th>
<th>Overall R-factor (Rw) (%)</th>
<th>Resolution (Å)</th>
<th>Molecules in unit cell</th>
<th>Space group</th>
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<td>0.93</td>
<td>2699</td>
<td>50</td>
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<td>19.7-12.45</td>
<td>99</td>
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<td>2538</td>
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<td>65</td>
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</tr>
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<td>3914</td>
<td>25</td>
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<td>19.7-12.75</td>
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Note: Values in parentheses correspond to the resolution limits.
Table 5-S2. Statistics of X-ray data collection and refinement for MBP mutants.

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<th>MBP\textsuperscript{216H/220H} -Zn</th>
<th>MBP\textsuperscript{310H/314H} -Zn</th>
<th>MBP\textsuperscript{310H/314H} -Zn</th>
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<td>P\textsubscript{2}\textsubscript{1}</td>
<td>P\textsubscript{2}\textsubscript{1}</td>
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<td>113.8, 115.7, 119.5</td>
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</table>

\textsuperscript{a} A.U. = Asymmetric Unit

\textsuperscript{b} Values in parentheses correspond to the highest resolution shell.

\textsuperscript{c} \(R_{\text{merge}} = \left(\sum |I-I'|-<\!|I-I'\!>|/\sum |I|\right)\)

\textsuperscript{d} \(R_{\text{work}} = \left(\sum |F_o-F_c|-<\!|F_o-F_c\!>|/\sum |F_o|\right)\)

\textsuperscript{e} \(R_{\text{free}}=\left(\sum |F_o-F_c|-<\!|F_o-F_c\!>|/\sum |F_o|\right)\), calculated using a random set containing 5% reflections that were not included throughout structure refinement.
Table 5-S3. Crystallization conditions for all the T4L and MBP mutants.

<table>
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<th>Protein</th>
<th>Conditions</th>
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<td>3.5 M NaCOOH pH 7.0</td>
</tr>
<tr>
<td>T4L^{61H/65H/76H/80H}-Cu (P2_{1}2_{1}2_{1})</td>
<td>0.2 M NH_{4}NO_{3}, 20% (w/v) PEG 3350</td>
</tr>
<tr>
<td>T4L^{61H/65H/76H/80H}-Cu (H3)</td>
<td>0.2 M KSCN, 25% (w/v) PEG 3350</td>
</tr>
<tr>
<td>T4L^{61H/65H}-Cu</td>
<td>3.5 M NaCOOH pH 7.0</td>
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<tr>
<td>T4L^{76H/80H}-Zn</td>
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</tr>
<tr>
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<td>0.2 M MgCl_{2}, 0.1 M Tris pH 8.5, 20% (w/v) PEG 8000</td>
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References


crystal structure of a basic "blue" copper protein from cucumbers. Science 241:806-811.


Chapter 6: Current Work

Chapter overview: This chapter represents my current work and future directions as I conclude my graduate studies.

Abstract
Recent studies of toxic amyloidogenic segments by X-ray crystallography have revealed a novel packing motif, consisting of out-of-register beta-sheets, that may play a role in disease pathogenesis. Here, we determined five new crystal structures of amyloidogenic segments from islet amyloid polypeptide, p63, and p73, in which three of these structures feature out-of-register fibril packing. Analysis of these structures reveals some notable differences in comparison to previously solved out-of-register fibrils; in particular they do not contain a weak interface of interstrand hydrogen bonds. Cell viability assays confirmed that fibrils formed from these structures exhibit only slight toxicity. The results suggest the importance of weak hydrogen bond interfaces in conferring toxicity for out-of-register fibrils.
Introduction

Recent studies have begun to unravel some of the structural details of the toxic entities that may play a role in the etiology of amyloid-related diseases\(^1\)\(^-\)\(^7\). In particular, the crystal structure of an 11-residue segment from alpha-B crystallin, termed cylindrin, offers the first atomic-resolution view of a toxic amyloidogenic peptide segment in its oligomeric state\(^8\). Since then, structural studies of amyloid \(\beta\)-sheet mimics (BAMs) and a hexameric segment from \(\beta\)2-microglobulin (\(\beta\)2m) have provided additional atomic-resolution models of toxic amyloid oligomers and fibrils\(^9\)\(^-\)\(^11\). Notably, two characteristics are shared in all of these structures. First, each structure is formed from out-of-register \(\beta\)-strands. This is in contrast to the classic in-register \(\beta\)-sheet packing seen in most studies\(^12\)\(^-\)\(^14\). Second, two interfaces of interstrand hydrogen bond networks are present: (i) a strong interface in which the hydrogen bond donors and acceptors within the peptide backbone are satisfied; and (ii) a weak interface that leaves unsatisfied donors and acceptors. Such segments were shown to be more toxic than segments that form classic in-register fibril structures; however the mechanism of their toxicity remains unknown\(^8\)\(^,\)\(^9\).

In this chapter, we present five new crystal structures of various amyloidogenic segments from proteins IAPP, p63, and p73. Interestingly, three of these segments formed out-of-register steric zipper structures. Analysis of these structures shows that, unlike the previously solved toxic segments that formed out-of-register beta-sheet structures\(^8\)\(^,\)\(^9\), the hydrogen bond network between each strand and its two neighboring strands is the same. Cell viability assays showed that fibrils formed from these structures exhibit only slight toxicity. While additional studies need to be performed on these
segments, the results presented here suggest the importance of a weak interface of interstrand hydrogen bonds in conferring toxicity for out-of-register fibrils, as we hypothesize that these weak interfaces allow fast conversion to toxic out-of-register oligomers.

Results

Additional steric zipper structures from IAPP

In chapter 1, we described the molecular basis for fibril polymorphism in Islet Amyloid Polypeptide (IAPP), whose amyloid deposits are implicated in Type II diabetes\textsuperscript{15}. We showed that full-length IAPP is capable of forming at least two different fibril morphologies that originate from distinct regions within the sequence. In addition, we solved crystal structures of six segments within residues 14-37 from IAPP, suggesting the large variety of steric-zipper spines that can form from the full-length sequence\textsuperscript{15} (Fig. 6-1). Here, we expand on the previous work, elucidating the atomic details of three more IAPP segments derived from its amyloidogenic regions, bringing the total number of polymorphs of IAPP amyloid fibrils to at least ten\textsuperscript{15}. Data collection and refinement statistics can be found in Table 6-1, and steric zipper statistics in Tables 6-3 and 6-4.

Two segments, located in the central region of the IAPP, crystallize as in-register steric zippers. The segment ANFLVH (residues 13-18) forms beta strands that are arranged as parallel, in-register beta sheets, with a dry steric zipper interface displaying a face-to-back orientation of the pair of sheets (Fig 6-1b). This is a class 2 steric zipper\textsuperscript{13}. The fibril core consists of hydrophobic interactions involving F15 and V17 of one sheet
interdigitating with L16 of the adjacent sheet. Both the strands and the sheets pack in a parallel orientation, with Phe and His residues stacking on one another along the sheets, adding to the stability of the fiber (Fig. 6-1b and c). The hexameric segment LVHSSN (residues 16-21) also forms an in-register steric zipper (Figure 6-1d and e). However, in this structure the strands stack in staggered, anti-parallel orientation, while the sheets are oriented parallel. Thus, the segment forms a class 7 zipper13.

Crystal structure of IAPP22-28 NFGAILS reveals an out-of-register steric zipper

We identified a third segment from IAPP, located in the very amyloidogenic C-terminal region, that interestingly forms a novel out-of-register steric zipper (Fig. 6-2). The segment NFGAILS (residues 22-28) forms anti-parallel beta strands arranged into parallel sheets, forming a class 7 steric zipper (Table 6-4). The glycine and alanine residues in the center of the segment allow space for the larger phenylalanine, leucine, and isoleucine residues forming the dry, highly complementary steric zipper interface (Fig. 6-2a).

Each strand within each sheet of NFGAILS is out of register by two residues (Fig. 6-2b), similar to a previously determined protofilament structure from β2m, KDWSFY (residues 58-63)⁹. Similar to the KDWSFY structure, the β-strands in the NFGAILS structure are not perpendicular to the fibril axis as seen for in-register steric zippers¹³; instead, each strand is at an angle of 40° from the perpendicular (Fig. 6-2b and Table 6-4). However, in contrast to β2m structure⁹, the beta sheets have no crossing angle since they run parallel to each other (Fig. 6-2b). This is the first out-of-register structure
determined in which the strands have no crossing angle. In addition, each antiparallel out-of-register $\beta$-sheet of NFGAILS contains the same number of hydrogen bonds within each interface, six to each of its neighboring strands (Fig. 6-2c). Previously determined out-of-register structures have revealed a weak and strong interface that alternate along each sheet; that is, one interface has contains more hydrogen bonds than the next interface$^{8,9}$. However, in the NFGAILS structure, there is no weak interface as the number of hydrogen bonds along each strand is the same.

Out-of-register steric zipper structures from p53 paralogs

In chapter 3, we determined the atomic details of the aggregating region of p53, showing that it is capable of forming in-register steric zippers and suggesting a mechanism by which mutant p53 aggregation results in loss of wild-type function. It was shown previously that mutant p53 can also recruit its paralogs p63 and p73 into amyloid fibrils, resulting in their loss of transcriptional activity$^{16,17}$. Because both p63 and p73 share a similar aggregation-prone sequence in their DNA-binding domains (Fig. 6-3a), we hypothesized that is the steric zipper interactions between these sequence segments that drive p53 aggregates to recruit wild-type p63 and p73. We thus set out to determine the atomic details of the aggregation-prone regions of the two paralogs.

Using the 3D-profile method$^{18,19}$ we threaded the sequences of p63 and p73 on to steric zipper profile to identify segments predicted to form steric zippers (Fig. 6-3b and c). Similar to region 251-258 of p53, region 321-328 of p63 and region 271-278 of p73 appear amyloidogenic. We examined various hexameric and heptameric segments within
each region and tested their ability to form fibers and crystals. The 7-residue segments that were studied formed fibers but failed to crystallize (data not shown). The 6-residue segments formed both fibers (Fig. 6-4a) and microcrystals (Fig. 6-4b); we successfully determined the crystal structure of each peptide using diffraction data collected from these crystals. Surprisingly, in contrast to the classic in-register steric zipper packing formed by the aggregating segment of p53 (253-TIITLE-258), both structures from p63 and p73 feature out-of-register fibril-like packing. Data collection and refinement statistics can be found in Table 6-2, and steric zipper statistics in Table 6-4.

Crystal structures of p63_{323-328} IIVTLE and p73_{273-278} IIITLE.

Segment 323-IIVTLE-328 from p63 and segment 273-IIITLE-278 from p73 form nearly identical steric zipper structures (Fig. 6-5 and 6-6). Both form anti-parallel, out-of-register β-sheets with a dry zipper interface in a face-to-face orientation\textsuperscript{13} (Fig 6-5a and 6-6a). Again, similar to previous out-of-register protofilament structures\textsuperscript{9}, the β-strands are not aligned perpendicular to the fibril axis, but are at an angle of 35° from the perpendicular (Fig. 6-2b and Table 6-4). Also, the β-strands from adjacent sheets form a crossing angle of 70°, instead of the 0° angle found in the NFGAILS structure and in-register steric zippers\textsuperscript{13} (Fig 6-5b and 6-6b). As a result, these structures contain very low buried area and shape complementarity (Table 6-4). Like the NFGAILS structure, each antiparallel out-of-register β-sheet from IIVTLE and IIITLE possesses six hydrogen bonds along each strand (Fig. 6-5c and 6-6c); therefore, these structures do not contain the weak interfaces found in other out-of-register fibrils\textsuperscript{9}.
Cell-viability assays suggest these segments are not as toxic as previously determined out-of-register protofilament structures

Previous studies showed that amyloidogenic segments that form out-of-register beta-sheet structures are cytotoxic to cultured cells\textsuperscript{8,9}. In addition, such segments tend to be more toxic than segments that form in-register fibril structures. We therefore examined the cytotoxicity of our out-of-register structures via MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based cell viability assays. NFGAILS (IAPP 22-28) fibrils prepared in water failed to show significant toxicity compared with in-register fibrils formed from other segments from IAPP\textsuperscript{15} (Fig. 6-7a). Since NFGAILS crystallized in the presence of salt (see materials and methods), we then attempted to mimic these conditions using PBS instead of water to prepare IAPP fibrils. In this solution, NFGAILS fibrils exhibited slight toxicity compared to in-register fibrils (Fig. 6-7b), though the result was not significant. We also compared the cytotoxicity of fibrils and monomers formed from homologous segments of p53, p63, and p73 (Fig. 6-8). Fibrils were prepared by dissolving the peptides in 10mM LiOH and incubating them at 37° for 18 days. While fibrils of p63 (IIVTLE) and p73 (IIITLE) induced slight toxicity compared to p53 fibrils (TIITLE) and monomers, this toxicity was neither significant nor dose-dependent (data not shown).
Discussion

The structures presented here suggest many amyloid proteins are capable of forming out-of-register fibrils, as we show a segment from the well-studied amyloid IAPP capable of forming an out-of-register steric zipper. This, in addition to the 2 in-register structures also determined, expands on previous work on IAPP segmental polymorphism, bringing the total number of possible steric zipper spines in IAPP to ten\textsuperscript{15}, and suggests that segments in IAPP may also be capable of forming out-of-register cyldrin-like oligomers\textsuperscript{8}. Furthermore, we show that segments from p63 and p73 are also able to form out-of-register zippers. The relevance of these structures in relation to the full-length proteins remains to be determined, as previous studies regarding aggregation of p63 and p73 involve co-aggregation with mutant p53. However, given these results, it appears that out-of-register packing of beta-sheets may be a common structural motif in amyloid proteins.

Previous work on out-of-register fibrils and oligomers described weak interfaces in out-of-register beta-sheet structures, which leave unsatisfied hydrogen bond donors and acceptors at the ends of the strands that are not engaged in interstrand hydrogen bonds (Fig. 6-9c)\textsuperscript{8,9}. Molecular dynamics (MD) simulations showed that the presence of these interfaces allows rapid interconversion between out-of-register fibrils and out-of-register oligomers, due to a small free energy difference between an out-of-register oligomeric state and an out-of-register fibrillar state\textsuperscript{9}. Thus, the relative instability of out-of-register fibrils makes them more prone to conversion to toxic, out-of-register cyldrin-like oligomers. Cell-viability assays confirmed that out-of-register fibrils that
form weak interfaces were indeed toxic\textsuperscript{9}. Interestingly, in the out-of-register structures presented here, instead of a weak interface containing two interstrand hydrogen bonds, each interface contains six interstrand hydrogen bonds; thus, the sheets are made up of only strong interfaces (Fig. 6-9a and b). Though we have not yet performed MD simulations for these fibrils, we hypothesize a significant energy change in the conversion of an out-of-register oligomer to this type of fibril, due to an absence of a weak interface. Therefore, conversion to toxic out-of-register oligomers is less likely to occur. Cell viability assays confirmed that the fibrils formed from the segments studied here exhibit little toxicity, despite the presence of high concentrations of the samples.

More information is required to understand the structural and pathological mechanisms involved in the formation of out-of-register oligomers and fibrils. Additional experiments, such as fibril diffraction, conformational antibody binding assays, histological-dye binding assays, and additional cell-viability studies still need to be performed on the segments studied here. However, the evidence currently presented suggest the importance of weak interstrand hydrogen bonds interfaces in conferring toxicity for out-of-register fibrils. As demonstrated here, we expect out-of-register fibrils that contain only strong interfaces to be nontoxic.

**Materials and Methods**

*Sample preparation and crystallization.* Peptides were synthesized at greater than 97% purity from CS. Bio (Menlo Park, CA) and Celtek Bioscience (Nashville, TN). All peptide solutions were filtered through a 0.1 µm Ultrafree-MC centrifugal filter device
(Amicon, Bedford, MA) prior to crystallization experiments at 18 °C via hanging-drop vapor diffusion.

**Crystallization conditions:**

**IAPP\textsubscript{13-18} 13-ANFLVH-18:** The segment was dissolved at 20 mg/ml in water and mixed with 10% (w/v) PEG-8000, 0.1 M Na/K phosphate pH 6.2, and 0.2 M NaCl.

**IAPP\textsubscript{16-21} 16-LVHSSN-21:** This segment was dissolved at 20 mg/ml in water and mixed with 0.09 M HEPES pH 7.5, 1.26M tri-sodium citrate, and 10% glycerol.

**IAPP\textsubscript{22-28} 22-NFGAILS-28:** This segment was dissolved at 7 mg/ml in water and mixed with 10% ethanol and 1.5M NaCl.

**p63\textsubscript{323-328} 323-IIVTLE-328:** The segment was dissolved at 6 mg/ml in 10mM lithium hydroxide (LiOH) 0.01M Cobalt (II) chloride 0.1M sodium acetate pH 4.6, and 1.0 M 1,6-Hexanediol.

**p73\textsubscript{273-278} 273-IIVTLE-278:** This segment was dissolved at 6 mg/ml in 10 mM LiOH and mixed 0.2M NaCl, 0.1M sodium acetate pH 4.6, 30% 2-methyl-2,4-pentadiol (MPD). Also present in this solution was a hexameric segment from p53, residues 253-258 (TIITLE), also dissolved at 6mg/ml in 10 mM LiOH. Upon structure determination, it was confirmed that this segment was not present in the crystals formed from this condition.

**Data collection and structure refinement.** Crystals of IAPP segments were mounted on 20-50 μm Mitegen LD (Ithaca, NY) loops in the presence of 20% glycerol and flash cooled in liquid nitrogen. Crystals of p63 and p73 segments were mounted on the ends of pulled glass capillaries in the presence of cryoprotectant. Data was collected at 100 K
using a microfocus beam (5x5 µm²) at beamline 24-ID-E of the Advanced Photon Source (APS) at Argonne National Laboratory. Data indexing, integration and scaling were performed using XDS/XSCALE and DENZO/SCALEPACK. The merged scaled data was imported into the CCP4 format with programs from the CCP4 program suite organized under the “CCP4i” interface. Molecular replacement solutions for the segments were obtained using the program PHASER, using a polyalanine beta-strand as the search model. Crystallographic refinements were performed with REFMAC, and PHENIX. Model building was performed with COOT and illustrated with PYMOL.

Transmission electron microscopy. p63 and p73 segments were dissolved in 10 mM LiOH at their respective crystallization concentrations. Segments were incubated at 37 degrees for seventeen days. The samples were fixed on carbon-coated EM grids (Ted Pella), and glow-discharged immediately before use. 5 uL samples were applied directly onto grids and allowed to adhere for 4 minutes. Grids were rinsed twice with 5 uL distilled water and negatively stained with 1% (wt/vol) uranyl acetate for 2 minutes. Specimens were examined in a FEI T12 electron microscope at an accelerating voltage of 120 kV. Images were recorded digitally by a Gatan 2K x 2K CCD.

Cell viability assays. The MTT assay was performed using the CellTiter 96 nonradioactive cell proliferation assay kit (Promega). The protocol follows that from a previous study of out-of-register fibrils (REF), using HeLa and HEK293 cells (CRL-1573; ATCC). Cells were maintained at 37 °C in 5%(vol/vol) CO₂, and were cultured in ATCC-formulated RPMI medium 1640 (30–2001; ATCC) with DMEM with 10% (vol/vol) FBS. For all cell viability experiments, 96-well plates (3596; Costar) were used.
Before reagent treatment, HeLa cells were plated out at 10,000 cells/well and cultured for 20 h at 37 °C in 5% (vol/vol) CO₂. For fibril sample preparation, all peptides were dissolved at a concentration of 5 mM in water or PBS for IAPP segments, or 10 mM LiOH for p63 and p73 segments, then incubated at 37 degrees with shaking for various periods of time. For the IAPP segments, incubation time was 7-14 days; for the p63 and p73 segments, incubation time was 17 days. Fibrils were confirmed by visualizing samples on EM grids. To start the assay, 10 μL sample were added to each well containing 90 μL medium; 15 μL Dye solution (G4000; Promega) was added into each well after 24 h of the incubation period followed by further incubation for 4 h at 37 °C in 5% (vol/vol) CO₂. Then, 100 μL solubilization Solution/Stop Mix (G4000; Promega) were added to each well. The absorbance was measured at 570 nm after 12 h incubation at room temperature to fully solubilize the dye molecules. The background absorbance was recorded at 700 nm. Four replicates were measured per sample. Abeta1-42 was used as a positive control at a final concentration of 0.5 μM. The results were normalized by using the buffer treated cell as 100% viability and cell treated with 0.2% SDS as 0% viability.
Table 6-1. Statistics of X-ray data collection and refinement for crystal structures of IAPP segments.

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<td>APS (24-ID-E)</td>
<td>APS (24-ID-E)</td>
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a. Values in parentheses correspond to the highest resolution shell.

b. \( R_{\text{merge}} = \frac{\sum |I - \langle I\rangle|}{\sum I} \)

c. \( R_{\text{work}} = \frac{\sum |F_o - F_c|}{\sum F_o} \)

d. \( R_{\text{free}} = \frac{\sum |F_o - F_c|}{\sum F_o} \), calculated using a random set containing 10% reflections that were not included throughout structure refinement.
Table 6-2. Statistics of X-ray data collection and refinement for out-of-register crystal structures of p53 homologous segments.

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<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20.0</td>
<td>20.9</td>
</tr>
<tr>
<td>No. atoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Ligand/ion</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Water</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Overall B-factors</td>
<td>13.6</td>
<td>15.6</td>
</tr>
<tr>
<td>R.m.s. deviation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond length (Å)</td>
<td>0.007</td>
<td>0.006</td>
</tr>
<tr>
<td>Bond angle (°)</td>
<td>1.23</td>
<td>0.98</td>
</tr>
</tbody>
</table>

a. Values in parentheses correspond to the highest resolution shell.
b. R<sub>merge</sub> = Σ |I - <I>| / Σ I.
c. R<sub>work</sub> = Σ |F<sub>o</sub> - F<sub>c</sub>| / Σ F<sub>o</sub>.
d. R<sub>free</sub> = Σ |F<sub>o</sub> - F<sub>c</sub>| / Σ F<sub>o</sub>, calculated using a random set containing 10% reflections that were not included throughout structure refinement.
Table 6-3. Features of the dry steric zipper interface of microcrystals derived from in-register fibril-forming segments of IAPP.

<table>
<thead>
<tr>
<th>Amyloid segment</th>
<th>Strand orientation</th>
<th>Steric zipper type</th>
<th>Area buried(^a) (Å(^2))</th>
<th>Shape complementarily(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-ANFLVH-18 (IAPP)</td>
<td>Parallel</td>
<td>Face-to-back In register β-sheets Symmetry class 2</td>
<td>258</td>
<td>0.80</td>
</tr>
<tr>
<td>16-LVHSSN-21 (IAPP)</td>
<td>Anti-parallel</td>
<td>Face=back Staggered β-sheets Symmetry class 7</td>
<td>160</td>
<td>0.50</td>
</tr>
</tbody>
</table>

c. Area buried was calculated using AREAIMOL\(^28\) with a probe radius of 1.4Å. The summation of the difference between the accessible surface areas of a) one β-strand alone and in contact with the opposite β-sheet, and of b) the β-sheet alone and in contact with the opposite β-strand, constitutes the reported area buried. In structures with anti-parallel β-strand orientation, as well as in parallel β-strand orientations with different conformations, the average area buried per β-strand is reported.
d. Lawrence and Colman’s shape complementarity index\(^29\).
Table 6-4. Features of the out-of-register steric zipper interfaces of microcrystals derived from various fibril-forming segments.

<table>
<thead>
<tr>
<th>Symmetry class</th>
<th>Number of register sheets</th>
<th>Free-fo-face</th>
<th>Anti-parallel</th>
<th>Anti-parallel</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D73)</td>
<td>35</td>
<td>166</td>
<td>70</td>
<td>0.25</td>
</tr>
<tr>
<td>(D65)</td>
<td>35</td>
<td>189</td>
<td>70</td>
<td>0.29</td>
</tr>
<tr>
<td>(1APD)</td>
<td>35</td>
<td>293</td>
<td>0</td>
<td>0.83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complementarity</th>
<th>Stereodependent type</th>
<th>Stereodivergent position</th>
<th>Stereoregion orientation</th>
<th>Stereoregion orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, A</td>
<td>A</td>
<td>Perpendicular</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>A, A</td>
<td>A</td>
<td>Perpendicular</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>A, A</td>
<td>A</td>
<td>Perpendicular</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>A, A</td>
<td>A</td>
<td>Perpendicular</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>
Figure 6-1. IAPP segments 13-ANFLVH-18 and 16-LVHSSN-21 form in-register steric zippers. (a) Sequence of human IAPP. Segments studied in this chapter are highlighted by a colored bar overlapping the segment. The color corresponds the predicted energy of that segment by the 3D profile method, shown previously. Segments colored from yellow to red have energies of −23 kcal mol$^{-1}$ or lower and thus are predicted to form amyloid-like fibrils containing a steric zipper interaction. (b and c) Crystal structure of segment ANFLVH. (b) View looking down the fibril axis reveals the steric zipper interface involving the phenylalanine, leucine, and valine residues. ANFLVH forms a parallel, class 2 steric zipper. (c) View perpendicular to the fibril axis reveals hydrogen bond network and stacking of aromatic residues Phe and His, which add to the stability of the fibril. (d) View looking down the fibril axis showing the steric zipper interface of segment LVHSSN. This peptide forms a class 7 steric zipper, in which the strands stack in an anti-parallel orientation, while the sheets pack parallel to each other. (e) View perpendicular to the fibril axis, showing the hydrogen bond network of LVHSSN.
Figure 6-2

Segment 22-NFGAILS-28 from IAPP forms an out-of-register steric zipper. (a) View down the fiber axis reveals the steric-zipper interface. NFGAILS forms a pair of anti-parallel beta sheets. (b) Cartoon representation parallel to the fiber axis shows the strands out of register with each other, resulting in the sheets arranged at an angle about 40° perpendicular to the fibril axis. This is different for in-register sheets, which run exactly perpendicular to the fibril axis. In addition, unlike previously solved out-of-register steric zippers, the sheets do not form a non-zero cross angle. (c) View of the hydrogen bond network between strands along a single sheet. Previously determined out-of-register β-sheet structures have revealed a weak and strong interface that run along the sheet; however, in this structure, all interfaces are strong by virtue of six hydrogen bonds formed between each strand instead of two.
Figure 6-3. Prediction of fiber-forming segments of the aggregating region of p63 and p73 by the 3D profile method. (a) Sequence alignment of p53, p63, and p73 shows a shared aggregation-prone region. Residues highlighted show the residue differences among the segments. (b and c) The Rosetta energy of steric zipper-like self-association was calculated by scanning through the sequence with a six-residue window. The profile for residues 321-328 of p63 (b) and residues 271-278 of p73 (c) is shown. Energies at or below -23 kcal/mol, indicated by a horizontal gray line, are predicted to form amyloid-like fibrils containing a steric zipper interaction\textsuperscript{16,17}. The calculated energies of segments are colored in rainbow from green to red for low to high predicted propensity for fiber formation. The segments studied in this chapter are highlighted in the arrows, the colors corresponding to the predicted energy score.
Figure 6-4. Fibrils and microcrystals of segment 323-IIVTLE-328 from p63 and segment 273-IIITLE-278 from p73. (a) Electron micrographs of IIVTLE and IIITLE after incubation at 37° for 24 hours. Both form typical amyloid-like fibrils as visualized under EM. (b) Microcrystals of both segments observed by light microscopy. These crystals were used for diffraction studies. The width of these crystals is about 5 µm.
Figure 6-5. 323-IIVTLE-328 from p63 forms an out-of-register steric zipper. (a) View down the fiber axis reveals the steric-zipper interface. IIVTLE forms a pair of anti-parallel beta sheets. (b) Cartoon representation parallel to the fiber axis showing each strand is two residues out of register with each other, resulting in the strands arranging at an angle about 35° perpendicular to the fibril axis. The strands along each sheet forms a 70° crossing angle with each other, similar to previously determined out of register fibrils. (c) View of the hydrogen bond network between strands along a single sheet. Previously determined structures have revealed a weak and strong interface that alternate along a sheet; however, in this structure, there is no weak interface as each pair of strands forms six main-chain hydrogen bonds.
Figure 6-6. 273-IIITLE-273 from p73 also forms an out-of-register steric zipper. Structure determination of this segment reveals almost identical packing as in segment IIVTLE from p63. (a) View down the fiber axis reveals the steric-zipper interface. IIITLE forms a pair of anti-parallel beta sheets. (b) Cartoon representation parallel to the fiber axis showing each strand is two residues out of register with each other, resulting in the strands arranging at an angle about 35 degrees perpendicular to the fibril axis. The strands along each sheet form a 70° crossing angle with each other. (c) View of the hydrogen bond network between strands along a single sheet, again showing no weak interface as the number of hydrogen bonds along each strand is six.
Figure 6-7. Comparison of cytotoxicity of an out-of-register fibril (NFGAILS) with in-register fibrils from IAPP. MTT-based experiments were performed on HeLa cells. (a) NFGAILS (in bold, residues 22-28) fibrils failed to show significant toxicity compared with in-register fibrils formed by NNFGAIL (residues 21-27), LVHSSN (residues 16-21), and SSTNVG (residues 28-33)\textsuperscript{12}. The concentrations of each sample is 500 μM. Fibril samples were prepared by dissolving them in water and incubating them at 37° for 7 d with shaking. (b) In conditions that mimicked crystallization conditions of NFGAILS, NFGAILS fibrils (in bold) show slight toxicity compared with in-register fibrils formed by NFLVHSS (residues 14-20) and SSTNVG (residues 27-32)\textsuperscript{12}. The concentration of each sample is 500 μM. Fibril samples were prepared by dissolving the peptides in PBS to mimic crystallization conditions that induced an out-of-register crystal structure, and incubating them at 37° for 14 d with shaking. Error bars represent 1 SD (n = 4).
Figure 6-8. Comparison of cytotoxicity of fibrils and monomers of homologous segments from p53, p63, and p73. MTT-cell viability assay was performed on HeLa cells in the presence of fibril and monomer samples from p53 (IIITLE, in grey), p63 (IIIVTLE, in green), and p73 (IIITLE, in blue). All concentrations are 600 μM (monomer equivalent). Fibrils of p63 and p73 induced slight toxicity compared with p53 fibrils. However, this toxicity was not dose-dependent (data not shown). Fibril samples were prepared by dissolving peptides in 10mM LiOH and incubating at 37° for 17 d with shaking. Freshly dissolved (monomer) samples did not show any toxicity. Error bars represent 1 SD (n = 4).
Figure 6-9. Comparison of the hydrogen bond network of various out-of-register fibrils. View of the hydrogen bond network between strands along a single sheet for (a) IIITLE (Fig. 6-6), (b) NFGAILS (Fig. 6-2), and (c) KDWSFY\(^9\) (residues 58-63 of \(\beta\)-2-microglobulin, PDB 4E0K). The structure of KDWSFY reveals a weak and strong interface that run along a sheet, in which the weak interface contains only two interstrand hydrogen bonds. However, in the out-of-register structures described in this chapter, each interface contains six interstrand hydrogen bonds; thus only strong interfaces are formed within a sheet. We hypothesize that this prevents equilibrium to shift towards out-of-register oligomers that are shown to be toxic\(^8,9\). Cell viability assays confirmed that fibrils formed from the structures determined in this chapter are not significantly toxic.
References


