A method for probing the mutational landscape of amyloid structure

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ABSTRACT

Motivation: Proteins of all kinds can self-assemble into highly ordered β-sheet aggregates known as amyloid fibrils, important both biologically and clinically. However, the specific molecular structure of a fibril can vary dramatically depending on sequence and environmental conditions, and mutations can drastically alter amyloid function and pathogenicity. Experimental structure determination has proven extremely difficult with only a handful of NMR-based models proposed, suggesting a need for computational methods.

Results: We present AmyloidMutants, a statistical mechanics approach for de novo prediction and analysis of wild-type and mutant amyloid structures. Based on the premise of protein mutational landscapes, AmyloidMutants energetically quantifies the effects of sequence mutation on fibril conformation and stability. Tested on non-mutant, full-length amyloid structures with known chemical shift data, AmyloidMutants offers roughly 2-fold improvement in prediction accuracy over existing tools. Moreover, AmyloidMutants is the only method to predict complete secondary structures, enabling accurate discrimination of topologically dissimilar amyloids conformations that correspond to the same sequence locations. Applied to mutant prediction, AmyloidMutants identifies a global conformational switch between a wild-type and highly-toxic ‘Iowa’ mutant in agreement with recent experimental work based on partial chemical shift data. Predictions on mutant, yeast-toxic strains of HET-s suggest similar alternate folds. When applied to HET-s and a HET-s mutant with core asparagines replaced by glutamines (both highly amyloidogenic chemically similar residues abundant in many amyloids), AmyloidMutants surprisingly predicts a greatly reduced capacity of the glutamine mutant to form amyloid. We confirm this finding by conducting mutagenesis experiments.

Availability: Our tool is publicly available on the web at http://amyloid.csail.mit.edu/.

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Supplementary information: Supplementary data are available at Bioinformatics online.

1 INTRODUCTION

Under optimum conditions, proteins with diverse primary sequence exhibit the ability to self-assemble into structurally varied, but highly ordered β-sheet aggregates known as amyloid fibrils (Dobson, 2003). Those forming amyloid under normal physiological conditions can have profound effects on biological systems— deleterious and beneficial. On the one hand, amyloids play a role in diseases such as Alzheimer’s, Parkinson’s and Huntington’s, as well as systemic amyloidosis. On the other, they serve vital functions in normal biology such as in human peptide hormone storage, biofilm formation and a mechanism of protein-only inheritance by yeast prions (Halfmann and Lindquist, 2010). However, the generic nature of the fold, the observation that most proteins do not form amyloid under normal conditions and the ability of many amyloids to adopt multiple amyloid structures from the same peptide sequence (structural strains) confounds standard sequence-specific models of protein folding (Ostapchenko et al., 2010). Moreover, sequences with only a small likelihood of forming amyloid can remain so given many mutations, or become abundantly amyloidogenic after only a single point change (Lie et al., 2004). Therefore, to better understand the sequence/structure relationship of amyloid fibrils, a meaningful predictive model is required that describes the relationship between a given sequence and its mutational neighborhood.

Countless experimental studies have been performed to probe the molecular mechanism of these enigmatic structures. However, most methods (developed primarily for globular proteins) are difficult to apply to amyloids due to their large size and insolubility. Techniques such as solid-state nuclear magnetic resonance (NMR) spectroscopy and hydrogen-deuterium exchange (H/D-exchange) have brought us the most information about fibril structure, but only through exhaustive work and complex experimental design (Luca et al., 2007; Lührs et al., 2005; Mukrasch et al., 2009; Vilar et al., 2007; Wasm et al., 2008). The high cost of such studies has prevented the kinds of large-scale investigations that can reveal the underlying sequence/structure relationships in functional and pathogenic amyloid fibrils.

Seminal work has shown that computational prediction of sequence amyloidogenicity can help guide and speed investigations of amyloid structure (Alberti et al., 2009; Bryan et al., 2009; Fernandez-Escamilla et al., 2004; Tartaglia and Vendruscolo et al., 2008; Trovato et al., 2007). These advances enabled new possibilities for genome-wide studies, such as the discovery of 19 new functioning amyloid proteins in yeast (Alberti et al., 2009). More specialized tools (Maurer-Stroh et al., 2010; Thompson et al., 2006) have been further developed that detail the structure
of one particular amyloid fibril conformation: ‘steric zippers’, a repeated, dry β-strand-β-strand packing consisting of a few amino acids (Sawaya et al., 2007). However, other more elaborate amyloid conformations such as β-sheet/cuboids (Wasmor et al., 2008) cannot be considered by these specialized methods. Unfortunately, while these techniques can generate high-resolution structural predictions, they can only predict structural detail for regions of ∼6 to 10 residues at a time due to the assumption of a steric zipper conformation. While such short segments may act as hot spots for amyloid formation, a full-peptide structure prediction cannot be made, which encompasses the size of amyloid sequences found in nature. In the opposite vein, earlier tools are able to predict the amyloidogenicity of sequences of any length, and agnostic to a particular molecular conformation to be distinguished from another.

In this article, we develop an algorithm, AmyloidMutants, which predicts amyloid fibril structural conformations, and the sequence mutations that stabilize, reconfigure and de-stabilize each fibril conformation. Like earlier tools, our approach handles sequence mutations that stabilize, reconfigure and de-stabilize which predicts amyloid fibril structural conformations, and the details of complete super-secondary structures, and do not capture the finer conformations that are preferentially induced by wild-type (WT) mutant amyloidogenicity. Indeed, in agreement with experimental highly accurate predictions of structure, strain conformations and as a single point mutation is made; at the same time, it provides efficient calculation all these possible states, and produce accurate, physically meaningful amyloid fibril predictions.

AmyloidMutants is sensitive enough to distinguish dramatic shifts from one amyloid conformation to another when as little as a single point mutation is made; at the same time, it provides highly accurate predictions of structure, strain conformations and mutant amyloidogenicity. Indeed, in agreement with experimental observations, our tool identifies separate, incompatible amyloid conformations that are preferentially induced by wild-type (WT) Aβ and the Aβ Iowa mutant (Tycko et al., 2009), as well as similarly distinct structures resulting from wild-type and yeast-toxic mutant strains of HET-s (Couthouis et al., 2009). AmyloidMutants also allows us to probe the amyloidogenic relationship between chemically similar residues such as Asn and Gln, which revealed a specific HET-s sequence sensitivity to Asn.

2 APPROACH

We present AmyloidMutants, a web-based tool for predicting the structural and mutational landscapes of amyloid fibrils using an ensemble algorithm. In an ensemble predictor, each peptide sequence is presumed to fold into a complete set of millions (or billions) of unique structural states, with a single energetic value calculated for each state according to its entire conformation (McCaskill, 1990). From this quantified set of all possible structures, clusters of low-energy states with similar conformations can be extracted as predictions of likely real-world structures, with relative probabilities of occurrence. A mutational ensemble predictor simply increases the dimensionality of this set by including sequence variation within each state. (Note, “ensemble” predictors differ from consensus predictors; the latter produces a single prediction based on the consensus of multiple authors’ algorithms.) The definition of an amyloid fibril ‘state’ greatly impacts the accuracy of an ensemble predictor: including atomic details would result in an intractable computation, while high-level representations that work in 1D sequence space can miss important steric and energetic details. To capture critical 3D elements while retaining efficiency, we choose to model super-secondary structural information—each state contains a sequence and a unique set of residue/residue β-strand backbone interaction pairs. But even so, calculating the energy of all mathematically possible interactions would introduce an exponential number of states as a function of sequence length.

We introduce ‘schemas’ as an algorithmic construct to solve this by partitioning fibrillar from non-fibrillar conformations, enforcing steric consistency and enabling energetic calculations over all amyloid fibril sequence/structure states. For efficiency and usability purposes, putative amyloid fibril states are separated into three largely distinct topology families: schemas P , A and S, which to our knowledge, together subsume the variation found in most published experimental and hypothetical amyloid fibril structure models (Fig. 1). These schemas also account for sequence variation through a simple user specification of the mutational possibilities that should be explored: e.g. ‘all Val can mutate to Ala, Leu, or Ile’. For example, schema P describes an abstract ‘β-sheet’ encompassing millions of structures with unique residue/residue interactions and varying numbers of β-strands, β-rungs, β-sheet width, coil location, residue orientation and residue packing neighbors (for example, HET-s A predictions in Section 4 calculate the energy of ∼4 billions states). Specific 2-, 3- and 4-sheet β-helices like structures are accounted for by the introduction of ‘kinks’ (Fig. 2). Similarly, schema S represents millions of possible full-length
3 METHODS

3.1 Calculating amyloid ensembles

AmyloidMutants models the structural effects of sequence variation by conceptually scoring all possible amyloid fibril conformations that any sequence (and its mutants) can attain. Using a statistical mechanical approach, all structures are members of a canonical ensemble, with each state’s energetic value assigned according to a Boltzmann distribution. Such an ensemble predictor differs fundamentally from existing techniques that perform an algorithmic search for an individual, lowest energy structure. However, computing the score of all possible states in atomistic detail is considered computationally intractable (Istrail, 2000), so our approach uses a Boltzmann partition function of structures within an ensemble.

The Boltzmann partition function $Z$ is given by:

$$Z = \sum e^{-E_s/kT}$$

for given sequence $s$ and a Boltzmann-distributed energy score $E_s$ of all possible states $s$ within the ensemble. The Boltzmann partition function $Z$ as: $Z = \sum e^{-E_s/kT}$, given sequences $\omega$ and structures $\epsilon$. This encodes statistical variations in protein structure as well as sequence, distributed according to the energetic likelihood of that sequence’s conformations. With this, one can identify energetically favorable sequence/structure assignments and quantitatively measure the energetic difference of between states.

AmyloidMutants is implemented using C++, with modular templates describing the recursively enumerable sequence and structure space. An analysis is performed on the sequence input to optimize the search across sequence/structure states, and a dynamic programming procedure is constructed that traverses and scores all possible states, tabulating these values. From this, $Z$ can be calculated via a simple traversal.

3.2 Amyloid schema definition

Schemas are generative rules restricting the exponential set of peptide conformations to only those that form amyloid fibrils. These are defined in two parts, a recursive encoding of structure space and a protocol giving a list of all allowed sequence mutations. To model a theoretically endless fibril we employ a concept of symmetry, representing an amyloid as the conformation of single peptide combined with two sets of inter-peptide β-sheet interactions up and down the axis. We detail here specific characteristics used to define a schema, beyond the qualitative description in Figure 1.

Structure space is defined as putative geometric arrangement of β-sheets at the resolution of (i) intrapeptide hydrogen bonds along the fibril axis; (ii) β-sheet-β-sheet packing perpendicular to the axis (e.g. steric-zipper packings, etc.); and (iii) peptide/peptide symmetry describing interpeptide hydrogen bonds. Residue side chain orientations are also included in the model to indicate inward (hydrophobically packed) and outward (solvent exposed) states. Thus, a single structure can tell you whether a residue is in a β-sheet or coil, its orientation, which other residue(s) it forms a hydrogen bonding pair with and which topologically specific β-sheet it is found in, indicating other β-sheets it may pack against. Finally, β-strand ‘kinks’ model two successive β-strand residues that have the same side-chain orientation (Fig. 2). Modeling kinks allows more precise energetic parameters when two sequentially adjacent β-strands form a sharp turn (as in many β-helices), since these junctions differ from coil-separated β-strands.

Sequence space is defined by a set of allowed mutations off a base sequence, per sequence position, per residue. For example, ‘position index 10 can either be Ala, Leu, or Val’, input via programmable macros. This level of specification is required to avoid an exponential computation, as there are $20^3$ residue permutations in a sequence of length $N$. At runtime, an analysis is performed to determine the minimum dynamic programming table dimension required to fit each possible mutation. Presently, deletion and insertion mutations are not supported due to limitations of the energy models.

Although not used for results in this article, schemas can be further refined to incorporate specific point knowledge into the ensemble, enabling a more profitable, iterative back-and-forth between predictions and experimentation. These refinements include: (i) limiting β-strand or coil length; (ii) enabling or disabling β-sheet ‘kinks’; (iii) requiring a minimum/maximum total-fibril β-sheet concentration; (iv) enabling or disabling fibril twist; (v) permitting N- and C-terminal coil asymmetries; and (vi) allowing user-defined residue/residue hydrogen bond contacts to be fixed.

3.3 Energy model for amyloid-like interaction

AmyloidMutants uses a potential energy scoring function derived from observing the frequency of specific residue/residue interactions in (non-sequence-homologous) PDB (Berman et al., 2000) protein structures. Many protein and RNA modeling tools (Bradley et al., 2001; Trovato et al., 2007; Waldspühler et al., 2008b; Zuker and Stiegler, 1981) have successfully used such statistical potentials because of two main advantages: (i) residue/residue interactions (or base pairs in RNA) can efficiently capture the important, energetically stabilizing features of 3D structure without the need of
molecular detail, and (ii) constructing an energetic scoring function from known PDB structures does not require a priori expert information, so as new structures are solved, typically accuracy increases. Note, such statistical potentials do not incorporate environmental conditions such as pH.

Traditional pairwise contact models calculate the frequency with which residues pair within a β-sheet (Bradley et al., 2001; Waldispühli et al., 2006). AmyloidMutants extends this by conditioning each probability by the local 3D environment, including amphipathicity and solvent accessibility, β-strand edge proximity, residue-stacking ladders, β-sheet edges and β-sheet twist [e.g. p(i, i+α)], discretizing higher resolution information important to amyloid structure. Accordingly, each residue position in every possible ensemble conformation has an associated scoring function that allows the search procedure to apply the correct energy. For example, residues/residue pairs facing toward the center of the β-soloid in schemas P and A would be considered solvent inaccessible. These β-sheet potentials are combined and scaled with potentials for consecutive coil residues (psk(i)), as well as an optional hydrophatic packing score describing the propensity for β-sheet faces to pack against another (Kyte and Doolittle, 1982). There is no explicit cost for the act of mutation, merely an energetic change due to a new bond registration, coil location and optionally combines sequence, secondary structure, energy score, hydrogen bond probability of any residue sheet hydrogen bond, given all the conformations in the ensemble. To remove

$$E_{i} = \sum \beta \text{RTlog}(p_{i}) - \beta \text{RTlog}(Z)$$ (Close and Backofen, 2000). The probability p_{i} thus represents the likelihood of observing a substructural state k, such as the propensity for two residues to pair within a β-sheet, and log(Z) serves as a statistical centering constant. Predicted states represent steady-state conditions and do not reflect folding kinetics.

3.4 Sampling and stochastic contact maps

The principal output of AmyloidMutants is a sampled set of unique sequence/structure states (a list of sequences and their corresponding conformations) that is statistically representative of the full ensemble. Prior work has demonstrated the higher predictive accuracy of ensemble sampling over minimum energy structures (Waldispühli et al., 2008a). To achieve this, a sampling procedure performs an energetically weighted stochastic backtrack over subsequence/substructure scores generated when computing Z. Populations of similar structures are separated via PAM clustering, taking as input the number of clusters, and using a distance metric that optionally combines sequence, secondary structure, energy score, hydrogen bond registration, coil location and β-strand overlap. A method is selected to represent each cluster. User-definable distance metric changes allow for independent analysis of specific structural or sequential features.

Another form of output, the stochastic contact map, describes the Boltzmann-weighted likelihood p_{j} that any two residues i and j will form a β-sheet hydrogen bond, given all the conformations in the ensemble. To remove schema bias, the null hypothesis probability of any residue i and j forming a bond is subtracted from p_{j} (Section 3 of Supplementary Material). This allows AmyloidMutants to identify small β-strand interaction motifs within the ensemble that may be hard to discern from full conformation sampling. Furthermore, contact maps scores can be used to predict structural properties such as X-ray crystallography B-values (Waldispühli et al., 2008a).

4 RESULTS

4.1 Secondary and super-secondary structure prediction

Even in the absence of mutation predictions, AmyloidMutants offers the highest structure prediction accuracy to date. We demonstrate

### Table 1. Summary of secondary-structure prediction results

<table>
<thead>
<tr>
<th>Method</th>
<th>Aβ</th>
<th>HET-s</th>
<th>Amylin</th>
<th>α-syn</th>
<th>Tau</th>
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<td>37</td>
<td>140</td>
<td>441</td>
</tr>
<tr>
<td>Correct β-regions</td>
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<td>3 of 4</td>
<td>3</td>
<td>5 of 5</td>
<td>7 of 8</td>
</tr>
<tr>
<td>Percent sensitive/specificity</td>
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<td>95/95</td>
<td>70/90</td>
<td>81/95</td>
<td>66/95</td>
</tr>
<tr>
<td>SOV measure</td>
<td>100</td>
<td>90</td>
<td>97</td>
<td>62</td>
<td>62</td>
</tr>
</tbody>
</table>

this by comparing predictions against experimental data for five of the best studied WT amyloid proteins: Aβ (Lüthi et al., 2005; Petkova et al., 2003) (39-42aa), HET-s (Wawer et al., 2008) (73aa), amylin (Kajava et al., 2005; Luca et al., 2007) (37aa), α-synuclein (Heise et al., 2005; Vilar et al., 2007) (140aa) and tau (Mukrasch et al., 2009; von Bergan et al., 2000) (144aa). This set covers pathogenic and functional amyloids found in nature for which there is a number of published structural experiments, including NMR secondary structure chemical shift and H/D exchange data. The ability to accurately predict the structure of such peptides could potentially help elucidate how native amyloid-related processes (such as biofilm formation) impact cellular function, and allow for targeted experimentation.

For these five proteins, AmyloidMutants correctly identifies experimentally observed β-sheet regions in 21 of 22 cases (Fig. 4, Table 1)—a per-residue-secondary-structure classification sensitivity/specificity of 82%/95% and an average SOV score of 82 (Zelma et al., 1999). Using the same comparison, the best of the available full-length amyloid prediction tools (Bryan et al., 2009; Fernandez-Escamilla et al., 2004; Maurer-Stroh et al., 2010; Tartaglia and Vendruscolo et al., 2008; Trovato et al., 2007) produced a classification sensitivity/specificity of 42%/90% (Zyggregator) (Fig. 3). Per-residue β-sheet classification is used for this comparison since it can be inferred as a common output of all tools; however, AmyloidMutants can provide more rich predictions including super-secondary residue/residue interactions. Therefore, a detailed analysis of each protein’s predictions is given to demonstrate these added benefits, along with a demonstration of how ensemble predictions can help identify alternate fibril conformations in agreement with published experimental data.

AmyloidMutants was run on each sequence for all three schemas P, A and S, with the schema that agreed best presented. An ensemble was calculated, and conformations were sampled and clustered, with the mediod structures reported (Section 1 of Supplementary Material). Although rough computational tests can be applied to evaluate the schema fitness (Section 3 of Supplementary Material), in a typical real-world scenario (and what has been applied thus far), an uncharacterized amyloid sequence is predicted using all schemas, and results are compared against the body of existing experimental data or used to guide further disambiguating experimentation.

Note, although atomic-resolution steric zipper structures have been solved for many short (~4 to 10aa) synthetic peptides (Maurer-Stroh et al., 2010; Sawaya et al., 2007), AmyloidMutants predictions on such short peptides are trivial. Schemas can predict the position and arrangement of steric zipper sites throughout a full-length peptide, but are not designed to distinguish side-chain rotamers (and are unrelated to steric zipper classes).
A

TANGO and PASTA tools most closely match our tool's ability to predict et al.

Moreover, brain-seeded fibrils have exhibited spatial proximity does not contain a β-sheet formed under quiescence and agitation differ, for instance, in the studies of Aβ heterogeneous, including many other structural arrangements, and accounts for 39% of the ensemble. Furthermore, recent experimental accounts for 55% of the ensemble. This cluster is more nearly identically matches the latter of these two models (Lührs et al., 2003). After clustering, the highest likelihood mediod structure observed structure of two possible Aβ1-42, D23N,Aβ16-22, 2005), 2003). Our tool predicts the experimentally observed structure of the peptide based on NMR, H/D-exchange and mutational analysis (Lührs et al., 2005; Petkova et al., 2005). The α-helical segment thought to favor a lipid-binding amphipathic α-helical structure (Section 1 of Supplementary Material).

Amylin: AmyloidMutants predictions for human amylin indicate two viable conformations: a 2-sheet β-solennoid forms 80% of the ensemble and agrees closely with NMR and microscopy results (Luca et al., 2007) (Fig. 4c); and a much less likely three-sheet serpentine model that aligns almost perfectly with an older model of amylin structure (Kajava et al., 2005). Interestingly, the experimental model identifies an interprotifibril interaction between Phe23 and Tyr37—something beyond the scope of our schema. However, our β-solennoid predictions clearly separate into two distinct populations, one incorporating Phe23 into a β-sheet and one that does not. This highlights the importance of an ensemble analysis: the existence of high-likelihood alternate structures may draw attention to an overlooked structural interaction.

α-Synuclein: five β-sheet regions in α-synuclein have been identified through substantial experimental effort (Heise et al., 2005; Vilar et al., 2007). AmyloidMutants ensemble predictions agree extremely well with these results, aligning all five β-sheet regions, and identifying other important experimental observations such as a β-sheet break around residues 67–68 (Fig. 4d). One of the predicted clusters does produce a false positive, however, identifying amphipathic β-strands in the N-terminal region, a disordered segment thought to favor a lipid-binding amphipathic α-helical structure (Section 1 of Supplementary Material).

Tau (β): NMR studies have shown this 441 amino acid long amyloid to form a mixture of up to eight transient β-sheet regions (Mukrasch et al., 2009), with two specific β-strands necessary for fibril

Fig. 3. AmyloidMutants per-residue β-strand assignments indicate amyloid core regions, comparable with existing per-residue amyloidogenicity predictors. AmyloidMutants predictions (green) outperform those tools available for testing when using their default settings and thresholds. BETASCAN (gold) (Bryan et al., 2009), ZYGREGATOR (blue) (Tarraglia and Vendruscolo et al., 2008), TANGO (cyan) (Fernandez-Escamilla et al., 2004), PASTA (red) (Tozato et al., 2007) and Waltz (purple) (Maurer-Stroh et al., 2010), when compared against experimental structure models supported by NMR, H/D-exchange and mutational analysis (black) (Luca et al., 2007; Lührs et al., 2005; Mukrasch et al., 2009; Vilar et al., 2007; Wasmer et al., 2008, 2010). Note, the BETASCAN, ZYGREGATOR, TANGO and PASTA tools most closely match our tool’s ability to predict full-length per-residue amyloidogenicity, whereas Waltz aims to predict short hotspots that could specifically adopt a steric zipper.
A AmyloidMutants is uniquely capable of identifying change in A\textsubscript{Iowa} mutation (Tycko et al., 2009), consistent with published experimental data. Predicted kink occurs because schema does not account for known D23K28 salt bridge, including most packing orientations. Predicted kink occurs because schema does not account for known D23K28 salt bridge (Lührs et al., 2005) (black arrows), including most packing orientations. Predicted kink occurs because schema does not account for known D23K28 salt bridge (Lührs et al., 2005) (bottom, black arrows) shows near identical match, including residue orientations and kink location. (c) Top two amylin predictions (solid, striped green arrows) align well to NMR model (Luca et al., 2007) (black arrows). Predictions differ only by their inclusion of Phe23 within β-sheet, a residue experimentally shown to form non-β-sheet interpeptide interactions not considered by schema. (d) Top two amylin predictions (ii,iv) agree very well with H/D exchange data (ii,iv) and NMR model (v) (Heise et al., 2005, Vilar et al., 2007). (c) Tau predictions identify 7/8 regions observed experimentally (Mukrasch et al., 2009). The highest AmyloidMutants scores (red boxes) specifically identify regions 274–279 and 305–310, positions believed crucial to fibril nucleation (von Bergan et al., 2000).

4.2 Prediction of a conformational switch in A\textsubscript{β} and HET-s mutants

AmyloidMutants is uniquely capable of identifying change in amyloid fibril conformation from one amyloid β-sheet topology to another. This distinction from tools that predict general amyloidogenicity is important as a structural change from one amyloid form to another can have a dramatic impact on oligomerization and nucleation rates (Kim and Hecht, 2008), disease infectivity (Tycko et al., 2009), and prion propagation (Alberti et al., 2009). We have used this ability to identify potential alternate, distinct amyloid fibril conformations that arise in the A\textsubscript{β} familial ‘Iowa’ mutation (Tycko et al., 2009) and yeast-toxic mutants of HET-s (Bertelot et al., 2009; Couthouis et al., 2009) (details and comment provided in Sections 2 and 3 of Supplementary Material). Described below are these AmyloidMutants results, highlighting consistencies with published experimental data.

A\textsubscript{β} Iowa mutant: recent studies (Tycko et al., 2009) suggest that A\textsubscript{β\textsubscript{1–40/D23N}} may form an antiparallel β-sheet fibril conformation that differs completely from known experimental models (Lührs et al., 2005; Petkova et al., 2003). This work suggests an antiparallel β-sheet around residues 16–22 (with unknown length), with an inter-fibril interface such that L17 bonds to A21 (designated ‘17+k↔21’ registry (Tycko et al., 2009)). Similarly, a second antiparallel β-sheet likely exists around positions 30–36, with L34 and F19 in close contact. Interestingly, this specific A\textsubscript{β\textsubscript{1–40}} registry has only previously been seen in the peptide fragment A\textsubscript{β\textsubscript{16–22}}, which lacks D23 (Tycko and Ishii, 2003), while the antiparallel forming fragment A\textsubscript{β\textsubscript{11–25}} shows inverted ‘17+k↔22’- and ‘17+k↔20’-registry predictions (Petkova et al., 2004) (Table 2).

To analyze this point mutant, we predicted ensembles for A\textsubscript{β\textsubscript{1–40}} and A\textsubscript{β\textsubscript{1–40/D23N}} using schema A (which allows antiparallel inter-peptide interactions). Detailed in Table 2, AmyloidMutants’ A\textsubscript{β\textsubscript{1–40/D23N}} predictions strongly preferred a ‘17+k↔21’ registry conformation, with predicted contacts between L34/F19, and very little variation within the ensemble. This arrangement agrees with observed A\textsubscript{β\textsubscript{16–22}} structures. Conversely, predictions for WT A\textsubscript{β\textsubscript{1–40}} are quite heterogeneous, although with the largest cluster of structures forming ‘17+k↔22’ registry, in agreement with observed A\textsubscript{β\textsubscript{11–25}} structure. More strikingly, the ‘17+k↔22’ registry conformation favored by A\textsubscript{β\textsubscript{1–40/D23N}} appears to be strongly disfavored by A\textsubscript{β\textsubscript{1–40}} (and A\textsubscript{β\textsubscript{1–40/D23N}} appears to disfavor ‘17+k↔22’ registry). These predictions and the divergence in ensemble makeup between A\textsubscript{β\textsubscript{1–40}} and A\textsubscript{β\textsubscript{1–40/D23N}} strongly supports the idea that the D23N mutation results in a singular energetically favorable conformational rearrangement from parallel β-sheets (in WT) to antiparallel β-sheets (in the D23N mutation). At the residue level, the adoption of this ‘17+k↔21’-conformation may be driven by both the alignment of oppositely charged K16 and E22 and the stacking arrangement of Q15 and N23 (Table 2).

HET-s yeast-toxic mutants: our technique is able to further predict putative conformational rearrangements between a set of HET-s mutants shown to exhibit toxicity in yeast. In recent studies (Bertelot et al., 2009; Couthouis et al., 2009), structural assembly (von Bergan et al., 2000) (positions 306–311 and 275–280). Predicted β-sheets align very closely with these observed regions in seven of eight cases. Moreover, AmyloidMutants identifies the two hexapeptides experimentally observed necessary for assembly by predicting their β-strand interactions as having the strongest score (Section 1 of Supplementary Material). Four false positive regions are predicted to contain β-strands, although similar to α-helices which the schema does not incorporate and may contain sequences with high β-sheet propensity. Overall, the sensitivity and specificity of our predictions over such a long sequence considerably advance the state of the art (Fig. 3).

**Fig. 4.** AmyloidMutants structure predictions match experimentally observed β-strand interactions of A\textsubscript{β\textsubscript{1–40}} (a), HET-s (b), amylin (c), α-synuclein (d) and tau (e). (a) Diagram depicts A\textsubscript{β\textsubscript{1–40}} β-sheet in gray, residues in blue (with in-out orientation) and β-sheet α-sheet packing as one β-sheet above another, packed residues facing center. Predicted structure (green arrows) mirrors NMR structure (Lührs et al., 2005) (black arrows), including most packing orientations. Predicted kink occurs because schema does not account for known D23K28 salt bridge. (b) Similar depiction of HET-s prediction (top, green arrows) compared with NMR model (Wasmer et al., 2008) (bottom, black arrows) shows near identical match, including residue orientations and kink location. (c) Top two amylin predictions (solid, striped green arrows) align well to NMR model (Luca et al., 2007) (black arrows). Predictions differ only by their inclusion of Phe23 within β-sheet, a residue experimentally shown to form non-β-sheet interpeptide interactions not considered by schema. (d) Top two α-synuclein predictions (ii,iv) agree very well with H/D exchange data (ii,iv) and NMR model (v) (Heise et al., 2005, Vilar et al., 2007). (e) Tau predictions identify 7/8 β-regions observed experimentally (Mukrasch et al., 2009). The highest AmyloidMutants scores (red boxes) specifically identify regions 274–279 and 305–310, positions believed crucial to fibril nucleation (von Bergan et al., 2000).
Table 2. AmyloidMutants predictions reveal conformational switch between Aβ1–40 and Aβ1–40/D23N in agreement with published data

<table>
<thead>
<tr>
<th>Pred. Aβ1–40 (%)</th>
<th>Pred. Aβ1–40/D23N (%)</th>
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<tr>
<td>69</td>
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<thead>
<tr>
<th>Pred. Aβ1–25 (%)</th>
<th>Obs. Aβ1–25 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Predictions show a significant change in the conformational landscapes of Aβ1–40 and Aβ1–40/D23N, in agreement with published experimental evidence (Tycko et al., 2009) of an antiparallel, ‘17+↔21-’ registry -sheet β-sheet (boldface). Sampled ensemble structures were classified into one of three categories of β-sheet registry, with the percent makeup of each provided: β-sheet registry is classified by residue/residue X pairing, depicted with X highlighting position 23. Check marks indicate experimentally observed registrations in Aβ1–25 (Petkova et al., 2004) and Aβ1–22 (Tycko and Ishii, 2003).

Table 3. AmyloidMutants ensemble predictions of HET-s sequence variants reveal the yeast-toxic mutant m8 to be unique

<table>
<thead>
<tr>
<th>Schema/class</th>
<th>WT</th>
<th>m4</th>
<th>m8</th>
<th>m3</th>
<th>m9</th>
<th>m11</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 2-rung (%)</td>
<td>75</td>
<td>95</td>
<td>72</td>
<td>13</td>
<td>49</td>
<td>55</td>
</tr>
<tr>
<td>P 1-rung (%)</td>
<td>25</td>
<td>5</td>
<td>28</td>
<td>87</td>
<td>51</td>
<td>45</td>
</tr>
<tr>
<td>A 2-rung-A (%)</td>
<td>45</td>
<td>42</td>
<td>81</td>
<td>44</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>A 2-rung-B (%)</td>
<td>25</td>
<td>43</td>
<td>0</td>
<td>36</td>
<td>22</td>
<td>40</td>
</tr>
<tr>
<td>A 1-rung (%)</td>
<td>30</td>
<td>15</td>
<td>19</td>
<td>20</td>
<td>22</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aggregation</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diff.</td>
<td>Minor</td>
</tr>
<tr>
<td>Severe</td>
<td></td>
</tr>
</tbody>
</table>

Ensemble conformational landscape predictions of six HET-s variants produced two general structure classifications for schema P and three general classifications for schema A (rows, relative percent makeup given). While other mutants do not favor one particular schema A structure, the yeast-toxic m8 mutant exhibits a strong energetic bias for A 2-rung-A. The differences in structure bias shown may suggest an increased likelihood that m8 adopts an antiparallel conformation (boldface). Observed phenotypic differences between mutants are summarized at the bottom (Couthouis et al., 2009).

4.4 Mutational landscapes predict experimental amyloidogenicity

AmyloidMutants’ ability to accurately predict amyloidogenicity is validated by comparing our results against a large number of experimentally characterized amyloid mutants. This includes an analysis of the 280-residue HET-s/HET-S natural homologs found in Panserina, a combination of three Aβ scanning mutagenesis properties, allowing us to study a more fundamental question: the role chemically similar residues Asn and Gln play in fibril structure. Given the high propensity of QN-rich peptides to form amyloid (Chiti and Dobson, 2006), the amyloidogenic potential of Asn and Gln has often been considered equal—however, recent evidence suggests that N-rich proteins may have a slightly higher tendency to form amyloid (Alberti et al., 2009) even though Gln mutations can improve stability (Gromiha et al., 1999). We study this question by considering the effect of four ladder-forming asparagine residues in Panserina HET-s (positions 226, 243, 262 and 279) which are believed important for fibril stabilization (Wasmcr et al., 2008), and whose regions are conserved in a Fagrinaceaean homolog. AmyloidMutants sequence/structure landscapes were calculated permitting these four residues to mutate to Gln (‘HET-s/4N→Q’), and the likelihood and corresponding energetic weight of each sequence within the ensemble was compared. The WT HET-s sequence was much more energetically unfavorable than HET-s/4N→Q, comprising ~96% of the ensemble, suggesting a greatly reduced ability of HET-s/4N→Q to form fibrils, and a putatively higher amyloidogenic potential of Ann over Gln. Stochastic contact map predictions further illustrate this difference between sequences (Supplementary Fig. S9). We tested these predictions experimentally, using purified recombinant WT and 4N→Q HET-s proteins (Section 4 of Supplementary Material). Denatured proteins were diluted into a physiological buffer and allowed to form amyloid. While the WT protein readily did so, as detected by the retention of detergent-insoluble aggregates on a non-binding membrane, the mutant protein was recalcitrant to amyloid formation (Fig. 5).

4.3 Identification of preferential Asn amyloidogenicity over Gln in HET-s

Beyond its ability to discriminate amyloid fibril structure states, AmyloidMutants accurately models more coarse amyloidogenicity over Gln in HET-s, with the prediction that Asn mutations can improve stability (Gromiha et al., 1999). We study this question by considering the effect of four ladder-forming asparagine residues in Panserina HET-s (positions 226, 243, 262 and 279) which are believed important for fibril stabilization (Wasmcr et al., 2008), and whose regions are conserved in a Fagrinaceaean homolog. AmyloidMutants sequence/structure landscapes were calculated permitting these four residues to mutate to Gln (‘HET-s/4N→Q’), and the likelihood and corresponding energetic weight of each sequence within the ensemble was compared. The WT HET-s sequence was much more energetically unfavorable than HET-s/4N→Q, comprising ~96% of the ensemble, suggesting a greatly reduced ability of HET-s/4N→Q to form fibrils, and a putatively higher amyloidogenic potential of Ann over Gln. Stochastic contact map predictions further illustrate this difference between sequences (Supplementary Fig. S9). We tested these predictions experimentally, using purified recombinant WT and 4N→Q HET-s proteins (Section 4 of Supplementary Material). Denatured proteins were diluted into a physiological buffer and allowed to form amyloid. While the WT protein readily did so, as detected by the retention of detergent-insoluble aggregates on a non-binding membrane, the mutant protein was recalcitrant to amyloid formation (Fig. 5).
is more amyloidogenic than HET-S. Although N-terminal mutations energetically favorable path for amyloid formation. predictions suggests a sequence bias in HET-s permitting a more formation, measured by WT/mutant of proline-, alanine- and cysteine-replacement on amyloid fibril residue C-terminus, and 13 overall (Coustou et al., 1999). conformation in its prion form, while the HET-S allele does not, found that 72% of the ensemble favored HET-s, indicating that it is more amyloidogenic than HET-S. Although N-terminal mutations can induce a prion state in HET-S (Coustou et al., 1999), our predictions suggests a sequence bias in HET-s permitting a more energetically favorable path for amyloid formation.

**HET-s/HET-S**: In *P.anserina*, the HET-s allele forms an amyloid conformation in its prion form, while the HET-S allele does not, despite differing by only three residues in the amyloid-forming 72-residue C-terminus, and 13 overall (Coustou et al., 1999). Predicting the joint HET-s/HET-S mutational landscape, AmyloidMutants found that ~72% of the ensemble favored HET-s, indicating that it is more amyloidogenic than HET-S. Although N-terminal mutations can induce a prion state in HET-S (Coustou et al., 1999), our predictions suggests a sequence bias in HET-s permitting a more energetically favorable path for amyloid formation.

**Aβ single-point proline mutagenesis**: scanning mutagenesis studies have been performed on Aβ to detect the sequence position effect of proline-, alanine- and cysteine-replacement on amyloid fibril formation, measured by WT/mutant ΔΔG (Shivaprasad et al., 2006; Williams et al., 2004, 2006). Although P, A and C-replacement ΔΔG values are difficult to interpret independently (due to experimental structural heterogeneity (Williams et al., 2006)), they support the broader conclusion that Aβ positions 18–21, 25–26 and 32–33 are particularly sensitive to P-replacement (Williams et al., 2006). AmyloidMutants’ predictions of the joint mutational landscape for individual proline replacements identified positions 16–25 and 31–35 as particularly disruptive in agreement with these studies. Supplementary Figure S12 plots this agreement along with similar predictions by TANGO and Zyggregator, although a direct one-to-one comparison between predictions and ΔΔG values would be inappropriate.

**Aβ multiple-residue mutagenesis**: AmyloidMutants predictions were also performed on a set of 74 Aβ mutants (Kim and Hecht, 2006, 2008; Wurth et al., 2002) whose relative aggregation levels were observed by GFP/fluorescence relative to WT. AmyloidMutants accurately identifies which mutants form amyloid more (or less) readily than WT in 81% of sequences (60 of 74, Supplementary Fig. S8). AmyloidMutants’ performance on such a large set further supports its general applicability.

5 DISCUSSION

AmyloidMutants provides the highest accuracy prediction to date of the full fibril structure of amyloid sequences, but its greater value is its unique ability to discover which mutations effect a change in amyloid structure(s), to predict what that structure is and to assign meaningful energetic weights comparing mutant conformations. This accuracy is due, in part, to the ability to model coarse, higher dimension spatial interactions, beyond simpler 1D sequence motifs. This is an important distinction from amyloidogenicity predictors that identify structurally homogeneous peptide sequences (Maurer-Stroh et al., 2010). While the latter can be helpful during an initial screen (searching for amyloid steric zippers in particular), AmyloidMutants can predict and provide insight into the full-length structure (Fig. 3) and residue/residue interactions of both β-solenoidal and serpentine steric zipper fibrils (Fig. 1), putatively identifying interactions critical to function or pathogenicity. Further, through the use of Boltzmann ensembles, our model of sequence/structure space is the only amyloid modeling tool that captures fibril structure variation and β-contact structural topology changes that may arise in vivo.

The exploration of mutational landscapes is an important step in understanding differences between amyloid topologies, how mutational variants arise in the wild, and to elucidate evolutionary relationships between related amyloid proteins. This capability depends on AmyloidMutants’ novel thermodynamic characterization of all points within a mutational landscape, and is necessary for the discovery of non-additive functional relationships between sequences and conformational epistasis (Ortlund et al., 2007). Further, we note that our tool provides additional features for experimentalists (not used in this article) that allows extra-segmental experimental data to be incorporated into the predictor (Supplementary Text Section 1)—as much or as little a priori knowledge as desired, enabling a new tactic for iterative tool re-use.

At face value, the ability of most proteins to form a characteristic cross-β-sheet amyloid structure *in vitro* (Dobson, 2003) seems at odds with the relatively small number of amyloid-forming proteins that have been identified *in vivo*, and the apparently high sequence dependence some amyloids show when compared against sequence homologs. Moreover, the existence of both beneficial functional amyloid sequences, and putatively pathogenic ‘misfolded’ amyloid proteins suggests a more complicated sequence/structure relationship than is found in standard protein folding models. The power to accurately predict amyloid structure from sequence, and to fully characterize the amyloidogenicity of an entire mutational landscape provides insight into this problem by identifying recurring sequence motifs, coarse 3D residue arrangements and putative mutational pathways linking the sequences of known amyloid structures. The immediate impact of this could improve our ability to identify amyloid structures from genomic data alone, to better understand familial mutations that intensify pathogenesis in diseases such as Alzheimer’s, to predict the interaction strength of fibril regions that may be involved in nucleation and to enable targeted peptide design to alter fibril structure or inhibit fibril formation.
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Conflict of Interest: none declared.

REFERENCES


