Introduction

Amyloids are protein aggregates with a cross-β structure. Amyloid folds are gradually being recognized as important components in the structural landscape of peptides and proteins. The amyloid fold has been envisioned as a generic primordial fold from which globular folds had to emancipate in prebiotic times to attain structural and functional diversification into globular proteins [1,2,3,4,5]. Amyloid folds also fulfill a variety of biological functions in relation to their specific structural properties [2,6,7].

Mutations in the latter region either positively or negatively affected prion formation. We thus identify a region that modulates prion formation although it is not part of the rigid cross-β core, an observation that might be relevant to other amyloid models.

Abstract

The [Het-s] prion of the fungus Podospora anserina represents a good model system for studying the structure-function relationship in amyloid proteins because a high resolution solid-state NMR structure of the amyloid prion form of the HET-s prion forming domain (PFD) is available. The HET-s PFD adopts a specific β-solenoid fold with two rungs of β-strands delimiting a triangular hydrophobic core. A C-terminal loop folds back onto the rigid core region and forms a more dynamic semi-hydrophobic pocket extending the hydrophobic core. Herein, an alanine scanning mutagenesis of the HET-s PFD was conducted. Different structural elements identified in the prion fold such as the triangular hydrophobic core, the salt bridges, the asparagines ladders and the C-terminal loop were altered and the effect of these mutations on prion function, fibril structure and stability was assayed. Prion activity and structure were found to be very robust; only a few key mutations were able to corrupt structure and function. While some mutations strongly destabilize the fold, many substitutions in fact increase stability of the fold. This increase in structural stability did not influence prion formation propensity in vivo. However, if an Ala replacement did alter the structure of the core or did influence the shape of the denaturation curve, the corresponding variant showed a decreased prion efficacy. It is also the finding that in addition to the structural elements of the rigid core region, the aromatic residues in the C-terminal semi-hydrophobic pocket are critical for prion propagation. Mutations in the latter region either positively or negatively affected prion formation. We thus identify a region that modulates prion formation although it is not part of the rigid cross-β core, an observation that might be relevant to other amyloid models.

Contribution of Specific Residues of the β-Solenoid Fold to HET-s Prion Function, Amyloid Structure and Stability

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Introduction

Amyloids are protein aggregates with a cross-β structure. Amyloid folds are gradually being recognized as important components in the structural landscape of peptides and proteins. The amyloid fold has been envisioned as a generic primordial fold from which globular folds had to emancipate in prebiotic times to attain structural and functional diversification into globular proteins [1,2,3,4,5]. Amyloid folds also fulfill a variety of biological functions in relation to their specific structural properties [2,6,7]. Importantly, amyloids represent the underlying cause of a number of age-related protein deposition diseases which impose a major burden to modern societies [8,9]. Yet, the determinants that govern amyloid folding and stability are much less well understood than in the case of globular proteins in part because of the scarcity of available high resolution structures of amyloid proteins.

Amyloids have the inherent property of being self-perpetuating and as such can represent the mechanistic basis for prion formation [10,11,12,13]. Many prions are amyloids that are self-perpetuating in vivo. Amyloid prions cause fatal neurodegenerative diseases in mammals and can be encountered as epigenetic elements in fungi [14,15]. The [Het-s] prion of the filamentous fungus Podospora anserina represents an example of such fungal prions [16,17,18]. Highly prevalent in nature, [Het-s] is involved in a non-self recognition process that takes place when cells of unlike genotype undergo fusion [16,19]. This process termed heterokaryon incompatibility leads to the cell death of the mixed fusion cells. The het-s gene exists as two incompatible alleles termed het-s and het-S. When a het-s and a het-S strain are confronted, the incompatibility cell death response leads to the formation of a macroscopic demarcation line termed barrage. It is proposed that this cell death reaction might have a more general function in fungal defense [20,21,22]. Strains of the het-s genotype exist as two alternate epigenetic states: [Het-s] (the non-prion-state) and [Het-s] (the prion state). Transition to the prion state can occur spontaneously at a low rate or can be induced systematically by contact with a prion-infected ([Het-s]) strain. The prion form then invades the fungal hyphal network at a rate an order of magnitude higher than the linear growth rate of the fungus [23]. Incompatibility is only triggered when HET-s is in the
Author Summary

Prions are infectious protein particles causing fatal diseases in mammals. Prions correspond to self-perpetuating amyloid protein polymers. Prions also exist in fungi where they behave as cytoplasmic infectious elements. The [Het-s] prion of the fungus Podospora anserina constitutes a favorable model for the analysis of the structural basis of prion propagation because a high resolution structure of the prion form of [Het-s] is available, a situation so far unique to this prion model. We have analyzed the relation between [Het-s] structure and function using alanine scanning mutagenesis. We have generated 32 single amino acid variants of the prion forming domain and analyzed their prion function in vivo and structure by solid-state NMR. We find that the PFD structure is very robust and that only a few key mutations affect prion structure and function. In addition, we find that a C-terminal semi-flexible loop plays a critical role in prion propagation although it is not part of rigid amyloid core. This study offers insights on the structural basis of prion propagation and illustrates that accessory regions outside of the amyloid core can critically participate in prion function, an observation that could be relevant to other amyloid models.

Sequence comparisons suggest an evolutionary conservation of the β-solenoid fold [35,36]. Characterization of a HET-S homolog of a different fungal species, the plant pathogen Fusarium graminearum revealed a conservation of the β-solenoid fold and of the prion formation ability [37,38]. Thus, the HET-s PFD sequence has been evolutionarily shaped to adopt this fold and the overall fold and the prion forming ability have been conserved over an extended evolutionary period. Perhaps, as a consequence of this evolutionary process, the HET-s PFD sequence does not only lead to the formation of amyloid polymorphs in physiological conditions as generally observed for disease causing amyloids or for yeast prions [13,39]. HET-s(218–289) adopts the same structure in vitro and when forming inclusion bodies during heterologous expression in E. coli [40]. Only at highly acidic pH, when the native β-solenoid fold cannot be attained, HET-s(218–289) adopts an alternate non-infectious amyloid structure [34,41,42,43,44]. Alanine scanning approaches have been widely used to analyze folding and stability of globular proteins and such approaches have also been used in the context of amyloid fibril formation and stability [45,46]. In the case of yeast prion proteins such as the well studied Sup35 model, the applicability of such approaches is complicated by the absence of a full structure model, the primary sequence independence in prion formation and the existence of multiple prion strains corresponding to different amyloid polymorphs [47,48,49]. Nevertheless, a recent study has revealed the importance of a glycine pair in [PS] prion formation as a structural determinant in the soluble form of the PFD [50]. The HET-s model constitutes a favorable system for such studies because of the lack of prion strain variants and the availability of a high resolution structure. A previous study has revealed the functional importance of the β-strand elements in the β-solenoid structure using a proline mutant approach [27]. Here, we relied on the more subtle alanine scanning approach to analyze the functional and structural role of specific residues and structural components of the HET-s PFD fold.

Results

Evolutionary conservation of the PFD region in various fungal species

Previous studies have analyzed the evolutionary conservation of the β-solenoid fold [21,35,36,37,38]. Due to the rapid increase in the number of fully sequenced fungal genomes, many additional HET-S homologs sequences have become recently available, we have thus conducted a database search for HET-s/HET-S homologs in current fungal genome sequences. We could identify a total of 51 het-s/het-S homologs (file S1). Based on residues found at 33 of the Helo domain which is known to define HET-s and HET-S allele specificities [51,52], the homologs found in the other species are of the HET-S, rather than HET-s-type (Figure S1), as suggested previously [18,21,50,37]. Some species like Neurospora crassa and some strains of the Fusarium oxysporum species complex contained up to 4 het-S paralogs. The majority of the sequences showed conservation of the 21 amino acid repeat regions (R1 and R2) but in a fraction of the sequences there was a two amino acid deletion in the first repeated motif (R1). Figure 1B shows an alignment of the 22 non redundant sequences for which both 21-amino acid repeat sequences are conserved. The level of identity between the sequences is in the range of 30% which is about the level of identity between the P. anserina HET-s(218–289) and F. graminearum FgHET-s(218–289) sequences that were previously found to display closely related structures [38]. It can thus be reasonably inferred that the sequences presented in the alignment share similar folds, as previously suggested by homology modeling.
The positions showing strict conservation are two of glycines (G242/G278, position 17 of the 21 aa repeat) located in the arc between the third and fourth β-strand of each rung, (Figure 1A and B). The N226/N262 asparagine pair (forming the asparagine ladder at the start of the first β-strand, position 1) was also conserved although some exceptions occurred. The second asparagine ladder (N243/N279, position 18) is not as strongly conserved as the first ladder. There is also a strong conservation of the hydrophobic residues in the core, large hydrophobic residues are found at core positions 231/267 (position 6), 239/275 (position 14) and 241/277 (position 16). There is an interesting trend of mutual exclusion of large hydrophobic side chains at position 228 and 264 (position 3), suggesting that the presence of large residues at both positions might lead to sterical hindrance. Interestingly, two sequences (Fo_4287-2 and Cg_HET-S) constitute exceptions in that regard with an isoleucine found both at 228 and 264. But in these sequences, an Ala residue is found in 231 which might allow for accommodation of the larger residues in position 3 (i.e. residue 231 in Figure 1A). Although the residues forming the three salt bridges identified in HET-s (in positions 4, 9 and 11) are not strictly conserved, there is an overall preference for favorable charge interactions at these positions. In the 22 listed sequences, favorable charge combination occurs in 13 and 15 sequences at position 4 and 9 respectively while repulsive interactions are never found. At position 11, favorable charge interactions are less conserved and occur in five sequences. Also of note, is the conservation of the glycine-rich C-terminal loop region encompassing the W287 and F286 aromatic residues. Conservation occurs not only on the aromatic positions but also the glycines and the charged residues (K284 and D288) are strongly conserved. This region is however entirely missing in a fraction of the sequences. Of note is the fact that the sequences missing this region are found in species that contain other HET-S paralogs that do display the loop region. The conservation is poor in the central loop region between R1 and R2. This region generally contains glycine residues and the length of the loop region varies from 12 to 18 residues. It was shown experimentally that three to five amino acid deletions in the loop approach the C-terminal loop region.}

Figure 1. Structure and sequence conservation of the HET-s prion forming domain. A. The structure of the first pseudo repeat (residue 226–246) as well as the second repeat and the C-terminal loop region (residue 262–289) are given (after PDB 2KJ3). The 32 residues that have been mutated are coloured. Colour coding is as follows, polar residues in green, hydrophobic in blue, aromatic in cyan, positively charge in red, negatively charged in magenta and glycine in yellow. B. Sequence alignment of the PFD region of various HET-S homologs from various fungal species. The 21 amino acid repeats are boxed. The position of the β-strands of the β-solenoid core is represented as blue arrows. C. Consensus sequence of the first and second repeats of the HET-S homologs presented in the alignment in B. The consensus was generated using MEME. In this graphical representation, the size of the letter reflects the level of conservation of the corresponding residue; the scale is given in information content measured in bits.

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loop region could be tolerated without affecting [Het-s] and HET-S function [26,27].

In order to achieve an overall comparison of the two repeats, we devised a separate consensus sequence for repeats R1 and R2 using MEME [53] (Figure 1C). The consensus sequence reveals some marked differences. In the turn at position 10, there is a preference for a Gly in R1 and a Gln residue in R2. In position 12, there is a preference for an Ala and Ser in R1 and R2 respectively, which is reflected by the reciprocal distribution in position 8. Also, there is a strong conservation of the Gly at the end of R2 in the region leading into the C-terminal semi-hydrophobic pocket. The charge complementary mentioned above is also apparent in these consensus sequences; with a preference in R1 for positive charges in position 4 and negative charges in 9 and the opposite preference in R2. This differential conservation of specific residues in the first and the second repeat suggest that both repeats are not strictly functionally equivalent.

Globally, many aspects of the sequence conservation and variation in HET-s homologs are well explained by the structural information available. Yet, the role of certain features such as for instance the conservation of the Arg residue at position 13 is not directly apparent.

Effect of alanine scanning mutagenesis on [Het-s] activity in vivo

We set out to analyze the structure-function relation in the HET-s PFD by generating a series of 32 alanine mutants along the HET-s PFD and by testing their prion activity in vivo in Podospora anserina. Based on the sequence conservation described above the β-strand region and the C-terminal pocket were specifically targeted (Figure 1A). The mutation coverage amounts to one mutation every 1.8 residue in these regions. Mutations were introduced in a plasmid expressing full length HET-s and the corresponding plasmid was introduced into a Ab hetero strain as previously reported for proline and deletion mutants of the PFD [27]. For each mutation, at least 24 individual transformants were assayed for [Het-s] incompatibility function and for their ability to infect a [Het-s+] strain. In this experimental setting, transformants express the transgene from ectopic integration sites and in multiple copies. This approach allows a rough categorization of the mutants. Results are presented in Figure 2. In most cases and as previously observed, the number of transformants able to infect a [Het-s+] strain is equivalent or higher than those producing a barrage reaction to [Het-S] [27]. This is likely due to the fact, that higher prion titers are required to produce an incompatibility reaction than to infect a [Het-s+] strain.

We have categorized the mutants into three functional classes: mutations that lead to percentages of active transformants in the range of wild-type (>60% of active transformants), those significantly reducing the number of transformants with [Het-s] activity (>30% and <60%) and those strongly affecting the number of active transformants (<30%). The majority of the mutations (18/32) did not significantly affect [Het-s] activity in this assay. Nine mutants were slightly affected and five were strongly affected. The latter corresponded to two group of mutants, the mutants of the glycine residue at the sharp turn in position 17 (G242A/G278A) and the aromatic F286 and W287 residues in the C-terminal loop. The Ala replacement of the strongly conserved Tyr residue ending the second rung (Y281) led also to a marked decrease in the number of active transformants. The other mutations having a lesser effect on activity were the mutation of the Asn pair forming the first asparagines ladder (N226/N262, position 1). Mutation of the second Asn pair (N243/N279, position 18) had no effect in agreement with the lower conservation of this second N-pair. In general, mutation of the core hydrophobic residues did not reduce activity significantly with the exceptions of I231A and K277A. Also the Ala replacement at positions E234, K270 and R236 affected the prion function. These residues are involved in the second and third salt bridges in the HET-s fold. Mutations in the residues forming the first salt bridge (E229A/E265A, position 4) had no detectable effect on function in this assay. The variant Q240A showed a reduced activity. Q240 is located close to W287 in the structure [28] and it might be that Q240A also affects this pocket region. Indeed, solid state NMR studies of the variants at the C-terminus results in chemical shift changes of Q240 (as discussed below and Figure S3 and S4). The D288 residue is also located in that spatial neighborhood. The mutational approach points to an instrumental role of this region in prion propagation although this region is not part of the β-solenoid core.

Based on this first series of mutants, we generated a second set of mutants to specifically test several hypotheses. First, because our results suggest that the salt bridges are not essential for activity, we generated a triple mutant that leads to inactivation of all three salt bridges (E234A, E236A, E265A) and found it to be active (Figure 2). In respect to this observation it is noteworthy that, there appears to be an overall conservation of the salt-bridges at position 4 and 9 of the repeat motif, but some HET-S orthologs such as _Cg_ HET-S lack these salt bridges completely (Figure 1B). Next, we have generated a double mutant with both N ladders replaced (N226A and N243A). This mutant lacked activity suggesting that at least one N-ladder is required for function and thus providing evidence for a functional role also for the second N-ladder. In both rungs there is a conservation of an Arg residue (R238/R274, position 13). Yet, single Ala replacements at these positions showed normal function. In a R238A/R274A double variant however the prion function is affected. This result could explain conservation at that position. Finally, we reasoned that if a reduced activity of I231A is caused by a reduction of the hydrophobicity of the core, one might be able to introduce a compensatory mutation increasing hydrophobicity elsewhere in the core. We thus changed the A228 residue of the core by a larger residue. We generated a I231A/A228V double variant and found that this double mutant recovered wild-type like activity.

We concluded that in this multicycopy assay, the function of HET-s appears to be very robust with very few mutations having a very strong effect on prion activity, namely G242A and G278A in the turns after the triangular core and P286A and W287A in the C-terminal pocket. Double mutants could also demonstrate the functional importance of other elements such as the N-ladders, the hydrophobic core (i.e. I231), and the Arg row at position 238/274.

Detailed analysis of selected mutants assayed as single copy integrants

The multicycopy assay allows an initial functional characterization of the mutants but does not allow for a detailed and sensitive analysis of phenotypic differences. We have thus selected a number of mutants for a more detailed functional characterization as single copy integrants. In order to have more subtle and sensitive assays for prion function, we have re-introduced the mutant allele at the resident het-s locus. We chose to further analyze a subset of seven single mutants N226A, Q240A, E272A, K284A, F286, W287, D288A and the R238A/R274A and T233A/S273A double mutants and the E234A/R236A/E265A triple mutant. Q240A, K284A, F286A, W287A and D288A were chosen to investigate the role of the C-terminal loop region. The triple E234A/R236A/E265A mutant was chosen to analyze in more detail the effect of the elimination of all three salt-bridges.
R238A/R274A and T233A/S273A double mutants were included to analyze respectively the role of the conserved Arg residues close to the loop and the role of the partner hydroxyl residues in the core.

Strains with the corresponding gene replacements were generated and tested for [Het-s] function. The three mutants F286A, W287A and R238A/R274A led to a null phenotype as single copy integrants. This result confirms the functional importance of the aromatic residues of the C-terminal pocket and also stresses the functional importance of the conserved Arg residues at position 13 of each repeat. The remaining mutants were able to express the [Het-s] phenotype (i.e. infect a [Het-s*] strain and produce a barrage reaction to het-S) and were tested for three other criteria of prion formation and propagation efficacy. First, we determined the spontaneous prion formation rate (Table 1). Secondly, we measured the rate of spreading of the prion infection in the mycelium, reflecting prion propagation efficiency (Figure 3). This propagation rate is measured in an experimental setting where the length of the barrage line corresponds to the distance of prion spreading in a given period of time [54] (Figure S2). Third, the rate of spore-killing in the sexual cycle (a measure of prion maintenance during the sexual cycle) was determined [24], (Table 2). These three criteria have been compared previously between wt and a D PaHsp104 mutant. All three were found to be diminished in this mutant background [55].

In these assays, all mutants were affected for at least one of the measured criteria. There was in general a good correlation between the effect of the mutations on propagation rate and spore killing rates. K284A was an exception as this mutation affected spore-killing rates although the propagation rate was close to wt. The E234A/R236A/E265A triple mutant lacking all three salt bridges was significantly affected in both assays, suggesting that the salt bridges participate in [Het-s] function although the effect of the mutation went unnoticed in the more basic multicopy assay (Figure 2). When spontaneous prion formation rates were measured, it appeared that N226A and K284A had significantly reduced spontaneous prion formation rate (Table 1). For K284A, 700 strains were tested but none had spontaneously acquired [Het-s]. Interestingly, we measured an increase in spontaneous prion formation rate in D288A and the T233A S273A double mutant. These mutants represent to our knowledge the first HET-s mutants with enhanced prion behavior (Table 1).

Solid-state NMR of amyloid fibrils of HET-s(218-289) variants

In order to get insights into the structural consequences of the Ala replacements, 2D 13C-13C dipolar assisted rotational resonance (DARR) solid state NMR spectra of amyloid fibrils of the 15N,13C-labeled HET-s(218-289) variants K229A, I231A, V239A, Q240A, L241A, N262A, V264A, V267A, E272A, S273A, R274A, I277A, G278A, N279A, F286A, D288A and the double variant F286A/W287A were recorded and compared with the corresponding spectrum of 15N,13C-labeled wild-type HET-s(218-289) amyloid fibrils [27,56]. Since the chemical shift is a highly sensitive probe of the electronic surrounding structure, the measurement of the 2D 13C-13C DARR spectrum can be regarded as a fingerprint of the 3D structure. Thus, a comparison of the DARR spectrum of wild-type and Ala-variant fibrils enables a straightforward analysis of the preservation or alteration of the β-solenoid fold (Figure 1A). This atomic-resolution analysis is of particular importance in the establishment of a structure-activity relationship of amyloids.
because many amyloidogenic systems are prone to polymorphism and thus a secondary structure analyses or structural comparisons conducted at the mesoscopic level may not be sufficient to reveal structural preservation or alterations.

As shown in Figure 4, the 2D DARR spectra of K229A, I231A, V239, Q240A, L241A, N262A, V264A, E265A, V267A, E272A, S273A, R274A, I277A, E279A, F286A, W288A, and D288A closely resemble the wild-type spectrum. This indicates that the overall structure of the listed variants is conserved. A detailed inspection of the individual spectra shows in general, small chemical shift differences of residues close in space to the replaced amino acid side chain (in addition to the lack of the signal of the replaced side chain), which is attributed to local structural changes. For example, in the 2D DARR spectrum of the N262A variant, cross peak changes of the spatially close residues I231, T261, S263, V244, I277, and Q240, making it difficult to assess whether the double variant forms the β-solenoid fold (Figure 4). In conclusion, the comparative analysis of the solid state NMR spectra suggests that all the single Ala variants with the exception of G278A form the β-solenoid fold. With the exception of G278A, all the single Ala variants can therefore be used to explore the detailed structure-infectivity relationship of the HET-s prion.

**Stability measurements of amyloid fibrils of HET-s(218–289) variants**

One of the consequences of a side chain replacement with Ala may be a change in the stability of the protein fold, which may influence function [57,58,59,60]. Applied to HET-s this would mean that a change of the stability of the β-solenoid may alter heterokaryon incompatibility, spontaneous prion formation, prion propagation and spore killing (Figure 2, Figure 3, Tables 1 and 2). In order to get insights into the individual contribution of the amino acid side chains to the stability of the HET-s prion, Ala variants K229A, I231A, V239A, Q240A, L241A, N262A, V264A, V267A, R274A, F286A, W287A were measured by a fibril denaturation assay using GuHCl (Figure 5 and Table 3) following the concept by Santoro and Bolen to study protein folding and unfolding [61]. However, here we studied only the fibril denaturation process. This was conducted at the mesoscopic level may not be sufficient to reveal structural preservation or alterations.

### Table 1. Spontaneous [Het-s] prion formation rate for alanine mutants of HET-s.

| Strain | (Het-s)/total tested | % of prion formation | p-value (significance of difference to wt)** |
|--------|----------------------|-----------------------|--------------------------------------------|
| WT     | 13/600               | 2.1%                  | -                                          |
| F286A  | 0*                   | n.a                   |                                            |
| W287A  | 0*                   | n.a                   |                                            |
| R234A R274A | 0*               | n.a                   |                                            |
| N226A  | 3/500                | 0.6%                  | <0.05                                      |
| Q240A  | 1/400                | 0.3%                  | <0.02                                      |
| E272A  | 15/400               | 3.7%                  | n.s.                                       |
| K284A  | 0/700                | 0                     | <0.0001                                    |
| D288A  | 21/500               | 4.2%                  | <0.05                                      |
| E234A R236A E265A | 6/500              | 1.2%                  | n.s.                                       |
| T233A S273A | 24/500             | 4.8%                  | <0.02                                      |

*these mutants are unable to propagate [Het-s] even after infection by wt [Het-s].

**p-value is calculated in a two-tailed Fisher’s test, n.s. non significant.

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Such a fibril denaturation reaction mechanism can be of complex nature but here a simplified two-state process was assumed for the quantitative analysis. HET-s fibril denaturation studies have been performed previously using Trp fluorescence as probe for fibril denaturation [41], because the unique Trp residue (W286) is located in the C-terminal loop rather than in the actual core, we favored the use of light scattering because it might constitute a better probe for the overall unfolding of the fibril since Trp fluorescence might be affected by local unfolding of the loop.

The individual denaturation curves in triplicates show a sigmoidal behavior (Figure 5). [61]. For most of the variants (i.e. wild-type, K229A, V239A, Q240A, L241A, N262A, V264A, V267A, E272A, S273A, F286A, D288A) the measurements appear to be reproducible. Only for G278A and possibly the double variant F286A/W287A a lack of reproducibility is observed (Figure 5). The lack of reproducibility is attributed to the altered, possibly polymorphic structure as evidenced by the solid state NMR spectra (Figure 4). A further qualitative inspection of Figure 5 suggests that the variants K229A, V239A, L241A, V264A, V267A, E272A, S273A, and D288A (labeled group 1 in the following) show sigmoidal denaturation curves similar to wild-type, while the denaturation curves of the variants I231A, Q240A, N262A, G278A, F286A (group 2), and the double mutant F286A/W287A have a different form and properties and are sometimes less reproducible (as just stated) (see also Table 3, fifth column).

The two-state analysis of the denaturation curves yields two parameters for the unfolding transition: the $m$-value and the Gibbs free energy $\Delta G$. The $m$-value is the steepness of the transition and is generally thought of as a measure of the change in solvent accessible surface area upon unfolding and reflects how cooperative the unfolding transition is [63,64,65]. The change in Gibbs free energy ($\Delta G$) is a measure of the contribution of the amino acid side chain to the protein unfolding energy (given that the structures of the folded and unfolded states as well as the unfolding pathway are otherwise unperturbed). The variants of group 1 can all be fitted with a two-state model, most of them with an unchanged $m$-value (i.e. the $m$-value of the wildtype HET-s(218–289) prion). All the group 1 variants had an unaltered $\Delta G$ value.

Table 2. Spore-killing activity of selected het-s mutants in a cross a with het-S male parent.

| maternal parent in cross | 2-spored asci/total counted asci | spore-killing (%) | p-value (significance of difference to wt)* |
|--------------------------|----------------------------------|------------------|-------------------------------------------|
| het-S                    | 0/272                           | 0                | <0.0001                                   |
| het-s wt                 | 445/632                         | 70.4             | -                                         |
| N226A                    | 7/664                           | 1                | <0.0001                                   |
| Q240A                    | 0/425                           | 0                | <0.0001                                   |
| E272A                    | 328/746                         | 44               | <0.0001                                   |
| K284A                    | 31/506                          | 5.8              | <0.0001                                   |
| D288A                    | 190/509                         | 37.3             | <0.0001                                   |
| T233A/S273A              | 311/836                         | 37.2             | <0.0001                                   |
| E234A/R236A/E265A        | 94/654                          | 14.4             | <0.0001                                   |

*p-value is calculated in a two-tailed Fisher’s test. doi:10.1371/journal.ppat.1004158.t002

Figure 3. Propagation rate of [Het-s] in single copy integrants of alanine mutants. A. For wt and each mutant, the rate of [Het-s] propagation was measured in the experimental setting in which the length of the barrage line gives the distance of [Het-s] spreading in a period of 10 hours. B. Mean propagation distance in at least 4 experiments with standard deviation. doi:10.1371/journal.ppat.1004158.g003
or were up to 2 kcal/mol more stable (Table 3). The positive ΔΔG value for K229A of 1.5 kcal/mol indicates that the salt bridge between K229 and E265 does not play a favorable stability effect on the β-solennoid structure. Similarly, the replacement E272A (removing the E272-R236 salt bridge) results in a positive stability effect of approximately 2 kcal/mol. The amino acid replacements in the hydrophobic core at position V239, L241, V264 also had a 1 kcal/mol positive effect on stability V267 had no effect. This is in line with the in vivo data showing that none of these variants were affected for the prion phenotype (Figure 2). In combination with the solid state NMR spectra (Figure 4 and see above), these unfolding measurements indicate that the Ala variants of group 1 are composed of the same β-solennoid 3D structure and the amino acid side chain replacement had no negative effect on its unfolding transition.

In contrast, the GuHCl denaturation curves of group 2 variants (Figure 5) are distinct from wild-type showing a flattened slope (lower m-value) (i.e. I231A, Q240A, and N262A) indicative of a decrease in unfolding cooperativity or a change of the surface. The latter potential explanation finds support in the surface location of the Q240. Some replacements comprise a complex unfolding transition exemplified by an immediate loss of light scattering at low GuHCl concentrations (i.e. I231A, Q240A, N262A, and F286A) or poor reproducibility (i.e. G278A and F286A/W287A), thus for these variants a Gibbs free energy calculation has not been considered (see Table 3). It is worth mentioning, that the less reproducible nature of the denaturation curves of G278A and F286A/W287A is reflected in the less well defined solid state NMR spectra (Figure 4). Together the data strengthen the notion that these two variants are not able to fold properly into the β-solennoid structure. The effect on the denaturation curve of N262A is attributed to the loss of the conserved Asn ladder. The I231A in the hydrophobic core replacement appears to influence the stability and unfolding pathway (Table 3). The NMR analyses also indicated an important structural role of this residue as the I231A replacement leads to a substantial amount of chemical shift perturbations in the β-solennoid fold (Figure 4, Figure S3 and S4). Most interestingly, with the exception of D288A there appears to be a perfect correlation between the decrease in prion phenotype formation in vivo determined by the heterokaryon incompatibility assay (Figure 2) and the change in the denaturation curve profile (Table 3).

The F286A and W287A variants of C-terminal pocket are null alleles but the corresponding proteins form infectious fibrils in vitro

The F286A and W287A mutants affecting the C-terminal semi-hydrophobic pocket stand out in virtue for their drastic effect on [Het-s] activity although the corresponding residues reside outside the rigid β-solennoid core and do not alter the β-solennoid structure significantly (Figure 4). Only the double variant F286A/W287A may be unable to form the β-solennoid structure as suggested by the solid state NMR studies (Figure 4) and the GuHCl denaturation curves (Table 3, Figure 5). Moreover, two further mutants in the same region K284A and D288A also modulate prion behavior in a subtle way as the first mutation decreases prion formation while the other in fact increases the prion formation rate (Table 2).

However, the D288A variant shows a wild-type-like solid state NMR spectrum and is ca. 1.2 kcal/mol more stable than wild-type, having otherwise a wild-type-like GuHCl denaturation curve (Figure 4 and 5). We chose to further analyze the mutants in that region for their ability to form aggregates in vivo as GFP fusion proteins. We found that neither F286A nor W287A led to the formation of dot-like aggregates in vivo, however when the mutant allele as GFP fusion proteins were introduced in a strain expressing wild-type HET-s (Figure 6), the mutant alleles were found to form dot-like aggregates presumably by being integrated into wild-type aggregates, a situation already described for the K284P mutant residing in the same conserved C-terminal loop region [27].

We also tested infectivity of the variants F286A and W287A fibrils generated in vitro and found them to be infectious (Table 4). The level of infectivity of the recombinant fibrils was at least as elevated as for wild-type fibrils. Infectivity could be detected in the same level of dilutions as for wild-type fibrils. These results indicate that once the amyloid fold is acquired, these mutants display wild-type seeding activity in vivo.

Discussion

The [Het-s] prion of the filamentous fungus Podospora anserina represents an example of a functional amyloid. The amyloid fold serves as an activation trigger for inducing the transformation of HET-S into a toxin [30]. We have conducted a mutational analysis of HET-s PFD to assess the role of specific residues on prion function and propagation, as well as amyloid structure and stability. It is observed here that only very few Ala point mutations are able to abolish prion function, alter significantly the β-solennoid structure and reduce fibril stability. Most mutants retain partial function indicative of a robust prion. These studies also revealed a previously unsuspected role for the C-terminal pocket region in prion function.

Structure-function of the HET-s fold

The determination of the HET-s β-solennoid structure exposed the existence of a number of structural elements defining this fold, such as the hydrophobic core, the asparagines ladders, the sharp turns connecting the β-strands termed arches [66], the salt bridges, the internal hydrogen bond between the Thr and Ser residing in different β-strand layers and the C-terminal loop folding back onto the core [28,29]. The functional importance of the individual β-strands was documented before in a mutational approach involving proline substitutions [27]. Here, it is shown that to various degrees, the other described structural determinants also participate in prion function.

Ala replacement of the strictly conserved G278, leads to a null prion phenotype and this variant is unable to form the β-solennoid structure as evidenced by the solid state NMR studies. This effect is very likely due to the importance of the glycine residue in the arc between β-strands β4a and β4b for the β-solennoid fold (Figure 1). Ala replacements in the asparagene ladder affected function in vivo and the N262A variant shows an altered denaturation curve. The hydrophobic core residues I231 and I277 appear to be important for prion infectivity (Figure 2) and in the case of I231A also for β-solennoid unfolding and structure (Table 3, Figure 4). Also, the
substitution of the highly conserved Thr/Ser pair leads to a detectable alteration of prion function but only in the spore-killing assay, while the S273A variant is 1.4 kcal/mol more stable. For the prion function in vivo, the solvent exposed salt bridges are important determinants, but their contribution to the β-solenoid stability is negative (i.e. E272A is 1.9 kcal/mol more stable than wild-type). Sequence comparisons show a preference for a positively charged residue in position 13 of each repeat (R238 and R274 in HET-s) (Figure 1). The functional significance of these conserved Arg residues is not directly evident. While the single Ala replacement did not change the prion activity, the R238A/R274A double mutant leads to a null phenotype thus providing a functional justification for the sequence conservation. A possible explanation of the role of these positively charged residues could be an interaction with the C-terminal loop and in particular the penultimate conserved Asp residue. In support of this hypothesis, is the fact that the two sequences that are exceptions to the conservation of positively charged residues at these positions also lack the C-terminal glycine-rich loop like Fc_Cl52-2 and Nh_mpfVI-1. (Figure 1B).

In the presented mutation scan, two mutants were found, for which spontaneous prion formation rate was increased rather than decreased, the D288A mutant and the T233A/S273A double mutant. This would indicate that the HET-s PFD does not have an optimal behavior in terms of prion formation rates. It might be that the increased prion formation rate of D288A is due to the higher stability of the variant (i.e. ΔG = +1.2 kcal/mol, Table 3) or to a modification of the properties of the C-terminal loop which is functionally important for a prion phenotype (see below). The T233A/S273A double mutation increases the overall hydrophobicity of the core. Possibly, this increase in hydrophobicity favors a hydrophobic collapse and subsequent prion nucleation and/or stabilizes the β-solenoid fold since already the amyloid of S273A alone is 1.4 kcal/mol more stable (Table 3). Interestingly, the D288 and the S273 residues (and to a lesser extend T233) are conserved positions (Figure 1B). Thus, one may hypothesize that conservation at these positions reflects the need to reduce spontaneous β-solenoid folding because a too strong propensity for spontaneous folding might lead to uncontrolled activation of the HET-s pore-forming toxin. Similarly, the fact that several replacements increase rather than decrease stability also suggests that the HET-s PFD sequence is not optimized for highest possible stability. The theoretical model of prion propagation by the Weissman group [67] and others [68,69] highlights that for the prion propagation, amyloid fibril fragmentation is necessary. Thus, highest possible amyloid stability may not be compatible with the function of the HET-s fold.

Modulating prion formation by a short sequence stretch located outside of the cross-β core

One finding of this study is the detection of the functional importance of a 7 amino acid residues C-terminal loop starting at

![Table 3. Gibbs free unfolding energy of HET-s(218-289) amyloid fibrils.](image)

**Table 3. Gibbs free unfolding energy of HET-s(218-289) amyloid fibrils.**

| Variant     | ΔG (kcal/mol) | ΔΔG (kcal/mol) | m-value | Reproducibility and denaturation curve shape when compared to wt | In vivo [Het-s] Property ([Het-s] activity/propagation rate/spore killing) |
|-------------|---------------|----------------|---------|---------------------------------------------------------------|-------------------------------------------------------------------------|
| WT          | 2.9±0.2       | 0.5±0.2        | wt      | y, wt                                                         | [Het-s] enhanced prion formation, not wt-like, wt-like                              |
| K229A       | 4.4±0.0       | 1.5±0.2        | wt      | y, wt-like                                                   | [Het-s] reduced/reduced propagation and spore killing                      |
| I231A       | n. d.         | n. d.          | not wt  | y, no plateau at low GuHCl conc.                            | [Het-s] reduced                                                            |
| V239A       | 3.9±0.5       | 1±0.5          | wt      | y                                                            | wt-like                                                                  |
| Q240A       | n. d.         | n. d.          | not wt  | n, no plateau at low GuHCl conc., not a two-state process    | [Het-s] reduced/reduced propagation and spore killing                      |
| L241A       | 3.9±0.1       | 1.0±0.3        | wt      | y, wt-like                                                   | wt-like                                                                  |
| N262A       | n. d.         | n. d.          | not wt  | y, not a two-state process                                   | [Het-s] reduced                                                            |
| V264A       | 4.0±0.2       | 1.0±0.3        | wt      | y, wt-like                                                   | wt-like                                                                  |
| V267A       | 2.9±0.1       | 0.2±0.3        | wt      | y, wt-like                                                   | wt-like                                                                  |
| E272A       | 4.8±0.1       | 1.9±0.3        | wt      | y, wt-like                                                   | wt-like/reduced propagation                                                |
| S273A       | 4.3±0.1       | 1.4±0.2        | wt      | y, wt-like                                                   | wt-like                                                                  |
| G278A       | 2.5±0.3(10)   | 1.3±0.1(10)    | not-wt  | n, no plateau at low GuHCl conc.                            | [Het-s] strongly reduced                                                  |
| F286A       | 4.1±0.1       | 1.2±0.3        | not wt  | y, not a two-state process                                   | [Het-s] strongly reduced                                                  |
| D288A       | n. d.         | n. d.          | not wt  | y, not a two-state process                                   | [Het-s] enhanced/enhanced prion formation                                 |
| F286A/W287A | n. d.         | n. d.          | not wt  | y, not a two-state process                                   | n.d.                                                                     |

(1) The ΔG value was obtained using the m-value of the wt unless stated in the fourth column that the curve fitting was improved with a free m-value different from the corresponding wt one.

(2) The ΔG value was obtained using a free m-value. Two different denaturation curves were obtained and their individual ΔG values are listed.

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the end of β-strand β4b and ending at the C-terminus of the protein (GKGFWDN, residue 283 to 289). The only previous indication of the functional importance of this region came for the observation that the K284P mutation affects HET-s aggregate formation in vivo [27]. Structurally, this region forms a semi-hydrophobic pocket that folds back onto the space delimited by the third and fourth strand of each rung [28]. This part of the structure is less well defined and appears to be more dynamic than the core region. The individual mutations of the two aromatic residues F286A and W287A have a very strong effect on activity. Actually, these two mutations are the most severe ones in our set of 32 mutants together with the mutations of the Gly in the arc positions (i.e. G242/G278). In addition, the mutations of the conserved charged residues in that region K284A, D288A (and the structurally nearby Q240A) also affect function. As noted above, the D288A mutation produced an unexpected effect on prion function as this mutation increased spontaneous [Het-s] prion formation. This is the first mutation found to increase [Het-s] prion formation. Of note however is that other criteria such as the spore-killing activity are in fact slightly reduced in that mutant.

A recent molecular dynamics simulation study of the HET-s(218–289) β-solenoid suggested that the F286A and W287A mutations reduce flexibility at the fibril ends and that this flexibility is an important determinant for prion fibril infectivity [70]. However, F286A and W287A are able to form fibrils in vitro that display the same levels of infectivity as wild-type fibrils suggesting that mutation of the aromatic residues does not critically affect templating activity and the 3D structure of the β-solenoid fold. Alternatively, the hydrophobic nature of F286 and W287 may be important for an initial non specific hydrophobic interaction of the incoming molecule with the prion template. This hypothesis is supported by the solid state NMR spectra of both variants indicating no significant structural changes of the β-solenoid fold. Rather it appears that this region might be required for the prion formation process without actually being part of the cross-β part of the fold. These interpretation illustrates that amyloid folding can be dramatically modulated in a functionally relevant way by a sequence stretch that is not actually part of the cross-β structure per se. Strikingly, this part of the HET-s PFD sequence turns out to be the region of the protein with the highest level of sequence conservation. While, the repeat region can accommodate a significant level of divergence and still retain the ability to adopt the β-solenoid fold, the ability for this short C-terminal region to act possibly as a folding inducer of the repeat region appears highly constrained at the sequence level. The β-solenoid forming region of HET-s/HET-S is thus located between the HeLo domain which can have a prion inhibitory function and the C-terminal pocket region which acts as a prion promoting region.

Figure 6. Expression of a HET-s-GFP F286A and W287A fusion proteins in P. anserine. HET-s-GFP F286A and W287A were expressed in Δhet-s P. anserina strain or the same strain over-expressing wild-type HET-s. Note that both mutants are unable to form dot-like aggregates in Δhet-s but can be incorporated in dot-like aggregates in the presence of wild-type HET-s.

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Table 4. Infectivity of recombinant HET-s(218-289) F286A and W287 fibrils.

| Amount of protein | [Het-s]-infected strains over total tested strains |
|-------------------|--------------------------------------------------|
| no protein        | 1/96                                             |
| HET-s(218–289) wt  |                                                  |
| 250 μM            | 63/96                                           |
| 50 μM             | 15/72                                           |
| 10 μM             | 1/48                                            |
| 2 μM              | 0/24                                            |
| HET-s(218–289) F286A|                                                  |
| 250 μM            | 69/96                                           |
| 50 μM             | 25/72                                           |
| 10 μM             | 5/48                                            |
| 2 μM              | 0/24                                            |
| HET-s(218–289) W287A|                                                  |
| 250 μM            | 68/96                                           |
| 50 μM             | 12/72                                           |
| 10 μM             | 2/48                                            |
| 2 μM              | 0/24                                            |

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These results revealing the existence of a short amyloid folding modulator stretch that is not directly involved in the cross-β core could be of general importance in the identification of sequence determinants for relevant amyloid formation and in efforts to control amyloid aggregation in disease related systems.

**Level of complexity of the β-solenoid fold**

Since many proteins are able to aggregate into amyloid-like entities and since the inter-molecular β-sheet formation with its many hydrogen bonds is an essential determinant of the cross-β-sheet motif, it has been suggested that the formation of amyloid fibrils is mainly a generic property of the polypeptide backbone and that the side chains play a minor role [71]. On the contrary, amyloid aggregation is highly amino-acid sequence specific as demonstrated by the intermolecular side chain interactions observed in the crystal structures [72,73,74] and the essential involvement of side chain interactions in the aggregation process as evidenced for example by the experimentally-derived scale of amino-acid aggregation-propensities [75,76,77]). These observations accompanied with the predictive power of several algorithms for the cross-β aggregation propensity of polypeptide sequences [78,79,80] suggests that the cross-β state is less complex than most structures of soluble proteins, indicating that the complexity of the cross-β fold may fall somewhere between a secondary and a tertiary structure [2].

This notion of intermediate folding complexity of amyloids is now supported by the stability measurements of the HET-s Ala variants. Indeed, our study reveals both the absence of destabilizing effect for many replacements but also the critical importance of certain key residues. While the loss of side chain hydrogen bonds, salt bridges and hydrophobic interactions in soluble proteins yields in general a ΔΔG loss of ~2 kcal/mol [58,59,60], in the case of the HET-s prion, the ΔΔG for the group 1 variants with K229A and E272A deleting a salt bridge, S273A getting rid of a side chain hydrogen bond, and V239A, L241A, V264A, and V267A reducing the hydrophobicity of the core, is either insignificant or with a positive value of up to +2 kcal/mol (Table 3). In particular, hydrophobic core residue replacements have little effect (+0.6 kcal/mol in average; Table 3) compared to the average ΔΔG of ~2 kcal/mol predicted from data of soluble proteins. Only a few side chains appear to be crucial for the formation of the β-solenoid structure (such as for instance I231 and N262). Side chain replacements of such residues destabilize the denaturation curve. Thus, it appears that alteration of the β-solenoid fold may fall somewhere between a secondary and a tertiary structure.

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**Materials and Methods**

**Strains, plasmids and media**

*P. anserina* strains used in this study were wild-type het-s, het-S, Ahet-s strains and the APaHsp104 strain [55]. Growth medium for barrage assays and prion transmission assays was standard corn meal agar DO medium. The Ahet-s strain was constructed by inserting the nat1 cassette from the pAPI508 plasmid [81] in place of the het-s ORF. The nat1 cassette was amplified with oligonucleotides 5’ CTTCCTGGACACTCTCTGACAC 3’ and 5’ ATCCTAGATGACTTAAGACGACAG 3’. The sequences upstream and downstream of the het-s ORF were amplified respectively with oligonucleotide pairs 5’ AAGCTTTCG- GAATTGCTCTTCAG 3’ and 5’ GGACGGATTTGACGG- GAAACGGAAG 3’ and 5’ GGACTAGTACCTCCTGAG- CAAGATAGC 3’ and 5’ GCACGCGGATCAGGGCATT- GCATCTGGG 3’. The fragments were ligated to create the het-s:nat1 cassette cloned as a HindIII-Nol fragment in a pSK plasmid. This cassette was used to transform a het-s APK670 strain (81). Nourseothricine resistant transformants compatible with a het-S tester were selected. Inactivation of het-s was verified by PCR using oligonucleotides 5’ CGACGATCACGACATACGCT- GTGG 3’ and 5’ ATCGGGCTCTCCCTGACCTGCTTC 3’.

The strain was then backcrossed once to a het-s wild-type strains and Ahet-s (NouK and het-s compatible) Ahet-s APK70 strains (NouK, PhleB and het-S compatible) were selected in the progeny.

**Prion propagation, prion formation and incompatibility assays**

Methods for determination of incompatibility phenotypes, prion formation and prion propagation were as previously described [54]. In brief, incompatibility phenotypes were determined by confronting strains of solid corn meal agar medium to [Het-s] and [Het-S] tester strains and visualizing the formation of barres (abnormal contact lines forming upon confrontation of incompatibile strains). [Het-s] prion propagation was assayed as the ability to transmit the [Het-s] prion from a [Het-s]-donor strain to a [Het- s*] prion-free tester strain after confrontation on solid medium. Prion formation rates of [Het-s] were determined by measuring the fraction of [Het-s*] or subcultures that spontaneously acquired the prion phenotype after 5 days of growth at 26°C.

**Microscopy**

For fluorescence microscopy, synthetic medium containing 2% (wt/vol) agarose was poured as two 10 ml layers of medium. *P. anserina* hyphae were inoculated on this medium and cultivated for 16 to 24 h at 26°C. The top layer of the medium was then cut out and the mycelium was examined with a Leica DMRXA microscope equipped with a Micromax CCD (Princeton Instruments) controlled by the Metamorph 5.06 software (Roper Scientific). The microscope was fitted with a Leica PL APO 100X immersion lens.

**Protein preparation and fibril formation**

HET-s(218–289), HET-s(218–289) Ala variants proteins were expressed in *E. coli* and purified as previously described [27]. Both proteins had a C-terminal 6 histidine tag and expressed as insoluble proteins and purified under denaturing conditions using Qiagen columns. Yields were in the range of 10 mg/L of culture. Proteins were eluted in 6 M GuHCl 50 mM Tris–HCl pH 8, 150 mM NaCl, 200 mM imidazole. Elution buffer was replaced by 175 mM acetic acid by passage on a 5 ml Hitrap column (Amersham). After lyophilisation the samples were stored at −20°C.

The protein variants were dissolved in diluted HCl (45 mM) and the concentration adjusted to 90 μM which was affirmed by UV absorption at 280 nm. The solution was filtered through a 0.2 μM filter and Buffer E (Tris [3 M]; NaCl [1 M] HCl [45 mM] 1/19 V) was added such that the end concentration of NaCl and Tris were 150 and 50 mM respectively. If necessary pH was adjusted to 7.4 using NaOH (4 M). Samples were rotated at 37°C on a rotator at constant speed for at least one week.
Protein transfection assays
Protein transfection experiments with amyloid fibrils of recombinant HET-s(218–289) variants were performed using a cell disruptor (Fast-prep FP120, Bio101, Qbiogen Inc.). For each test, ~0.5 cm² of [Het-s] mycelium grown on solid medium is sheared (run time 30 s, speed 6) in 500 µl of STC50 buffer (0.8 M sorbitol, 50 mM CaCl₂, 100 mM Tris HCl pH 7.5) and the sonicated HET-s(218–289) amyloids assembled at pH 7 (20 µl at 1 mM) in a 2 ml screw cap tube. The sheared amyloid is then diluted with 600 µl of STC50 buffer and then plated onto DO~0.8 M sorbitol medium and incubated at 26°C until being confluent (7–8 days). Several implants (at least two per mycelium) are checked for the [Het-s] phenotype in barrage tests.

Denaturation measurements of amyloid fibrils of HET-s(218–289) variants
The fibril containing samples of the various HET-s(218–289) variants were concentrated after centrifugation (20000 g; 10 min) to 500 µM. The fibrils were then diluted in the different GuHCl stock solutions (between 0–8 M in 0.5 M steps) to an end concentration of 20 µM. It is important to note that the fibril samples were added to the GuHCl solution by an Eppendorf pipette with a single extrusion. The samples were incubated at room temperature without shaking for one day or if stated for one week, respectively. The extent of amyloid disassembly was monitored by the measurement of the optical density at 500 nm (OD₅₀₀) on a JASCO V-650 spectrophotometer with quartz cuvettes and an adaptor from Eppendorf. The OD₅₀₀ is believed to be an indirect measure of the amount of protein aggregation. Each experiment has been measured three times starting in part from different sample preparations. The analysis and extraction of the ΔG and m-value was done by the software package MATLAB following the mathematical formula and procedures of Santoro and Bolen [61].

As stated above the samples were incubated before the OD₅₀₀ measurement at the various concentrations of GuHCl for one day in order to reach equilibrium conditions. Longer incubation periods such as one week did either not alter the measurements as demonstrated for the V267A variant in Figure S5, or a flattening of the denaturation curve was observed as shown for the V264A variant (Figure S5). The flattening of the curve is interpreted as a restructuring of the amyloid aggregate during the long incubation time of one week, which let us concentrate on the use of the denaturation measurements with one day incubation for the quantitative analysis. Although in the case of V264A the extracted ΔG value did not alter between the two type of measurements (Figure S5), the change of the denaturation curve indicates that in GuHCl only a pseudo-equilibrium is obtained. Another point worth mentioning is that highly reproducible denaturation curves were obtained by incubating the protein in GuHCl solution without shaking, while in presence of shaking the reproducibility was only moderate (data not shown).

Solid state NMR of amyloid fibrils of ¹⁵N,¹³C-labeled HET-s(218–289) variants
All the solid-state NMR experiments were conducted on a AVANCE II Bruker 600 MHz [14, 1 T] spectrometer using a Bruker 3.2 mm probe, DARR spectra with 20 ms mixing time were obtained from ¹⁵N,¹³C-labeled HET-s(218–289) variants [82]. All the spectra were processed using Topspin 2.0 (Bruker Biospin), using a shifted cosine squared window function as indicated in the Figure captions and so called zero-filling was done to the next power of two in both dimensions. Automated baseline correction was applied in the direct dimension. For spectral analysis the software package CcpNMR Analysis [83] was used and the chemical shift list was taken from [56].

Sequence analysis
HET-S homologs were recovered from available genome databases at ncbi (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and jgi (http://genome.jgi-psf.org/) using BLAST searches. Multiple alignments were performed with CLUSTALW2 at http://www.ebi.ac.uk/Tools/msa/clustalw2/. The consensus sequence of the R1 and R2 repeats were generated using MEME at meme.nbcr.net/ [53]. The MEME output gives a graphical representation of the consensus sequence as a weighted consensus in which the size of the letter designating a given amino acid is proportional to the conservation of the residue in the different sequences used to generate the motif. The size of the character reflects the information content measured in bits.

Supporting Information
Figure S1 Alignment of N-terminal region of HET-s/ HET-S homologs. The N-terminal regions (1–34/35) of the sequences listed in File S1 have been aligned with ClustalW2. The HET-s and HET-S sequences are boxed in red. At position 33 (H3 in HET-S and P33 in HET-s), no sequence is of the HET-s type and most sequences are of the HET-S type. In addition to H, amino acids found at that position are R, Q and N. It was shown that H33R, H33Q and H33N HET-S variants retain het-s specificity [51,52]. The sole exception is the Nh-mpIV-4 sequence from Neetia haematococca showing a L at that position. The H33L mutation in HET-S leads to the het-s specificity [51,52]. In addition this sequence contains a deletion in the N-terminal region, making it possible that this sequence corresponds to a HET-s homolog.

(TIF)

Figure S2 Experimental setting used to measure [Het-s]-propagation rate. A row of 6 wild-type and mutant [Het-s] subcultures are inoculated on solid medium and the first strains of the row is infected with wild-type [Het-s] (grey arrow) and confronted to HET-S tester strains, the prion propagates from one subculture to another. The barrage line in the confrontation zone with the HET-S tester shows the progression of the prion infection at the time of contact with the HET-S tester, that is about 10 hours after infection of the first strain in the row. The distance measured between the two white diamonds is the distance reported in Figure 3. (TIF)

Figure S3 2D Solid state NMR spectra of Ala variants of HET-s(218–289) amyloid fibrils for variants K229A to V267A. The 2D DARR solid state NMR spectra of ¹⁵N,¹³C-labeled Ala variants of HET-S(218–289) amyloid fibrils are shown on top of the corresponding spectrum of wild-type HET-s(218–289) amyloid fibrils, the latter which contour lines are color-coded black. These are the same spectra as in Figure 4 with some cross peak assignment. The comparison between the 2D DARR of the Ala variant amyloid fibrils and the wild-type HET-s(218–289) amyloid fibrils indicates the preservation of the β-solenoid fold of all the Ala variants with small structural changes close to the replaced amino acid side chains as evidenced by small chemical shift perturbations upon Ala replacement. A significant chemical shift change is documented if the cross peak did shift approximately half a line width or more. In the following for each Ala variant these chemical shift perturbations are described: for
K229A there is only a slight chemical shift change observed for resonances of V264. For I231A a substantial amount of spatially local chemical shift perturbations are observed including A228, K229, D230, T233, V239, I241, T246, V267, W268, V275, and I277, as well as the spatially not close residues E235, V244, A247, A248, V267, and I277. Also, D288A did show small chemical shift perturbations. For V239A only the spatially close residues I231, T233, R274, and I271 show chemical shift perturbations. For Q240A only R274, V239, V244, and I277 show slight chemical shift changes; for L241A: A229, K229, I231, E235, Q240, and I277, as well as the structurally further away positioned S273 and V264; for N262A: V264, I231, T261, S263, and I277, as well as the structurally further away positioned T266, and K270; for V264A: only A238 and I277; and for E265A nothing significantly.

For the hydrophobic core residue V267A perturbed chemical shifts are of the core residues K229, I231, I277, V268, T233, and T266 and the spatially not close residue V244; For E272A no significant shifts are observed, for the Thr/Ser ladder residue V264A: only A228 and I277; and for E265A nothing significantly. For the single alanine scanning mutagenesis of the HET-s(218–289) amyloid fibrils for variants E272A to D288A. The 2D DARR solid state NMR spectra of $^{15}\text{N, }^{13}\text{C}$-labeled Ala variants of HET-s(218–289) amyloid fibrils are shown on top of the corresponding spectrum of wild-type HET-s(218–289) amyloid fibrils, the latter which contour lines are color-coded black. These are the same spectra as in Figure 4 with some cross peak assignments. (PDF)

**Figure S4** 2D Solid state NMR spectra of Ala variants of HET-s(218–289) amyloid fibrils for variants E272A to D288A.
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