Prion 4:1, 9-12; January/February/March 2010; © 2010 Landes Bioscience

New insights into the molecular mechanism of amyloid formation from cysteine scanning

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Our laboratory recently reported the identification of a peptide region, QVNI, within the prion domain of the yeast protein Ure2 that may act as an initiation point for fibril formation.[1] This potential amyloid-forming region, which corresponds to residues 18–21 of Ure2, was initially identified by systematic cysteine scanning of the Ure2 prion domain. The point mutant R17C, and the corresponding octapeptide CQVNIGNR, were found to form fibrils rapidly under oxidative conditions due to the formation of a disulfide bond. Deletions within the QVNI sequence cause the fibril formation ability of R17C Ure2 to be inhibited. The aggregation propensity of this region is strongly modulated by its preceding residue: replacement of R17 with a hydrophobic residue promotes fibril formation in both full-length Ure2 and in the corresponding octapeptides. The wild-type octapeptide, RQVNIGNR, also forms fibrils, and is the shortest amyloid-forming peptide found for Ure2 to date. Interestingly, the wild-type octapeptide crystallizes readily and so provides a starting point towards obtaining high resolution structural information for the amyloid core of Ure2 fibrils.

Key words: yeast, prion, amyloid, cysteine scanning, disulphide bond, Ure2, Ure2p, peptide, crystal, initiation site

Submitted: 09/02/09

Accepted: 11/16/09

Previously published online: www.landesbioscience.com/journals/prion/article/10670

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Unravelling Amyloid Structure

The association of amyloid-like fibrils with a number of human diseases[2] as well as with other biological processes[3] has provoked considerable research interest in understanding the mechanism by which fibrils form and how this process can be controlled. Obtaining detailed structural information about how the protein chains are arranged within amyloid fibrils has been the work of several decades, and only recently has significant progress been made.[4-6] NMR has provided important insight into the fibrillar structure of the fungal prions Het-s[7,8] and Sup35.[9,10] Atomic resolution X-ray structural data was obtained first from microcrystals of a Sup35 peptide[11] and this approach has since been extended to a range of amyloid peptides related to human diseases.[12] Techniques such as solid-state NMR[13,14] and H/D exchange combined with mass spectrometry[15] have also been applied to Ure2; however, to date, these have yielded only limited structural information.[5,6] The availability of activity assays for Ure2 has yielded insight into the overall architecture of the protein monomers within the fibrils, indicating that the globular GST-like C-domains have native structure[16,17] and are arranged as dimers within the fibrillar arrays.[18] However, the precise arrangement of the amyloid-like core is unknown and is a topic that has attracted some controversy.[19,20] The slow rate of progress in defining the molecular structure of Ure2 fibrils may be attributed in part to several factors, including heterogeneity of the fibrils,[21-24] the presence of a long flexible linker region between the N- and C-domains,[14] and the lack of identifiable short amyloid-forming peptides which might provide a starting point to obtaining high-resolution structural data.[1]

Using Cysteine Scanning to Gain Insight into Amyloid Fibril Structure

Cysteine scanning, and introduction of disulphides and other linkers, are other

Figure 1. The prion domain sequence of Ure2. The Gln/Asn-rich region of the 354-residue Ure2 protein extends from residues 1–89.[43] The residues mutated to Cys and tested for amyloid-forming ability under oxidizing conditions[1] are underlined. R17 is shown in bold.

approaches that have been used to gain insight into the structure of amyloids,[25] including fibrils of Sup35[26] and Ure2.[27,28] Accessibility of cysteine residues[26,29] or formation of disulphides or other linkages[26-28] in preformed fibrils can provide insight into the packing structure of the monomeric polypeptide chains within the fibrils. However, the introduction of exogenous linkers, especially when combined with fluorescent or other labels, could potentially disrupt the packing within fibrils and so prevent the very interactions that are being probed by the technique. In general, introduction of disulphide-forming cysteine residues into amyloid-forming proteins has been found to inhibit formation of fibrils.[21,26-28] Therefore, our discovery of a unique site within the Ure2 prion domain (R17) where the introduction of a disulphide bond dramatically increases the rate of fibril formation was particularly unexpected and interesting.[1] Importantly, the disulphide-containing fibrils are indistinguishable morphologically from the wild-type (WT) fibrils and are able to seed the WT protein to form fibrils, suggesting that the presence of the disulphide bond constrains the polypeptide chains in a way that promotes fibril formation and is compatible with the WT fibril structure.[1] The R17C mutation was also identified in a previous in vivo screen for prion inducing mutations in Ure2: this mutant was functional and was found to increase the prion induction rate, though only when combined with other mutations in the N and/or C domains.[30] In our in vitro study, R17C had no effect on the fibril formation rate under reducing conditions[1] i.e., under conditions that mirror the reducing environment of the cytosol, and so our results obtained in vitro are consistent with the previous observations in vivo.[30]

Understanding the Driving Force for Amyloid Formation

In order to understand the mechanism of fibril formation, one of the pressing issues is to identify the driving force, or the trigger, for this reaction i.e., the first event during protein conformational change from the soluble state to amyloid fibrils. This information might allow us to control the initiation of amyloid formation in both prion diseases and other neurodegenerative diseases, such as Alzeimer and Parkinson. The "amyloid stretch hypothesis" proposes that a short peptide stretch bearing a highly amyloidogenic motif might supply most of the driving force needed to trigger the self-catalytic assembly of a protein into amyloid fibrils.[31] This hypothesis is consistent with the finding that many amyloidogenic polypeptides contain shorter sequence regions that readily form amyloid;[12,32,33] further, the insertion of an amyloidogenic peptide stretch into a non-amyloid-related protein can induce the protein to form amyloid.[34] Currently available computational algorithms for predicting amyloid-forming regions have shown good agreement with experiment, at least for hydrophobic amyloid-forming sequences.[33,35,36] The yeast prion proteins including Sup35, Ure2, Rnq1 and Swil1 are different in the fact that they lack continuous stretches of hydrophobic residues, but instead are rich in Gln and Asn,[37] analogous to the Gln-rich sequences seen in some disease-related human proteins such as the Huntingtin protein.[38] Hydrogen bonding between these polar residues is suggested to form the basis for their tendency to aggregate.[11,39]

Defining a Potential Amyloid Stretch within Ure2

In our study[1] we systematically mutated every residue within the first 70 residues of the Ure2 prion domain that was neither a Gln nor an Asn residue, producing a total of 35 mutants (**Fig. 1**). Each of these point mutants of the full-length Ure2 protein was expressed in *E. coli*, purified and then incubated under oxidizing conditions (i.e., in the presence of 3 mM H_2O_2). Under these conditions, R17C formed fibrils significantly more rapidly than the WT protein (i.e., within hours instead of days when allowed to stand at 8°C), and was the only cysteine mutant that formed fibrils on a measurable time scale. (Reduced R17C or R17S formed fibrils on a similar timescale to WT). The fibrils of R17C were examined by electron microscopy and far-UV CD, and were found to be morphologically and structurally indistinguishable from WT fibrils, before and after proteinase K digestion. Further, the fibrils of R17C could seed the formation of WT fibrils, suggesting that the packing within the two types of fibrils is identical.

Having established that linking the polypeptide chains with a disulphide bond at site 17 dramatically facilitates fibril formation of Ure2, we then made a series of deletions in the surrounding residues, in order to identify which residues within Ure2 are critical for fibril formation.[1] We narrowed the crucial sequence region down to a 4-residue stretch immediately following on from site 17, QVNI (residues 18–21), as deletion of any one of these residues completely removed the ability of oxidized R17C to form fibrils, whereas even quite substantial deletions in other parts of the prion domain (e.g., Δ1-16, Δ22, Δ23, Δ24-42 or Δ42-81) still allowed fibril formation of R17C.

We then characterized a series of short peptides derived from the R17C Ure2 sequence, each containing the CQVNI sequence.[1] This pentapeptide had extremely low solubility and no fibrils could be formed from it, but the hexa-(CQVNIG), hepta-(CQVNIGN) and octa-(CQVNIGNR) peptides could all form fibrils; the octapeptide, as the most

soluble, was characterized further. This octapaptide formed fibrils within minutes under oxidizing conditions, and like full-length R17C, was also able to seed fibril formation of WT full-length Ure2. In order to further understand the potential role of site 17 in controlling fibril formation for Ure2, a series of substitutions were made in the octapeptide. We found that when the cysteine residue of the octapeptide (equivalent to site R17C in Ure2) was substituted with a hydrophobic residue (V, L, I, F or M), the octapeptide readily formed fibrils (i.e., within days when incubated with agitation at 8°C at a peptide concentration of 1–4 mg/ml), and these fibrils were likewise able to seed fibril formation of WT Ure2. In contrast, while the WT octapeptide (RQVNIGNR) was eventually able to form fibrils under these conditions, this required a much longer incubation time (i.e., several months). Interestingly, however, the WT octapeptide, which is predicted to be an intact β-strand in a model for Ure2 fibrils,[40] readily formed crystals when incubated at higher concentration overnight (**Fig. 2**). Therefore the identification of this peptide provides a starting point for further structural characterization of Ure2 fibrils.

Our peptide studies suggest that the charged residue, Arg, at site 17 of Ure2 actually reduces dramatically the amyloid forming propensity of the adjacent QVNI sequence.[1] In order to confirm a similar role for this residue in the full-length protein, we introduced the equivalent hydrophobic substitutions at site 17. It was found that replacement of R17 with a hydrophobic residue increased the rate of fibril formation for Ure2, whereas substitution with another charged residue (e.g., Lys) inhibited fibril formation to a similar extent as the WT Arg. Deletion of residue 17, which brings a Leu residue adjacent to the QVNI sequence, had a similar effect as the other hydrophobic substitutions in promoting fibril formation.

Taken together, these results suggest that residue R17 in Ure2 acts as a gate-keeper residue for the downstream potential amyloid stretch QVNI, limiting its propensity to form fibrils. This is consistent with the likelihood that the prion state of Ure2 only conveys an advantage to the organism under rare and limited circumstances, and so it is likely that the Ure2 sequence has evolved to limit its tendency to switch to the prion state.[5,41,42] The dramatic increase in the ability of the protein to form fibrils when disulphide cross-linked at site 17 suggests that this site and its downstream residues may act as an initiation point for assembly of the polypeptide chains into Ure2 fibrils, and that tethering at site 17 brings the polypeptide chains together in a manner that is not only compatible with the WT Ure2 fibril structure, but promotes its formation, thus mimicking the trigger for fibril formation in this protein.[1]

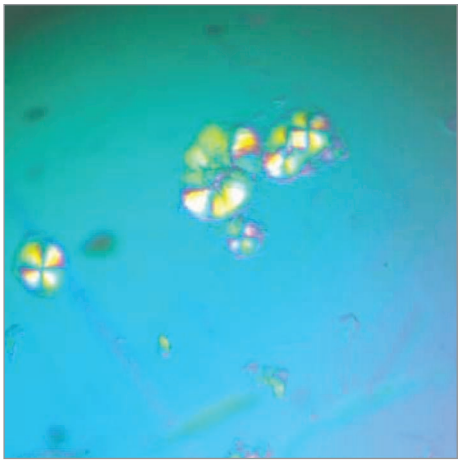

Figure 2. Crystals of the wild-type Ure2 octapeptide RQVNIGNR viewed under the polarized microscope. The crystals were formed by incubating the octapeptide solution (10 mg/ml in Tris buffer pH 8.4 with 200 mM NaCl) at 4°C overnight.

Acknowledgements

Work in the Perrett laboratory is supported by grants from the National Natural Science Foundation of China (30620130109, 30670428, 30870482), the Chinese Ministry of Science and Technology (2006CB500703, 2006CB910903) and the Chinese Academy of Sciences (KSCX2-YW-R-119). We thank members of the Perrett laboratory for critical reading of the manuscript.

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