Hierarchical Organization in the Amyloid Core of Yeast Prion Protein Ure2*1

Sam Ngo, Lei Gu, and Zhefeng Guo1

From the Department of Neurology, Brain Research Institute, Molecular Biology Institute, University of California, Los Angeles, California 90095

Formation of amyloid fibrils is involved in a range of fatal human disorders including Alzheimer, Parkinson, and prion diseases. Yeast prions, despite differences in sequence from their mammalian counterparts, share similar features with mammalian prions including infectivity, prion strain phenomenon, and species barrier and thus are good model systems for human prion diseases. Yeast prions normally have long prion domains that presumably form multiple β strands in the fibril, and structural knowledge about the yeast prion fibrils has been limited. Here we use site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy to investigate the structures of amyloid fibrils of Ure2 prion domain. We show that 15 spin-labeled Ure2 mutants, with spin labels at every 5th residue from position 5 to position 75, show a single-line or nearly single-line feature in their EPR spectra as a result of strong spin exchange interactions. These results suggest that a parallel in-register β structure exists at these spin-labeled positions. More interestingly, we also show that residues in the segment 30–65 have stronger spin exchange interactions, higher local stability, and lower solvent accessibility than segments 5–25 and 70–75, suggesting different local environment at these segments. We propose a hierarchical organization in the amyloid core of Ure2, with the segment 30–65 forming an inner core and the segments 5–25 and 70–75 forming an outer core. The hierarchical organization in the amyloid core may be a structural origin for polymorphism in fibrils and prion strains.

Prions are infectious proteins that underlie the transmissible spongiform encephalopathies in humans and other mammals. Transmissible spongiform encephalopathies are a group of fatal neurodegenerative diseases, including Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle. According to the protein-only hypothesis (1, 2), prions can undergo a conformational transition from a cellular form to a disease form, which is the agent that causes transmissible spongiform encephalopathies. The disease form of prion can further serve as a template to convert the cellular form to the disease form, enabling prion propagation. Prions belong to a large class of amyloid-forming proteins involved in various human disorders such as Alzheimer disease and type II diabetes (3). Amyloid is a fibrillar form of protein aggregates with some common characteristics such as staining with the dyes Congo red (4) and thioflavin T (5) and a cross-β x-ray diffraction pattern (6). The protein-only hypothesis for prion diseases is generally accepted, although some skeptics remain (7). In yeast and other fungi, prions are the basis of protein-based inheritance of several non-Mendelian phenotypes (8, 9). Despite differences in sequences from their mammalian counterparts, yeast prions share similar features with mammalian prions including infectivity, prion strain phenomenon, and species barrier (8, 9). Therefore, yeast prions have been used as a model system to address questions such as the protein-only hypothesis of prion transmission, prion strains, and involvement of molecular chaperones in prion diseases.

Ure2 protein is one of the best characterized prions in yeast Saccharomyces cerevisiae (10). The cellular function of Ure2 protein is to suppress the expression of enzymes and transporters involved in catabolizing poor nitrogen sources when a good nitrogen source is present (11). When Ure2 switches to its prion state, URE3, nitrogen catabolism genes for poor nitrogen sources are expressed even in the presence of a good nitrogen source (12). The full-length Ure2 protein contains 354 amino acids. The N-terminal domain (residues 1–89) is the prion domain, and C-terminal domain (residues 90–354) is a globular domain that is necessary and sufficient for its cellular function (13, 14). The structure of the C-terminal domain has been solved with x-ray crystallography (15, 16). The prion domain is required for the URE3 prion phenotype in vivo (17) and for amyloid fibril formation in vitro (18). Amyloid fibrils prepared from purified Ure2 prion domain are infectious to yeast cells, providing direct evidence for the protein-only hypothesis (19).

Structural studies of amyloid fibrils are not amenable to x-ray crystallography and solution NMR because of the insoluble and non-crystalline nature of the amyloid. X-ray crystallography is limited to the studies of microcrystals of 4–7-residue peptides from various amyloid proteins but has revealed a lot of atomic details of packing and side chain interactions that may be relevant to amyloid fibrils (20–22). Solid-state NMR (23) and electron paramagnetic resonance (EPR) spectroscopy (24) are two major techniques that provide residue-level structural details of amyloid fibrils. Solid-state NMR has been used to study the amyloids of a number of proteins including Aβ (23), islet amyloid polypeptide (25), α-synuclein (26, 27), Ure2 (28), Sup35 (29), Rnq1 (30), and HET-s (31). In parallel, EPR has also played a significant role in the structural studies of amyloid fibrils. Based on EPR studies, a parallel in-register β structural model...
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has been suggested for the fibrils of Aβ (32), islet amyloid polypeptide (33), Tau (34, 35), α-synuclein (36, 37), and human prion protein (38), consistent with structural models based on solid-state NMR studies. In these studies the EPR spectra of spin-labeled amyloid proteins show a single-line feature, whereas normally the EPR spectrum of a spin-labeled protein has three spectral lines. The single-line spectrum is a result of strong spin exchange interactions between stacked spin labels in the fibril (24, 39). This single-line feature is a convenient and powerful signature to identify the parallel in-register β structure in amyloids. With rapid development in the area of distance measurements ranging from 8 to 70 Å, EPR is capable of determining amyloid structures at a resolution of at least backbone fold.

Solid-state NMR studies of Ure2 prion domain 13C-labeled at backbone carbonyl sites of Leu or Val residues or at the side-chain methyl sites of Ala residue show intermolecular 13C-13C distances of ≈5 Å (28). This result is consistent only with a parallel in-register β-sheet structure. However, structural information was limited to eight residue positions at 9, 12, 15, 16, 19, 43, 58, and 81, and only one residue at position 58 was investigated between residues 44 and 80. Solid-state NMR studies of a shorter Ure2 prion domain fragment including residues 10–39 also show a parallel in-register β structure (40). Similar solid-state NMR studies have been performed on yeast prions Sup35 and Rnq1 with 13C-labeled Tyr, Leu, Phe, or Ala residues (29, 30), and a parallel in-register β structure was proposed for both proteins. The parallel in-register structural model is also supported by mass per unit length measurements (41) and sequence scrambling of prion domains (42). However, the detailed packing of the amyloid core has been poorly understood regardless the existence of parallel in-register structure.

In this work we use site-directed spin labeling and EPR to study the structure of fibrils formed by Ure2 prion domain. The general strategy of site-directed spin labeling is to introduce a cysteine residue at a selected site by site-directed mutagenesis. Then the cysteine residue is modified to generate a spin label side chain. The spin label used in this work is named R1 (supplemental Fig. S1). We show that a parallel in-register β structure exists at every 5th position from residue 5 to residue 75. Our results also show that residues 30–65 form an inner core with a different local environment from the outer core segments 5–25 and 70–75, suggesting a hierarchical organization in the amyloid core of Ure2 fibrils. The inner core residues may contribute to the overall stability of the fibril more than the outer core residues. Therefore, varying the size of the inner core may be a structural basis for amyloid polymorphism and prion strains, which have been shown to have different stabilities for yeast prions (43).

EXPERIMENTAL PROCEDURES

Protein Preparation and Spin Labeling—The construct of Ure2 prion domain was kindly provided by Dr. Susan Lindquist (Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology). The details of this construct have been described previously (44). For protein expression, the Ure2 construct was transformed into C41(DE3) cells (Lucigen) and induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside when cells grew to an absorbance of A600 = 0.6 at 37 °C. The induction was allowed to proceed at 28 °C for 4–6 h. The cells were harvested by centrifugation and resuspended in PSU buffer (50 mM phosphate, 0.3 M NaCl, 8 mM urea, pH 8.0). The cells were then sonicated, and the cell debris was pelleted by centrifugation. The supernatant was filtered using 0.45-μm filter (Whatman) and loaded onto a 5-ml HiTrap column (GE Healthcare) equilibrated with PSU buffer. Proteins were eluted with a linear imidazole gradient (50–500 mM) in 10 column volumes. Protein concentration was determined by UV absorption at 280 nm using an extinction coefficient of 6.97 × 10^4 M⁻¹ cm⁻¹ (45). For spin labeling, dithiothreitol was added to protein solution and allowed to incubate at room temperature for 20 min to break any disulfide bonds and then was removed with a HiTrap desalting column (GE Healthcare). For spin labeling, MTSSL2 (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate; Enzo Life Sciences) was added at 10× molar excess immediately after dithiothreitol treatment and incubated at room temperature for 1 h. MALDI-TOF mass spectrometry was performed on the spin-labeled proteins to ensure that the mass was correct and the extent of spin labeling was more than 95%. After spin labeling, proteins were precipitated with methanol, air-dried, and stored at −80 °C.

Fibril Formation—Proteins precipitated by methanol were first dissolved in 15 mM sodium phosphate, 7 mM guanidine hydrochloride, pH 6.8, to a concentration of 1 mM. To start fibril formation, proteins were diluted 20-fold to PBS buffer (50 mM sodium phosphate, 140 mM NaCl, pH 7.4). For the quiescent condition, the sample was left on the bench at room temperature (~22 °C). For the agitated condition, the sample was continuously shaken at 1200 rpm at room temperature. The process of fibril formation was monitored with thioflavin T binding.

Fibril Characterization—For electron microscopy, fibril samples were applied onto glow-discharged copper grids covered with 400 mesh Formvar/carbon film (Ted Pella) and stained with 1% uranyl acetate. Samples were examined under a JEOŁ JEM-1200EX electron microscope with an accelerating voltage of 80 kV. For a Congo red binding assay, Congo red (Sigma) was dissolved in PBS and filtered with 0.22-μm filter. The concentration of Congo red solution was determined using an extinction coefficient of 3.7 × 10^4 M⁻¹ cm⁻¹ at 498 nm. The final concentration of protein was 5 μM in the presence of 9 μM Congo red. After incubating at room temperature for 20 min, a wavelength scan of the absorbance was performed on a JASCO V-630 spectrophotometer. For thioflavin T binding assay, thioflavin T (Sigma) was dissolved in PBS and filtered with a 0.22-μm filter. Proteins were diluted to a final concentration of 1 μM in PBS containing 50 μM thioflavin T. A fluorescence scan was performed immediately on a JASCO FP-6200 spectrofluorometer. Excitation was at 480 nm (5-nm slit width), and emission was scanned from 460 to 650 nm (5-nm slit width).

EPR Spectroscopy and Spectral Analysis—EPR measurements were performed at X-band frequency on a Bruker EMX

2 The abbreviations used are: MTSSL, (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate; GdnHCl, guanidine hydrochloride; NiEDDA, Ni(II) ethylenediaminediacetic acid.
spectrometer fitted with the ER4102ST cavity at room temperature using a microwave power of 20 milliwatts and a modulation amplitude optimized to the natural line width of each individual spectrum. For 20 and 100% labeled fibrils, the modulation amplitude was ~2 and 4 gauss, respectively. Fibril samples were collected using Amicon Ultra filter with a 100-kDa cutoff (Millipore) and were placed in glass capillaries (VitroCom) sealed at one end. EPR spectra in each figure panel were normalized to the same number of spins.

Subtraction of mobile component was performed as previously described (46). Briefly, the mobile spectrum was simulated and subtracted from original spectrum using a LabVIEW program. The fraction of the mobile component, hyperfine splitting, and line width was adjusted to match the mobile component in the experimental spectrum. We estimate that the error in the fraction of the mobile component is ±10% that of the reported value.

For spectral simulations, experimental EPR spectra were simulated using a LabVIEW (National Instruments) interface (47) of the program NLSL developed by Freed and co-workers (48, 49). A microscopic order macroscopic disorder (MOMD) model was used as previously described (49). A least-squares fit of the user-defined spectral parameters was performed on the experimental spectra using the Levenberg-Marquardt algorithm. The values of the A and g magnetic tensors were fixed as $A_{zz} = 6.2$, $A_{yy} = 5.9$, and $A_{xz} = 37.0$, and $g_{xx} = 2.0078$, $g_{yy} = 2.0058$, and $g_{zz} = 2.0022$, which were determined previously for $R_1$ (50). Each EPR spectrum was assumed to contain one spectral component corresponding to one spin label state. The number of fitted parameters was kept at a minimum, which in this work includes the isotropic rotational diffusion constant ($R$) and Heisenberg exchange frequency ($\omega$). We found that satisfactory fits were obtained with only these two parameters. Rotational correlation time ($\tau$) can be calculated using $\tau = 1/(6R)$. Values of $\tau$ and $\omega$ are reported in supplemental Table S1. Error for exchange frequency was estimated by changing the value from its best fit until a significant deviation in simulated spectra was observed. The error was plotted in Fig. 4 as error bars. The correlation time at different labeling sites has a narrow range of 5–7 ns, which is comparable with buried sites in model proteins such as T4 lysozyme. Previous studies have found that the correlation time is ~4–5 ns for buried sites 118 and 131 in T4 lysozyme (51). In T4 lysozyme, the motion of the spin label at buried sites is highly ordered (51). Therefore, we also performed simulations with an anisotropic model by including an order parameter. For anisotropic simulations, diffusion tilt angles were fixed to $(\alpha,\beta,\gamma) = (0,36',0)$ for $yz$ axis anisotropy as previously reported (50). The diffusion tilt angles are the Euler angles relating the axes of the diffusion tensor and the magnetic tensor. We found that the fitted exchange frequency using anisotropic model is extremely similar to those obtained with isotropic model. Therefore, only parameters from isotropic models are reported here.

For guanidine hydrochloride (GdnHCl) treatment experiments, fibrils formed with only spin-labeled Ure2 proteins were collected and then mixed with PBS buffer containing 7 M GdnHCl to obtain the desired GdnHCl concentration. We found that dissociation of monomers from fibrils in the presence of GdnHCl reaches equilibrium within half an hour. To estimate the extent of monomer dissociation, we compare the center line amplitude of the EPR spectrum normalized to the same number of spins because the center line amplitude faithfully report the dissociation event in the presence of GdnHCl (Fig. 5B). Another possible analysis of monomer dissociation is to separate the mobile spectrum from the fibril spectrum, but we found this analysis is more prone to errors than using amplitude. The protein concentration in the final fibril sample was kept within 30% of one another based on the $A_{280}$ nm value of the solution after fibril formation. Our analysis shows that protein concentration variations by 30% in the presence of 3 M GdnHCl do not significantly change the center line amplitude.

For solvent accessibility measurements with Ni(II) ethylenediaminediacetic acid (NiEDDA), EPR spectra of 20% labeled fibril samples were measured in the presence of 30 mM NiEDDA. The NiEDDA-broadened EPR spectrum was fitted as a convolution of the EPR spectrum in the absence of NiEDDA and a Lorentzian broadening function as previously described (52). Collision frequency with NiEDDA is obtained from fitting. The factors that affect this fitting procedure contribute to the error of measurement. These factors include the signal-to-noise of the EPR spectrum, goodness of fit to the Lorentzian broadening function, and accuracy of NiEDDA concentration. Because it is difficult to estimate the contribution to measurement error from each individual factor, S.D. from two independent measurements were reported in Fig. 6 as error bars. These errors are generally less than 5% that of the reported value.

RESULTS

Fibril Formation of Ure2 Prion Domain—The construct of Ure2 prion domain (residues 1–89) in this work has the M domain (residues 124–253) of yeast prion protein Sup35 attached to the C terminus (44) and is designated as Ure2p$_{1-89}$ M here. The purpose of Sup35 M domain is to increase the solubility of Ure2 prion domain during sample preparation. Previous studies have shown that attachment of Sup35 M domain to various yeast prion domains, including Ure2, has no apparent effects on the amyloid formation (44, 53). Electron microscopy shows that under quiescent conditions, Ure2p$_{1-89}$ M forms elongated unbranched filaments, with an average width of ~12 nm (Fig. 1A). Under agitated condition (i.e. continuous shaking at 1200 rpm), Ure2p$_{1-89}$ M forms short fibrils with lengths between ~15 and 100 nm, and the width is similar to quiescent fibrils (Fig. 1A). The morphology of agitation fibrils could result from mechanical fragmentation of long fibrils, increased nucleation rate as a result of agitation, or different polymorphic structures. The quiescent fibrils bind Congo red, leading to a red shift of the absorption spectrum (Fig. 1B) and also bind thioflavin T, giving rise to a characteristic emission peak at ~482 nm (Fig. 1C). The agitated fibrils have similar binding properties to Congo red and thioflavin T. Therefore, we conclude Ure2p$_{1-89}$ M forms fibrils with amyloid properties.

Spin-labeled Ure2 Forms Amyloid Fibrils Similar to Wild Type Proteins—To investigate the structure of Ure2 fibrils, we introduced spin labels, 1 at a time, at every 5th residue from
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**FIGURE 1.** Ure2 prion domain forms amyloid fibrils. A, electron micrographs of fibrils formed by Ure2p1–89-M under quiescent and agitated conditions are shown. Bars, 100 nm. B, binding of Congo red to the quiescent Ure2 fibrils is shown. Congo red binding leads to a red shift of the absorption spectrum, characteristic of amyloids. C, binding of thioflavin T to the quiescent Ure2 fibrils is shown. Thioflavin T binding results in an emission peak at 482 nm. AU, arbitrary units.

**FIGURE 2.** Electron micrographs of the amyloid fibrils formed by Ure2 prion domain with the indicated residues replaced by spin label R1. The 100% labeled sample contains only spin-labeled proteins. The 20% labeled sample is a mixture of spin-labeled Ure2 and wild type Ure2 at 1:4 molar ratio. The fibrils are formed under either quiescent or agitated conditions. Bars, 100 nm.

position 5 to position 80. Supplemental Fig. S2 shows the amino acid sequence of the Ure2 prion domain together with the positions for spin labeling. Electron microscopy shows that spin-labeled Ure2p1–89-M proteins form similar fibrils to wild type proteins (Fig. 2). We also performed fibril formation on 20% labeled samples, in which spin-labeled Ure2 is mixed with wild type Ure2 at a 1:4 ratio. The fibrils of 20% labeled samples are similar to 100% labeled samples (Fig. 2), suggesting that spin-labeled Ure2 can form fibrils interchangeably with wild type proteins. All the fibrils bind to thioflavin T. Fibrils formed under different conditions may have different underlying structures (43, 54). It is not clear if the Ure2 fibrils formed under quiescent and agitated conditions have the same molecular structure. In this work we arbitrarily choose to study the structure of the fibrils formed under agitated conditions with EPR spectroscopy.

**EPR Spectra of Spin-labeled Ure2 Fibrils Suggest a Parallel In-register β Structure**—Normally spin-labeled proteins give rise to EPR spectra with three spectral lines. In a parallel in-register β structure, each position in one protein stacks upon the same position in the adjacent protein. When the fibrils are formed by proteins spin-labeled at a specific site, the parallel in-register structure results in the stacking of spin labels in the fibrils. The stacked spin labels have strong spin exchange interactions, leading to the collapse of the three spectral lines to form a characteristic single-line EPR spectrum (24, 39). Therefore, this single-line spectrum provides a convenient and powerful way to detect parallel in-register β structure in the amyloid fibrils. Due to a lack of quantitative description, we define the single-line spectrum as the spectrum whose center line is broadened to engulf the first and third spectral lines and whose first and third spectral lines are flattened to become small “bumps” on the broadened center line. At the same time, because EPR can measure distances ranging from ~8 to 70 Å, other fibril structures including antiparallel or β-helix can also be studied with EPR. The strength of spin exchange can be expressed by spin exchange frequencies. To observe the single-line feature, the exchange frequency must be greater than the hyperfine interaction, which determines the separation between spectral lines in the three-line spectrum. This translates into an exchange frequency of >100 MHz. Margittai and Langen (24) have estimated that if spin exchange is mediated through aqueous solution between two electrons, an exchange frequency of 100 MHz corresponds to a distance of ~6.6 Å. This represents an upper limit for a detectable spin exchange interaction via X-band continuous-wave single-line EPR spectrum. In comparison, another common spin-spin interaction, dipolar interaction, leads to the broadening of the three spectral lines when two spin labels are within ~20 Å but does not give rise to single-line feature.

We performed EPR measurements on the agitated fibrils of Ure2p1–89-M proteins. As shown in supplemental Fig. S3A, all the EPR spectra consist of two spectral components: a major component and a minor component. The major component is the single-line spectrum characteristic of stacked spin labels in the fibrils. The minor component has sharp spectral line shape and narrow separation between the two outmost lines, characteristic of fast motion. With a subtraction procedure, we subtracted the mobile component from the EPR spectrum of each sample (supplemental Fig. S3B). From subtraction, we have determined that the proportion of the mobile component is ~0.5–1.5% of the total spectrum from position 5 to position 65 and 2.4–3.8% for positions 70, 75, and 80. We reason that the minor component is not a result of structural perturbation by spin labeling for the following reasons. (i) The proportion of the
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mobile component stays in a narrow range of 0.5–3.8%. If spin labeling disrupted fibril structure, one would expect a wide range for the relative population of the mobile component when five different residues, including Asn, Ser, Ala, Ile, Thr, and Arg, have been replaced with spin labels. These residues have been shown to have widely different amyloid aggregation propensities (55). (ii) We also observed the mobile component in the spectra of 20% labeled Ure2 fibrils, and the proportion of the mobile component ranges from 0.6 to 3.2%, similar to 100% labeled fibrils (supplemental Fig. S4). If the minor component is due to structural perturbation of the spin label, we would expect to see less structural perturbation when 80% of the protein is wild type. The structural origin of the mobile component may be “imperfect packing” in the fibril core. In other words some disordered structures may exist in the ordered amyloids. This hypothesis is supported by the fact that the proportion of the minor component is higher at either end of the prion domain. It is reasonable that the local stability at the end of the prion domain is lower, leading to increased proportion of the mobile component. Another possible structural origin for the mobile component is soluble proteins dissociated from fibrils, which are largely disordered and thus would give rise to fast-motion spectrum. The results here demonstrate that EPR has the capability to resolve different structural populations in the sample.

After subtraction of the mobile component, all the 100% labeled Ure2 mutants except T80R1 give rise to a single-line or nearly single-line feature (red spectra in Fig. 3). In comparison, the EPR spectra of 20% labeled fibrils are devoid of the single-line feature (gray spectra in Fig. 3). The spectrum of 100% labeled T80R1 can be explained by either weak spin exchange interactions or dipolar interactions. Because the single-line spectrum requires strong spin exchange interactions (i.e. an interspin distance < 6.6 Å) between multiple spin labels (24), the results of single-line spectra at multiple positions across the full sequence of Ure2 prion domain are consistent only with a parallel in-register β structure model. Therefore, the results here suggest that a parallel in-register β structure exists at all 15 spin-labeled sites from position 5 to position 75. The absence of a parallel structure at T80R1 is consistent with its location at the C terminus of the prion domain. Previously only 1 residue at position 58 was characterized within the long segment 44 – 80 (28), and here we show that the segment 45 – 75 adopts parallel in-register β structures.

Hierarchical Organization in the Amyloid Core of Ure2 Prion Domain—A closer examination of the EPR spectra of fully labeled Ure2 shows that these spectra can be categorized into three groups based on their positions in sequence and similarity in spectral features: residues 5–25, 30–65, and 70–75 (Fig. 4A, different line colors). The EPR spectra are similar within each group, and the spectra at positions 5–25 are similar to the spectra at positions 70–75. In contrast, the spectra at positions 30–65 are different from other spectra, and they show a single-line feature, whereas other spectra show more pronounced peaks at the positions of the first and third spectral lines. To check the reproducibility of the different spectral features, we collected another set of EPR spectra on several mutants. Supplemental Fig. S5 shows that these spectral features are highly reproducible and thus truly reflect inherent structural environment at the labeling sites.

The difference in spectral features could be due to the strength in the spin exchange interactions among the spin labels. To test this idea, we simulated the fibril spectra to obtain the spin exchange frequencies. Amyloid fibrils are often found to be polymorphic, adopting different conformations in the same sample preparation. The spectra of 20% labeled Ure2 fibrils show that most sites have more than one spectral component, suggesting the existence of multiple states. This poses a challenge in spectral simulations because the parameters defining each conformation are not known. We assume for the purpose of simplifying simulation that only one conformation is adopted by the spin-labeled mutants. The exchange frequency obtained from simulation represents the average frequency from all conformations. Because we are concerned only with the differences among different residue positions, this one-conformation assumption is sufficient in providing a quantitative measure. Simulated spectra are shown in Fig. 4A (gray lines), and spin exchange frequencies obtained from simulations are
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plotted in Fig. 4B. It is clear from Fig. 4B that residues 30–65 have stronger spin exchange interactions than residues 5–25 and 70–75. From spin exchange frequencies, the distance between spin labels can be estimated if the mechanism of spin exchange is known. Based on modeling and temperature studies, it was suggested that the spin exchange interaction in the fibril is mediated through static contact rather than rapid collisions, and the unpaired electron is exchanged through oxygen–oxygen or oxygen–methyl group on the nitroxide (24). The crystal structure of the nitroxide spin label MTSSL shows a parallel stacking of spin labels, and the oxygen atom is closest to the methyl group of the neighboring nitroxide (56). Because a parallel stacking of spin labels is suggested in the fibrils (24), the crystal structure of MTSSL may represent a possible atomic organization in the fibrils. This suggests that in fibrils the spin exchange may take place through oxygen and a methyl group on the nitroxide. The exchange integral \( J \) decreases exponentially with increasing distance between spin labels and can be described as \( J(r) = J_0 \exp(-\alpha(r-r_0)) \), where \( J(r) \) is the exchange integral at distance \( r \), \( J_0 \) is the exchange integral at van der Waals contact distance \( r_0 \), and \( \alpha \) is a damping constant. Exchange integral \( J_0 \) is on the order of \( 10^{12} \) Hz (39), and \( \alpha \) is estimated to be \( \sim 3.2 \) Å\(^{-1} \) (24). Therefore, we can estimate that the distance between spin labels is \( \sim 5.2 \) Å, close to an inter-β-strand distance of \( \sim 4.8 \) Å. The distance calculated from simulation should be viewed as the “average distance” of different conformations. More importantly, we can estimate that a difference in exchange frequency by \( \sim 50 \) MHz as observed between the segment 30–65 and other segments (Fig. 4) corresponds to a difference in distance by 0.1 Å. Based on simulations, we conclude that the spectral difference may indicate a difference in interstrand distance between inner and outer core residues.

To further investigate the difference between segment 30–65 and the rest of the prion domain, we compare the local stability at selected residue positions using chemical denaturant GdnHCl. Fig. 5A shows that in the presence of GdnHCl only two spectral components exist in the EPR spectrum. The component with sharp lines is consistent with fast motion of disordered or soluble Ure2 proteins. With the increasing concentration of GdnHCl, the amplitude of the mobile component increases exponentially, implying a sigmoid curve for GdnHCl concentration. Fig. 5C shows that in the presence of 3 M GdnHCl, Error bars for Ure2 spin-labeled at positions 35, 65, and 75 are S.D. from two independent experiments. Error was not estimated for other mutants. The dotted line is drawn to aid for comparison. Note that overall the segment 30–65 has a smaller increase in center line amplitude, suggesting higher stability.

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Solvent accessibility is measured as spin exchange frequency between spin labels and 30 mM NiEDDA in the sample solution. The dotted line is drawn to aid for comparison. Note that the segment 30–65 in general has lower solvent accessibility, suggesting tighter intersheet packing interactions.

Furthermore, we studied the solvent accessibility at different labeling sites by measuring the spin exchange frequency between the spin label and the paramagnetic reagent NiEDDA. We chose NiEDDA because it is relatively large (\( M_r = 232 \)) and thus provides good contrast between solvent exposed and inaccessible sites. Previously, it was shown that the exchange frequency for a surface site in T4 lysozyme was \( \sim 13 \) MHz in the presence of 30 mM NiEDDA (52). In this work we obtained exchange frequencies from 14 to 16 MHz for labeling sites at both N- and C-terminal ends of the Ure2 prion domain (Fig. 6), suggesting that these residues are completely exposed to solvent. Some sites have very low exchange...
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frequencies, suggesting that they may be located in the middle of the β-strand. Intersheet packing interactions would shield the side chains of these residues from solvent. When comparing different segments, comparisons should be focused on the overall trend rather than individual residues. In general, Fig. 6 shows that the segment 30–65 has lower solvent accessibility than the segments 5–25 and 70–75. This observation suggests that the intersheet packing interactions are stronger in the segment 30–65. Residue 55 has higher solvent accessibility than other residues in the segment 30–65. We speculate that it may be located in a turn region, consistent with its fibril EPR spectrum showing weaker spin exchange interactions than its neighbors (Fig. 4). Fig. 6 also shows that the segment 70–75 has high exchange frequency with NiEDDA, suggesting that this segment does not pack tightly with the rest of the structure in the fibril. In addition, spin labels at positions 65 and 67 also have high accessibility to NiEDDA, suggesting that these two residues are located on the solvent-exposed surface of the β-sheet.

DISCUSSION

A parallel in-register β structure has been suggested for the Ure2 prion domain (residue 1–89) in a previous solid-state NMR study (28), but structural information was limited to eight residue positions at 9, 12, 15, 16, 19, 43, 58, and 81 (i.e. all the Leu, Val, and Ala residues). With EPR spectroscopy, here we show that nearly the entire Ure2 prion domain (residues 5–75) adopts the parallel in-register β structure in the fibrils (Fig. 3). Spin labels introduced at every 5th residue from position 5 to position 75 all show strong spin exchange interactions, suggesting that the amyloid core spans position 5 to position 75. With the minimum fibril width being ∼4 nm (57), such a long core region would certainly be comprised of multiple β strands. More interestingly, we show that the organization of the amyloid core in Ure2 is not uniform. The strands in the segment 30–65 and the strands in the segments 5–25 and 70–75 have different structural properties. Residues in the inner core have tighter interstrand packing or higher structural order and thus stronger spin exchange interactions (Fig. 4), higher local stability, and thus more resistant to chemical denaturant such as GdnHCl (Fig. 5) as well as stronger intersheet packing and thus lower solvent accessibility (Fig. 6). Therefore, we propose a hierarchical organization in which the segment 30–65 forms the “inner core,” and the segments 5–25 and 70–75 form the “outer core” of the Ure2 fibril. The inner core and outer core residues likely contribute differently to the stability of the fibril.

The results in this work provide a structural explanation for several previous in vivo and in vitro findings regarding the properties of Ure2 prion domain. In vivo studies have shown that deletion of the residues 1–65 abolished prion induction, and overexpression of the segment 1–65 lead to a 6000-fold increase in the rate of prion induction (17). Overexpression of the residues 1–80 resulted in a higher rate of prion induction (58). In vitro studies show that Ure2p1–65 and Ure2p1–80 form amyloid fibrils that are capable of converting yeast cells to prion state (19). Our work shows that the inner core of the Ure2 fibrils ends approximately at residue 65, providing a structural explanation for the importance of residues 1–65. In addition, we show that residues 70 and 75 also form part of the amyloid core, and this could explain the higher prion induction rate by Ure2p1–80 than Ure2p1–65. Previously, it was shown that Ure2 with a deletion of residues 15–42 could still form amyloid fibrils, albeit with an increased lag time (59). Because this region is conserved among different yeast species, it was surprising to see that deletion of this region did not abolish amyloid formation. Our work suggests that, even with a deletion of residues 15–42, the inner core of amyloid fibrils (residues 30–65) is still largely intact, explaining the capability of this deletion mutant to form amyloids.

This work does not provide sufficient data to build a unique structural model for the Ure2 fibrils but is consistent with a super-pleated β-sheet structure for the reasons below. First, our results show that parallel in-register β structure exists at every 5th residue from position 5 to position 75 (Fig. 3). This essentially excludes the existence of disordered segment in this region, because a three-residue stretch in between two adjacent labeling sites is unlikely to be a disordered segment. Previous work showed that the Ure2p1–65 fibrils have a width of 4–4 nm (57). Therefore, Ure2 fibrils must contain multiple β strands. Second, x-ray diffraction of amyloid fibrils gives rise to a cross-β pattern (60), suggesting that multiple β-sheets are stacked together. Packing between multiple β-sheets has been revealed by solid-state NMR studies on amyloid fibrils (23) and x-ray crystallographic studies on short peptides (20). Third, our solvent accessibility studies show that only N- and C-terminal residues have high accessibility, and all residues from 7 to 60 show some protection from solvent (Fig. 6). The solvent accessibility results are not consistent with any models that expose long stretches of non-terminal residues to solvent. Fourth, from a theoretical point of view, a super-pleated β-sheet structure maximizes the number of interresidue interactions and thus may be more favorable than other models. Based on currently available data, it is not clear how many strands are in the Ure2 prion domain and where are the exact boundaries of each β strand. A parallel super-pleated β sheet model has been proposed previously for Ure2, and each β-strand repeat containing the connecting loop is seven residues long (57). In contrast, solid-state NMR studies of Ure2p10–39 suggest that the segment 10–39 contains only two β strands (40). A model for the Ure2 fibril structure is shown in Fig. 7. Further investigation is required for a complete understanding of the detailed structure of Ure2 fibrils.

A parallel in-register β structure has been proposed for the amyloid fibrils of a number of proteins based on solid-state NMR (23) and EPR studies (24). Structural knowledge for fibrils with each protein forming more than two β strands has been limited to the general description of a “parallel in-register β structure.” Here we found that in the structure of Ure2 fibrils, which contain multiple β strands, the organization in the amyloid core is not uniform. There is a distinction of inner core and outer core with different structural properties. The inner core residues may have stronger interstrand and intersheet packing interactions and higher local stability. As a result, the size of the inner core may determine
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FIGURE 7. A schematic representation of a super-pleated β structure model for Ure2 fibrils. Segments 5–25, 30–65, and 70–75 are colored black, red, and green, respectively. The number of β strands and the length of each strand are approximate. Only residues with experimental information are drawn on the model. Residue 55 is labeled at two adjacent proteins to illustrate the parallel in-register stacking at the same residue positions. The location of residue 55 at a turn is supported by its low spin exchange frequency (Fig. 4) and are both highly solvent-accessible (Fig. 6). The β strand containing residues 70–75 is packed loosely with the rest of the amyloid core, supported by high solvent accessibility of residues 65, 70, and 75.

the overall stability of the fibril. We speculate that the size of the inner core may be varied, leading to amyloid fibrils with different stabilities. It has been shown that different strains of yeast prion Sup35 correspond to different stabilities of prion fibrils (43). Therefore, the hierarchical organization may be a structural origin for the structural polymorphism of amyloid fibrils and prion strains.

Acknowledgments—We thank Dr. Susan Lindquist at the Massachusetts Institute of Technology for providing the construct of Ure2 prion domain, Drs. Wayne L. Hubbell and Christian Altenbach for providing LabVIEW programs for EPR analysis and the reagent NiEDDA, Drs. Wayne L. Hubbell and David B. Teplow for insightful discussions, and Vicky Chiang, Elaine Ho, and Linh Le for help with preparation of spin-labeled proteins and fibrils.

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AUGUST 26, 2011 • VOLUME 286 • NUMBER 34

JOURNAL OF BIOLOGICAL CHEMISTRY 29699

Hierarchical Organization in the Amyloid Core of Yeast Prion Protein Ure2
Sam Ngo, Lei Gu and Zhefeng Guo

doi: 10.1074/jbc.M111.269092 originally published online July 5, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.269092

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