Shaking Alone Induces De Novo Conversion of Recombinant Prion Proteins to β-Sheet Rich Oligomers and Fibrils

Carol L. Ladner-Keay1,2,3, Bethany J. Griffith1,3, David S. Wishart1,2,3*

1 Department of Computing Science, University of Alberta, Edmonton, Alberta, Canada, 2 Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, 3 National Institute for Nanotechnology, Edmonton, Alberta, Canada

Abstract

The formation of β-sheet rich prion oligomers and fibrils from native prion protein (PrP) is thought to be a key step in the development of prion diseases. Many methods are available to convert recombinant prion protein into β-sheet rich fibrils using various chemical denaturants (urea, SDS, GdnHCl), high temperature, phospholipids, or mildly acidic conditions (pH 4). Many of these methods also require shaking or another form of agitation to complete the conversion process. We have identified that shaking alone causes the conversion of recombinant PrP to β-sheet rich oligomers and fibrils at near physiological pH (pH 5.5 to pH 6.2) and temperature. This conversion does not require any denaturant, detergent, or any other chemical cofactor. Interestingly, this conversion does not occur when the water-air interface is eliminated in the shaken sample. We have analyzed shaking-induced conversion using circular dichroism, resolution enhanced native acidic gel electrophoresis (RENGAE), electron microscopy, Fourier transform infrared spectroscopy, thioflavin T fluorescence and proteinase K resistance. Our results show that shaking causes the formation of β-sheet rich oligomers with a population distribution ranging from octamers to dodecamers and that further shaking causes a transition to β-sheet fibrils. In addition, we show that shaking-induced conversion occurs for a wide range of full-length and truncated constructs of mouse, hamster and cervid prion proteins. We propose that this method of conversion provides a robust, reproducible and easily accessible model for scrapie-like amyloid formation, allowing the generation of milligram quantities of physiologically stable β-sheet rich oligomers and fibrils. These results may also have interesting implications regarding our understanding of prion conversion and propagation both within the brain and via techniques such as protein misfolding cyclic amplification (PMCA) and quaking induced conversion (QuIC).

Introduction

Prion protein (PrP) is a highly conserved membrane-bound protein that is particularly abundant in the neuronal cells of vertebrates. While the physiological function of properly folded and processed PrP is not yet clear, it is now clear that misfolded PrP can cause a variety of fatal neurodegenerative diseases in both animals and humans. These include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, as well as Kuru, Creutzfeldt Jacob Disease (CJD) and Fatal Familial Insomnia (FFI) in humans [1]. Prions cause disease by converting from a native, helix-rich cellular form (PrPc) to an infectious β-sheet rich form (PrPsc) that is insoluble, protease resistant and highly pathogenic [2]. An abundance of misfolded PrPsc proteins on the neuronal cell surface or within endosomes leads to the accumulation of extracellular amyloid protein deposits that eventually lead to cell death and the manifestation of neuronal disease. To better understand this physiological process, a number of cell-free, de novo methods have been developed that allow recombinant (rec) PrPc to be converted to a β-sheet rich isoform. In these methods the conversion of recPrP to a β-sheet isoform is performed through the addition of denaturants and cofactors such as urea, copper ions, acid, nucleic acids, lipids and lipopolysaccharides [3,4,5,6,7,8,9,10,11]. Of course, conversion of PrP alone is not sufficient to cause prion diseases or to create infectious prion particles. Another critical component of prion disease is the occurrence of template directed replication of the infectious PrPsc isoform [2,12]. Several cell-free, in vitro systems have been developed that not only convert but also propagate or amplify infectious PrPsc molecules. These include protein misfolding cyclic amplification (PMCA) [13,14,15] and quaking-induced conversion (QuIC) [16]. In these prion amplification methods, small amounts of prions (PrPsc) are added to large amounts of native PrP (recombinant or brain-derived) and the mixed samples are shaken or sonicated for days. Over time this mixing and high energy input leads to template-directed conversion of the native PrP to infectious prions (PrPsc). This conversion can be serially propagated (repeatedly adding small amounts of seed PrPsc to large amounts of native PrP) to generate detectable amounts of
His6x-tags were expressed in *E. coli* K (PK) resistant PrPsc isoform and the spontaneously generated recMoPrP 90–231, recMoPrP 120–231 and recCePrP 94–233) with may occur incidentally. Protein misfolding cyclic amplification (PMCA) and quaking causes conversion to Furthermore, propagation of (POPG) and RNA [18], or urea and guanidine HCl [19]. Furthermore, propagation of *de novo* generated misfolded proteins, also requires additional cofactors including 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and RNA [18], or urea and guanidine HCl [19]. Additionally, 0.1 mM PMSF was added to the desalted was added to the eluted PrP fractions. Protein samples were then dialyzed first into 50 mM sodium acetate pH 5.5, followed by 18Q water and then lyophilized. The purity of all constructs was confirmed to be greater than 98% by SDS-PAGE. The protein concentration was determined using absorption extinction coefficients determined by Protparam (ExPASy) and confirmed by a Bradford protein assay (Biorad Laboratories Canada, Mississauga, Canada).

Removal of the His6x purification tag was performed with bovine thrombin (Sigma-Aldrich Canada, Oakville, Canada) at a ratio of 1:2000 in 10 mM Tris pH 7.1 for 6 hrs. The reaction was then stopped by removing thrombin with Pierce SBTI-agarose (Thermo Scientific, Rockford, USA) or p-aminobenzamidine-agarose (Sigma-Aldrich Canada) and then applying the sample to a PD10 desalting column equilibrated in 50 mM sodium acetate pH 5.5. Additionally, 0.1 mM PMSF was added to the desalted sample which was then dialyzed into water, snap frozen and lyophilized.

### Materials and Methods

**RecPrP** purification

Truncated recombinant prion proteins from Syrian hamster, mouse and white-tailed deer (cervid) constructs (recShPrP 90–231, recMoPrP 90–231, recShPrP 120–231 and recCePrP 94–233) with His6x-tags were expressed in *E. coli* and purified as previously described [9,22]. In addition, a full-length recMoPrP 23–231 construct was generated similarly by inserting MoPrP 23–231 into a pET15b expression plasmid containing an N-terminal fusion tag attached to a His6x tag, a thrombin cleavage site and an enterokinase cleavage site (MGSSHHHHHHSSGLVPRGSHMDDD). All constructs were purified on Ni-NTA (Qiagen Canada, Toronto, Canada) as previously described [22] and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA) added to eluted PrP fractions at a dilution of between 100X and 25X, from a tablet dissolved in 1 mL of water. In addition, for the full-length MoPrP 23–231 1 mM EDTA was added to the eluted PrP fractions. Protein samples were then dialyzed first into 50 mM sodium acetate pH 5.5, followed by 18Q water and then lyophilized.

### Shaking-induced conversion of recPrP to oligomers and fibrils

Selected samples of recPrP constructs from each species were shaken under various conditions to convert them to a β-sheet rich isoform. Standard conditions for conversion of all PrP constructs involved using 0.4 mL of a 0.5 mg/mL recPrP solution in 20 mM sodium acetate, pH 5.5 with 0.02% sodium azide, being shaken at either 350 rpm at 37°C in a 1.5 mL polypropylene MaxyClear microcentrifuge tube (Corning Life Sciences –Axxygen, Union City, USA). Different shaking speeds had to be used because the orbital incubator used at 350 rpm broke and was replaced with a shaker with a different orbital diameter. Samples shaken at 350 rpm, were placed on a Lab-line 3527 orbital shaker with a 0.75 orbit diameter; whereas, samples shaken at 250 rpm were placed on a Lab-line Orbit 3590 shaker with 2 orbit diameter. We also show that the propagation of fibrils occurs by seeding with shaking-induced fibrils. In addition, we show that shaking-induced conversion occurs for a wide range of full-length and truncated constructs of mouse, hamster and cervid prions. We propose that this method of conversion can generate milligram quantities of physiologically stable β-sheet rich oligomers and fibrils. The cofactor free nature of this conversion method makes it applicable to the screening of potential small molecule prion inhibitors. Furthermore our results have interesting implications in protein misfolding cyclic amplification (PMCA) and quaking induced conversion (QuIC), where agitation-induced conversion may occur incidentally.
conversion is found when shaking recMoPrP \(^{90-231}\) at 75 rpm for up to 4 days. When shaking recMoPrP \(^{90-231}\) at 150 rpm we found essentially no conversion after 3 days, then <1% oligomerization/ aggregation after 4 days and <2% oligomerization after 7 days of shaking (result not shown). However shaking recMoPrP \(^{90-231}\) at 200 rpm on the 2” orbit shaker does generate oligomers and fibrils of a similar distribution to that seen at 250 rpm. We also tested shaking induced conversion on an incubator with an orbit diameter of 1” (Lab-line 3520). For this incubator we found that shaking in the range from 250 to 350 rpm was necessary to generate oligomers and fibrils (results not shown).

For comparison we generated oligomers from recMoPrP \(^{90-231}\) and recShPrP \(^{90-231}\) using more conventional prion conversion conditions: 3 M urea, 20 mM sodium acetate, pH 4 and 200 mM NaCl [4]. We also generated prion fibrils using standard conditions [23] by shaking recMoPrP \(^{23-231}\) with a protein concentration of 1 mg/mL in 1 M guanidine HCL, 3 M urea, 50 mM HEPES and 150 mM NaCl, pH 7 at 350 rpm and room temperature for 3 days. Fibrils were then dialyzed into 20 mM sodium acetate pH 5.2 for further analysis. Sonications of recMoPrP \(^{90-231}\) and recMoPrP \(^{23-231}\) was conducted on an Ultrasonic 3000 Homogenizer (BioLogics Inc., Manassas, VA, USA) with a 3.8 mm micro tip or a 12.7 mm tapped tip, as indicated. Buffer conditions and prion concentrations were the same as used for shaking-induced conversion. For sonications using a micro tip, the end of the tip was placed directly in the prion protein solution. For sonication with the regular, tapped tip, the end of the probe was placed in the water bath, immediately at the side of a 0.2 mL PCR tube containing the prion sample. To mimic PMCA-like sonication, prion samples were sonicated for 1.2 minutes (2 minutes, pulsed at 60%) every 30 minutes for 24 hrs, in a 0.2 mL thin-walled PCR tube. Were sonicated for 1.2 minutes (2 minutes, pulsed at 60%) every 30 minutes for 24 hrs, in a 0.2 mL thin-walled PCR tube.

Resolution Enhanced Acidic Gel Eletrophoresis (RENAGE)

The size of the prion monomers, oligomers and fibrils were analyzed using a specially developed technique called RENAGE and sized via a PrP PICUP ladder (a ladder consisting of different covalently linked PrP oligomers). Gels were prepared using a 8% acrylamide pHi 4.3 running gel and a 3% acrylamide pHi 5.2 stacking gel as previously described [22]. The running buffer consisted of 0.35 M \(\beta\)-alanine and 0.14 M acrylamide at pH 4.3. Gels were pre-run at 30 mAmp per gel for 20 minutes and then 5 \(\mu\)g of the prion sample was loaded in dissolving buffer. The dissolving buffer contains 37% glycerol, 128 mM aceta-KOH, pH 5.2 and 0.01% crystal violet (Sigma-Aldrich Canada). The gels were run at 30 mAmp for 75 to 85 minutes with reverse polarity. Gels were stained with colloidal coomassie blue for approximately four hours and destained in water [24]. The PICUP ladder was generated by cross-linking recMoPrP \(^{90-231}\) at 1 mg/mL by photo-induced cross-linking of unmodified protein PICUP [25] as described above. PrP samples were then scanned in a refrigerator until the completion of time course study and then the RENAGE analysis was performed. Quantitation of monomers, oligomers and fibrils was performed by first scanning the gel image and then converting the gel lanes to a band-intensity chromatogram using ImageJ [http://rsbweb.nih.gov/ij/index.html]. Chromatograms were then plotted using the Origin software package (OriginLab Corp., version 9) and the peaks manually marked and integrated, using Origin’s “peak analyzer” module. Percentages of each oligomer class were then calculated from the area of each peak compared to the total integrated area. The RENAGE fibril peak areas were plotted versus time and fitted to a sigmoidal function \((y = a/(1+exp(-k*(x-xc))))\) or exponential function \((y = A*exp(x/t)+y0)\) using the Origin software package.

Circular Dichroism

The secondary structure of each PrP construct and PrP oligomers of recShPrP \(^{90-231}\) and recMoPrP \(^{90-231}\) was determined using CD. Spectra were acquired on a Jasco J-810 circular dichroism spectropolarimeter in a 0.1 mm quartz cell with samples dissolved in 20 mM sodium acetate, pH 5.5 or water at pH 5.5. Spectra were recorded as the average of three scans from 190 to 260 nm, acquired with a scan rate of 20 nm/min and smoothed with a Savitzky–Golay window of 9 or 11 points. The secondary structure was determined using CDPro [26] with the CONTINLL program [27] using the SP22X reference protein set.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were acquired on a Varian FTS-7000 infrared spectrometer (Varian) equipped with a DTGS (deuterated triglycine sulfate) detector. MoPrP \(^{23-231}\), MoPrP \(^{90-231}\) and MoPrP \(^{23-231}\) fibril samples (50 \(\mu\)L of 2–3 mg/mL) were dried onto a CaF\(_2\) plate under nitrogen. Spectra were acquired from 96 scans at a sensitivity of 2 and a resolution of 2 cm\(^{-1}\). First a background scan was run with a CaF\(_2\) plate in place and then a blank spectrum was acquired with buffer dried onto the CaF\(_2\) plate. Spectra were smoothed with a Savitsky-Golay window of 9 points using the Varian Resolution Pro software. FTIR spectra were further processed using Origin (Version 9) to assist with spectral deconvolution and secondary structure quantification. The second derivatives of the smoothed spectra were calculated using Origin (Version 9) with 2nd order Savitsky-Golay smoothing and a 9-point window. Before deconvolution, the baseline was subtracted from each absorbance spectrum. Absorbance spectra were then deconvoluted using Origin’s multiple peak fit, employing non-linear Lorentz curve fitting by fixing the wavenumber of the peak maxima from the second derivative spectra and using a peak width of 20 cm\(^{-1}\). The deconvoluted spectrum was then fit to multiple Gaussian curves by fixing the peak wavenumbers using the secondary derivative of the deconvoluted spectra and fixing the peak width to 13 cm\(^{-1}\).

Negative stain electron microscopy

PrP converted isoforms were applied to UV irradiated 300 mesh copper grids with a support film of Formvar with carbon (Ted Pella, Inc., Cat# 01753-F, Redding, CA, USA). The samples were diluted to 2 \(\mu\)M PrP in water and 10 \(\mu\)L of the prion solution was spotted onto Parafilm. The grid was placed on top of the droplet for 1 minute. Grids were washed once with 10 \(\mu\)L water and stained two times with 10 \(\mu\)L of 4% uranyl acetate, whereby one time the residual solution was wicked away using filter paper. Micrographs were acquired on a Philips/FEI (Morgagni) transmission electron microscope.

Thioflavin T fluorescence time course study

The progression of PrP conversion to fibrils during shaking was monitored by Thioflavin T (ThT) fluorescence enhancement. MoPrP \(^{90-231}\) and MoPrP \(^{23-231}\) were converted by shaking at 250 rpm and 37°C as described above. PrP samples were then removed at different time points and mixed with 20 mM sodium acetate pH 5.5 and 100 \(\mu\)M ThT (Sigma) to give 10 \(\mu\)M PrP (monomeric concentration). Emission spectra were acquired on a QuantaMaster 400 spectrophluorimeter (Photon Technology Inter-
was found to be 25%. Secondary structure content of these shaking-induced oligomers barely enters the stacking gel as seen for recMoPrP 90–231 (marked as formation of larger oligomers ([22]).

Shaking the PrP oligomer samples for longer times caused the 

Techniques: 3 M urea and 200 mM NaCl at pH 4 ([22] (Fig. 1).

Oligomers of ShPrP90–232 and recMoPrP 90–231 and confirmed by

presence of NaCl ([28]). Interestingly, this size distribution is distinct from what is seen when oligomers of heptamers and larger are formed using more conventional techniques: 3 M urea and 200 mM DTT, 50 mM Tris, pH 6.8, 0.01% bromophenol blue and boiled at 95°C for 5 min. Samples were run on a 12% SDS-PAGE gel with a Tris-Glycine buffer, pH 8.8 system.

Proteinase K digestion

The susceptibility/resistance to PK digestion was tested for recMoPrP 90–231, shaking-induced recMoPrP 90–231 fibrils and sonicated recMoPrP 23–231 samples using a method adapted from Atarashi et al. ([15]). For digestion, samples at 0.5 mg/mL PrP in 20 mM sodium acetate pH 5.5 were diluted 2 fold into 50 mM Tris pH 8. Then proteinase K (Promega, Madison, WI, USA) was added at ratios of 1:100, 1:200 and 1:50, PK to PrP (gg). The sample was digested for 1 hr at 37°C. After digest, ~4 M urea was added to individual samples along with Laemmli sample buffer (2% SDS, 5% glycerol, 2 mM DTT, 50 mM Tris, pH 6.8, 0.01% bromophenol blue) and boiled at 95°C for 5 min. Samples were run on a 12% SDS-PAGE gel with a Tris-Glycine buffer, pH 8.8 system and visualized with colloidal coomassie blue.

Results

Shaking alone induces recPrPc to form β-sheet rich oligomers

RENAGE ([22]) was initially used to discover the formation of oligomers induced by shaking recShPrP 90–232 and recMoPrP 90–231 at 37°C and 350 rpm, in buffer at pH 5.5. The size of these recMoPrP 90–231 oligomers follows a Gaussian or near-Gaussian distribution ranging from octamers to dodecamers as determined using a specially prepared PrP PICUP ladder (Fig. 1). This PrP PICUP ladder is generated by non-specifically cross-linking MoPrP 90–231 using the PICUP reagent ([22]). The same size and distribution of shaking-induced oligomers was produced from shaking recShPrP 90–232 (results not shown). Interestingly, this size distribution is distinct from what is seen when oligomers of heptamers and larger are formed using more conventional techniques: 3 M urea and 200 mM NaCl at pH 4 ([22] (Fig. 1).

Shaking the PrP oligomer samples for longer times caused the formation of larger oligomers (>16 mers) and a protein band that barely enters the stacking gel as seen for recMoPrP 90–231 (marked as the fibril band, Fig. 1, lane 4). This result is also seen for all recPrP constructs that we studied (results not shown). Formation of these shaking-induced oligomers occurs with or without the addition of 150 mM NaCl (results not shown). This is in contrast to urea induced conversion, where conversion to oligomers is dependent on the presence of NaCl ([28]).

CD was used to analyze oligomers that were formed by shaking recShPrP 90–232 and recMoPrP 90–231 and confirmed by RENAGE. This showed that ShPrP 90–232 oligomers have a β-sheet structure (Fig. 2). An aliquot of the same sample used for CD was analyzed by RENAGE and exhibited a bimodal size distribution with smaller oligomers (octamers to dodecamers) and larger oligomers (>16-mers), but no or little fibril band. Secondary structure content of these shaking-induced oligomers was found to be 25% β-sheet and 12% α-helix for recShPrP 90–231 as calculated by CDPko. A similar CD spectrum was acquired for a shaken recMoPrP 90–231 sample with approximately equal abundance of oligomers and fibrils (by RENAGE). These MoPrP 90–231 oligomers were also found to be β-sheet rich (24% β-sheet from CDPko). In contrast, monomeric recShPrP 90–232 and recMoPrP 90–231 contained 42% α-helix and 10% β-sheet. This result is consistent with previous findings that the appearance of discreet oligomer bands seen by RENAGE match with having a β-sheet structure ([22]).

Shaking-induced oligomers were also found at pH 6.2 using recShPrP 90–232 and recMoPrP 90–231 (result shown for recShPrP 90–232 in Fig. 3A). The distribution of oligomers formed at pH 6.2 is similar to that formed at pH 5.5. However at pH 6.2 there is an increase in high molecular oligomers (16 to 20-mers) relative to oligomers at 8 to 12-mers (Fig. 3A). Furthermore shaking-induced conversion occurred when recShPrP 90–232 and recMoPrP 90–231 were shaken at room temperature. In contrast

Figure 1. RENAGE indicates that shaking recPrPc generates oligomers. A PrP PICUP ladder (lane 1) is used to size the oligomers formed by shaking recMoPrP 90–231 in pH 5.5 buffer at 350 rpm and 37°C for 1 day (lane 2). Shaking-induced oligomers are predominantly a distribution of 8-mers to 13-mers. In comparison oligomers formed in urea and salt exhibit a bimodal size distribution of 7-mers to 12-mers (lane 3). Longer periods of shaking recMoPrP 90–231 (shaking at 350 rpm, 37°C for 2 days) will also generate a fibril band and bimodal distribution of 8-mers to 12-mers and larger oligomers (>16-mers) (lane 4). doi:10.1371/journal.pone.0098753.g001

Figure 2. Circular dichroism (CD) indicates that shaking-induced oligomers contain significant quantities of β-sheet. Shaking recShPrP 90–232 at 350 rpm (in pH 5.5 water and 150 mM NaCl) induces conversion from an α-helical protein (red line) to a β-sheet rich structure (blue line). The inset, on the right, shows the corresponding RENAGE gel of the same sample, indicating a preponderance of oligomers. CDPro analysis for native PrPc gives 43% α-helix and 10% β-sheet, and for oligomers it yields 16% α-helix and 24% β-sheet. doi:10.1371/journal.pone.0098753.g002
when shaking-induced conversion was tested at pH 7.4 the oligomers formed are predominantly large oligomers (>16-mers) and included a large oligomer band referred to as a fibril band on the RENAGE gels. Prion protein constructs of different lengths for MoPrP were assessed for their ability to convert to oligomers after 24 and 48 hrs shaking at pH 6.2, using RENAGE. Shaking-induced conversion occurs for full length recMoPrP 23–231 in a manner similar to truncated recMoPrP 90–231 although apparently with different kinetics (Fig. 3B). In contrast, shaking the C-terminal domain (recMoPrP 120–231) causes faster conversion as seen at 24 hrs and then after 48 hrs only large oligomers are visible by RENAGE (Fig. 3B). In this recMoPrP 120–231 sample which was shaken for 48 hours, there is a loss in the total amount of protein deposited on the gel in addition to the formation of a visible precipitate in the sample tube. This could indicate that after (C-terminal domain) oligomers form they preferentially progress to aggregates rather than fibrils. Conversion of these three different lengths of MoPrP occurred similarly at pH 5.5, except the C-terminal domain (recMoPrP 120–231) causes faster conversion as seen by RENAGE. Furthermore CD analysis of the same sample, shaken with no air-water interface, showed that there was no conversion to a β-sheet structure. All of the results presented in this paper were from shaking-induced conversion performed with a 1.5 mL centrifuge tube place on its side (unless otherwise stated). Experiments were conducted in this manner because it was found that conversion occurred faster when the tube was on its side, rather than when it was placed upright on a shaking platform (result not shown). This increase in conversion speed could be due to an increase in the water-air surface area.

In addition to CD analysis of ShPrP 90–232 and MoPrP 90–231 oligomers, the FTIR of the amide I band was used to characterize MoPrP 23–231 oligomers. The full-length construct was used so that we could focus on the characterization of the more physiologically relevant full-length recMoPrP 23–231 construct. The FTIR spectrum along with its second derivative of the oligomer sample shows the presence of different peaks compared to those found for the predominantly α-helical recMoPrP 25–231. Spectral deconvolution was performed on the FTIR absorbance spectra to determine the secondary structure composition of these oligomers (Fig. 4B). The areas of the resulting Gaussian peaks seen in Fig. 4B were used to determine the structural content (Table 1). The FTIR data shows that the recMoPrP 23–231 sample transitioned from 33% α-helix in the recPrP 94–233 sample to only 16% α-helix in the oligomeric sample. In its place the structure of the oligomers has transitioned to 18% β-sheet with significant turn and loop peaks (∼1662 and 1679 cm−1).

In this study shaking-induced conversion was tested on three different shaking incubators. Initially a shaker with a 0.75″ orbit diameter was used at 350 rpm. In experiments, where indicated, a shaker with a 2″ orbit diameter was used at 250 rpm. These two shaking speeds used with their respective orbit diameters (350 rpm; 0.75″ orbit and 250 rpm; 2″ orbit) generated oligomers of a similar distribution between oligomers and fibrils. To assess the reproducibility of shaking conversion with other orbit radii we also shook recMoPrP 90–231 with a 1″ orbital diameter. When comparing shaking speeds of 200 rpm, 300 rpm and

Figure 3. RENAGE of shaking-converted prions under various conditions. A) ShPrP 90–232 oligomers are formed by shaking in pH 5.5, 6.2 and 7.4 buffers, at 350 rpm and 37 °C. B) RENAGE after shaking at 350 rpm for 1 day with full length recMoPrP 23–231 (lane 1), truncated recMoPrP 90–231 (lane 2) and C-terminal domain recMoPrP 120–231 (lane 3) and after 2 days with recMoPrP 23–231 (lane 4), truncated recMoPrP 90–231 (lane 5) and C-terminal domain recMoPrP 120–231 (lane 6). Samples in panel B were shaken at 350 rpm and 37 °C in pH 6.2 buffer. C) Shaking a 0.6 mL solution of recShPrP 90–232 in a 0.6 mL centrifuge tube without any air or bubbles for two weeks (lane 1) as compared to the same sample of 0.4 mL in a 1.5 mL centrifuge tube (i.e. with air), shaken for one week (lane 2). doi:10.1371/journal.pone.0098753.g003
350 rpm we found that shaking at all three speeds generated the typical bimodal oligomer pattern, with more low molecular weight oligomers (8 to 12-mers) than high molecular weight oligomers (16 to 20-mers). Conversion to fibrils proceeded the fastest at 300 rpm with complete conversion after just 4 days, based on RENAGE (results not shown).

Characterization of shaking-induced β-sheet fibrils

To characterize the progression of PrP monomers to prion fibrils throughout the shaking period, we analyzed the shaking-converted isoforms by negative stain electron microscopy (EM). These samples were generated using different shaking conditions and different time points to provide an oligomer sample free of fibrils and a fibril sample free of oligomers. A sample of prion oligomers was generated by shaking recMoPrPc 90–231 monomers at 350 rpm, at room temperature for 1 day. The sample was shown by RENAGE to contain only oligomer bands and no fibril band. The sample was shaken at room temperature to enrich for oligomers and avoid the formation of fibrils, which was routinely found when shaking recPrP at room temperature, rather than 37°C. EM analysis of this sample showed that the oligomers were ~20 nm disc-like structures (Fig. 5A). It should be noted that there is an enrichment of high molecular weight oligomers (~20-mers) in this sample that likely aided in visualizing the oligomers by EM. EM characterization also confirmed what the RENAGE analysis initially showed: that the sample contained PrP oligomers only and no detectable fibrils. In contrast, PrPc that was shaken for 5 days at 350 rpm at 37°C, showed only a fibril band on RENAGE and contained abundant rod-like fibrils as seen by EM (Fig. 5B). The dominant species seen on the grid were these rod-like fibrils with no significant patches of the oligomeric structures that are seen in panel A. EM was also performed for recMoPrPc and recMoPrPc fibril samples (based on RENAGE) and showed the formation of similar rod-like fibrils (results not shown). However EM of shaking-induced conversion of MoPrPc did not show any rod-like fibrils, but rather only showed round clusters consistent with amorphous aggregates. However EM cannot rule out that fibrils are formed by shaking this C-terminal construct. This is because the fibrils may have been stuck to the tube and were at low abundance.

FTIR spectroscopy was also used to characterize the fully converted, shaking-induced fibrils. The extent of their conversion and fibril content was confirmed by RENAGE. Figure 6A shows the FTIR absorbance spectra and second derivative of both the full-length, native recMoPrPc 23–231 and the same protein fully converted to fibrils via shaking. The negative peaks seen in the

**Table 1.** Secondary structure composition of shaking-induced oligomers as determined from deconvolution and curve fitting of the FTIR amide I band.

| Assignment               | Wavenumber (cm⁻¹) | Secondary structure (%) |
|--------------------------|-------------------|-------------------------|
| Intermolecular β-sheet   | 1626              | 10                      |
| β-pleated sheets         | 1639              | 8                       |
| Random coil              | 1645              | 7                       |
| α-helix                  | 1657              | 16                      |
| Turns                    | 1662 to 1668      | 21                      |
| Turns/loops              | 1679              | 16                      |
| Anti-parallel β-sheet/turn| 1684              | 7.8                     |
| Anti-parallel β-sheet     | 1693              | 12                      |

DOI:10.1371/journal.pone.0098753.t001
second derivative spectra were used to assign the secondary structure components based on previously assigned PrPsc FTIR spectra [29] and de novo PrP fibril FTIR spectra [30]. For the shaking-induced PrP fibrils, the prominent peaks are a 1627 cm$^{-1}$ peak assigned to intermolecular hydrogen bonds characteristic of $\beta$-sheets and a 1634 cm$^{-1}$ peak assigned to $\beta$-pleated sheets. In comparison, in the recMoPrP 23–231 spectrum the predominant peak is 1652 cm$^{-1}$, which is the characteristic absorbance of $\alpha$-helices (Fig. 6A). FTIR spectral deconvolution was used to determine the percentage of secondary structure components from the shaking-induced fibril FTIR spectrum (Fig. 6B). Table 2 shows the full peak assignment and secondary structure percentages determined from Gaussian deconvolution of the FTIR spectrum. From this spectral deconvolution analysis it was determined that shaking-induced fibrils contained 38% $\beta$-sheet and 5% $\alpha$-helix (Table 2). In addition, we compared the FTIR spectrum obtained for shaking-induced fibrils with those from fibrils generated by shaking PrP in 2 M guanidine HCl (GdnHCl) and 1 M urea, pH 7 as described by Baskakov and colleagues [4,30] (Fig. S2A). Differences between the fibrils formed by the two conversion methods are very slight, but there appears to be small differences in the amount of intermolecular hydrogen bonding (1627 cm$^{-1}$) and intramolecular $\beta$-pleated sheet (1634 to 1637 cm$^{-1}$). In particular the GdnHCl/urea-formed fibrils exhibit a slight increase in the amount of the intermolecular hydrogen bonding (1627 cm$^{-1}$) compared to the shaking-induced fibrils. (Fig. S2 and Table S1).

Amyloid-like properties in shaking-converted prion fibrils

The time course of shaking-induced conversion of recMoPrP 23–231 was followed using RENAGE (Fig. 7A). This allowed us to quantify the amount of monomer, oligomer and fibril throughout the conversion process, using a single technique. As seen in Fig. 7A there is a loss of monomer that is concurrent with the formation and loss of oligomers, followed by the abrupt formation of fibrils. A time course for recMoPrP 90–231 also showed a loss of monomer concurrent with the formation of oligomers and a shift to fibrils (result not shown). We also used ThT to probe for the formation of the characteristic cross-$\beta$ structure found in amyloids [23,31]. Previously we determined that shaking-induced fibrils enhance ThT fluorescence (results not shown). Consequently, we monitored the time course changes in ThT fluorescence during fibril
formation, by shaking alone. Plotting the time course of ThT fluorescence over time we show a sigmoidal growth in the number of fibrils (Fig. 7B). On the same plot we also show that the growth of the fibril band in RENAGE was also sigmoidal (Fig. 7B). This suggests that the RENAGE fibril band is a suitable way to follow the kinetics of PrP fibril formation. Furthermore, the ability to overlay the growth of ThT fluorescence with the RENAGE fibril band growth indicates that it is the fibrils that are responsible for the characteristic cross-β structure of PrP amyloid fibrils. The fact that the fibrils (and not oligomers) exhibit amyloid-like structure was further confirmed when we found that PrP oligomers formed by urea conversion do not enhance ThT fluorescence (result not shown).

In addition to testing the amyloid character of shaking-induced fibrils, we also tested if shaking-induced fibrils could seed and propagate fibril growth. For this we conducted a serial dilution study where small amounts of shaking-induced fibrils were added to fresh recMoPrP 23–231. These serial dilution studies showed that if the sample is not shaken, fibril formation could not be propagated upon dilution of 5% fibril into fresh recPrP (data not shown). However, if the sample was shaken, fibril formation occurred faster when fresh PrP was seeded with 5% fibrils, than if no seed was added (Fig. 8A,B). The time dependence of the fibril formation as determined from RENAGE of seeded and unseeded fibril growth was fitted to exponential and sigmoidal functions, respectively (Fig. 8C). Later time points are not shown in Fig. 8C because of a loss of fibril content after the end point of the sigmoidal growth. We attribute this to loss of sample due to either fibril-fibril aggregation or adsorption of the fibrils onto the plastic container [32]. We have repeated the propagation of fibril formation by seeding fresh PrP with the shaking-induced prion fibrils for five generations (i.e. five 1:20 serial dilutions). During these propagation steps the kinetics seen by RENAGE did not change.

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].

Table 2. Secondary structure composition of shaking-induced fibrils as determined from deconvolution and curve fitting of the FTIR amide I band.

| Assignment          | Wavenumber (cm<sup>−1</sup>) | Secondary structure (%) |
|---------------------|------------------------------|-------------------------|
| Intermolecular β-sheet | 1616                        | 11                      |
| Intermolecular β-sheet | 1627                        | 16                      |
| β-pleated sheets    | 1634                         | 11                      |
| Random coil         | 1645                         | 16                      |
| α-helix             | 1653                         | 5.4                     |
| Turns               | 1662                         | 18                      |
| Turn/loops          | 1675                         | 13                      |
| Anti-parallel β-sheet/turn | 1683                   | 4.8                     |
| Anti-parallel β-sheet | 1694                        | 4.7                     |

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].

Proteinase K resistance of shaking-induced fibrils

Naturally occurring infectious prions, as well as many in vitro converted fibril forms, are known to exhibit PK resistance [33,34]. In fact, PK resistance is considered to be a hallmark for the presence of PrP<sup>P<sub>Sc</sub></sup>. As expected, we found that shaking-induced fibrils (from recMoPrP 23–231) are PK resistant (Fig. 9A,B). As seen in this gel there are three bands of approximately 12, 13 and 14 kDa corresponding to PK resistant fragments. Interestingly, we also observed the presence of low levels of a ~17 kDa PK resistance fragment. This may be the same, previously published, resistance fragment of PrP<sup>P<sub>Sc</sub></sup>, as described by others [35].
17 kDa band that is found in PrP\(^{sc}\) that has been PK digested after deglycosylation [15]. In fact, a 17 kDa band is also seen in recombinant PrP\(^{sc}\) generated via PMCA and POPG/RNA that has been PK digested [18]. However in these cases, the 17 kDa fragment, from PK digested PrP\(^{sc}\), is often as abundant at the 12/13 kDa bands.

Given that the 17 kDa PK resistant fragment seems to be characteristic of infectious prions and given that the 12/13 kDa fragments are often found in non-infectious prions, we are now working on modifying our shaking conversion protocol to see if we can enhance the proportion of the 17 kDa fragment. This could lead to the generation of a self-propagating form similar to that described by Deleault et al., [21]. We also tested the PK resistance of fibrils generated after five serial propagations, but found that the PK resistance of the resulting fibrils did not change (results not shown).

**Sonication versus shaking**

Because sonication (as opposed to shaking) is commonly used for PMCA, we also tested the effect of sonication, alone, on oligomer formation. In our first experiment we investigated what sonication would do to a solution (0.5 mg/mL) of recPrP without the usual detergent additives of SDS or Triton X-100. Figure 10 shows that sonication (for 8 cycles of a 10 sec pulse) using a microprobe directly in the sample of recMoPrP\(^{23–231}\) results in the formation of a mixture of large oligomers (>14-mers; 25%), 7 to 12-mers

![Figure 8. Growth of shaking-induced fibrils with seeding is exponential. A) RENAGE gel of the time course of shaking induced conversion of recMoPrP\(^{23–231}\) at 250 rpm and 37°C. B) RENAGE gel of shaking induced conversion of the same recMoPrP (same batch) under the same conditions except with seeding using 5% MoPrP\(^{23–231}\) fibrils into fresh recMoPrP\(^{23–231}\). C) The chromatogram profile of each gel lane was acquired to determine the fibril content. Time dependent fibril content growth is shown plotted against time and has a sigmoidal dependence when starting with only fresh recMoPrP\(^{23–231}\) (open squares, black line). Upon seeding with 5% PrP fibrils the fibril content grows logarithmically (grey circles, grey line). doi:10.1371/journal.pone.0098753.g008](#)

![Figure 9. Shaking-induced fibrils have Proteinase K resistance. SDS-PAGE of recMoPrP\(^{23–231}\) (panel A) and fibrils (panel B) without (PK-) and with PK at 1:50, 1:200 and 1:400 (PK:PrP, g:g) shows that shaking-induced fibrils have 12, 13, 14 and 17 kDa resistance bands. doi:10.1371/journal.pone.0098753.g009](#)
(23%) and monomers (49%). This suggests that sonication is a much more powerful and a far faster approach to prion conversion to oligomers than shaking. However, the sonication-induced conversion under these conditions does not convert all of the monomeric recPrP, even after 10 cycles of sonication (for a total sonication time of 100 sec). We also tested whether repeated sonication, using a similar scheme as in PMCA, will increase the level of prion oligomerization. We sonicated a sample of 0.5 mg/mL recMoPrP23–231 at pH 5.5 in a 0.2 mL PCR tube for 2 min every 30 min over a 24-hour cycle. We found a small amount of oligomer (~20%) formed when the sample was sonicated with the horn outside of the thin-walled PCR tube, and more oligomers (30%) were found when a micro tip was placed directly inside the tube, using a 24-hour cycle (Fig. 9). In this latter sample, sonication-induced conversion generated a sample of 51% large oligomers (>14-mers), 38% small oligomers (7 to 12-mers) and 1% fibrils, with 11% monomer remaining. We also tested for PK resistance in the sonicated recMoPrP23–231 material but found that the samples were not PK resistant (data not shown). This is consistent with the very low PK resistance (in comparison to fibrils) found for β-oligomers [34]. Furthermore it indicates that the material generated from sonication, without detergents, does not generate the same prion isoform which forms spontaneously from PMCA [15].

Discussion

Our results clearly show that shaking-alone can convert recombinant PrP to β-sheet rich oligomers and fibrils. This is the first demonstration that the conversion of native recombinant PrP to β-sheet oligomers and fibrils can occur under physiological conditions (i.e. without the addition of detergents, denaturants, low pH, or high temperatures). Previously the only other de novo conversion method that approached physiological conditions was a conversion protocol that used a pH 4 buffer with shaking at 8 rpm [35]. Essentially, all other de novo methods used to convert PrP to misfolded prions employ detergents (SDS, Triton X-100), denaturants (urea, guanidine, high temperatures, extreme pH), lipid surfactants (POPG), large cofactors (RNA), or combinations of the above [3,4,5,9,11,15,18,36,37]. Because shaking-induced conversion is free of these chemical contaminants or cofactors, we believe the characterization of misfolded prions generated by shaking-induced conversion will potentially provide a “cleaner” framework with which to understand the prion conversion and propagation process. In particular, we believe that the shaking-induced conversion protocol we have developed could be used as a new model for cell free, de novo PrP conversion that could be used to identify or characterize prion inhibitors. In fact, we are currently optimizing a shaking-induced conversion assay to screen known and potential prion small molecule inhibitors. All previously published cell-free assays for screening small molecule inhibitors for prion conversion used denaturants [23,38,39]. However, it has been noted that the presence of urea and other denaturants can significantly change the mode of action and effectiveness of small molecules in prion conversion [39]. The ability to generate de novo prion oligomers and fibrils under simple, physiological conditions could not only improve the accuracy of small molecule screening assays but also renew interest in finding small molecule inhibitors through cell free conversion assays [23]. Furthermore, the ability to routinely generate milligram amounts of stable oligomers and fibrils, in a simple, uncontaminated physiological buffer, will certainly enhance the opportunities for high-resolution structural and biophysical studies of prions.

It is important to distinguish our findings and our methods from some of the better-known protocols for prion conversion, propagation and detection – namely PMCA and QuIC. In PMCA, sonication is used to generate or amplify PrP* from brain-derived or recombinant PrP* [13,15]. During the initial development of PMCA, spontaneous formation of a protease resistance isoform was found to occur during the sonication of recombinant PrP* in 0.1% SDS but it could be prevented by the addition of 0.1% Triton X-100 [15]. Consequently, the standard protocol for all modern PMCA methods involves the addition of Triton X-100 (a detergent). The use of detergents and extended periods of sonication in PMCA makes the technique relatively non physiological. It also prevents the technique from being used in ligand screening assays or in generating material for biophysical or high-resolution structural studies. In QuIC, a finely tuned protocol with intermittent shaking is used to amplify PrP* via the addition of external, recombinant PrP* [16,40]. With QuIC, spontaneous formation of a protease resistant PrP isoform can occur with shaking using a pH 7 buffer with 0.1% SDS and 0.1% Triton X-100 [16]. This kind of spontaneous amyloid formation can lead to false positives, which can be prevented in QuIC by using an optimized recombinant PrP* concentration, an optimized sample volume and an optimized temperature [16]. Rather than preventing this spontaneous conversion to a protease resistant amyloid isoform, we have identified conditions to robustly convert recPrP* to PrP* like isoforms. Overall, our demonstration that shaking, alone, can create PrP oligomers and fibrils with the characteristic biophysical features (fibril structure, β-rich, ThT binding, amyloid character, PK resistant, serially propagating) seen in prions generated by PMCA or QuIC or in vitro prion detection methods has some interesting implications. In particular, our data shows that shaking PrP at higher concentrations (0.5 mg/mL) than standardly used in QuIC [16,41,42], can result in the spontaneous formation of β-sheet rich isoforms that exhibit PK

Figure 10. Sonication of PrP generates oligomers. RENAGE of recMoPrP90–231 sonicated for 8 cycles of 10 seconds each, show that oligomers are generated (lane 1). Furthermore sonication of recMoPrP23–231 in a single PMCA-like round generates oligomers (lane 2). The formation of oligomers in a PMCA-like round is enhanced by placing the probe inside the solution (lane 3).

doi:10.1371/journal.pone.0098753.g010
resistance. This spontaneous/shaking-induced conversion can lead to false positives in prion detection assays.

Our observation that shaking-induced prion conversion required air or an air-water interface (Fig. 3C) suggests a possible mechanism by which the α-helical PrP^C is converted into a β-sheet rich isoform. In particular, the presence of an air-water interface appears to provide a denaturing (i.e. hydrophobic) environment that causes partial unfolding and clustering of the prion protein. Several reports have recently appeared describing the importance of an air-water interface in protein denaturation, in protein aggregation and in amyloid conversion for myoglobin, Aβ and insulin [32,43,44]. These data are consistent with our results showing that conversion does not occur when PrP is shaken without the presence of a small layer of air above the solution (Fig. 3C). While air-water interfaces are easy to generate in the laboratory, they are not particularly common physiologically. However, air-water interfaces with significant levels of turbulence and shaking are found in the stomach, the large intestine and rumen of mammals. Given that prion proteins occur throughout the body (including the gut) and that prion diseases are mostly transmitted via consumption of prion-infected material, it is not hard to imagine that the initial, infectious prion seeds could be generated in the gut prior to moving to the brain.

Another mechanism explaining prion protein conversion by shaking suggests that it is due to hydrodynamic forces caused by vortexing. Vortexing leads to protein denaturation by sheering forces as well as secondary seeding effects [45]. Previously it was found that vortexing insulin solutions causes a decrease in CD ellipticity at 210 nm, which is concurrent with the formation of an insulin amyloid [45]. A similar effect is also seen in generation of denaturant-induced (urea/guanidine HCl) prion fibrils formed with shaking [30]. These prion fibrils have a 205 nm feature that may correspond to a superhelical structure [30]. The effect of hydrodynamic forces on protein aggregation suggests that shaking-induced conversion of PrP is physiologically relevant because these forces can be found in vivo with biological fluids, such as blood [46] or in the rumen, stomach, or intestine of mammals.

Supporting Information

Figure S1 RENAGE of oligomers formed with and without a His6 tag.

(PDF)

Figure S2 Fourier transform infrared spectroscopy of shaking-induced fibrils compared to GdnHCl/urea formed fibrils.

(PDF)

Table S1 Secondary structure composition of GdnHCl/urea formed fibrils as determined from deconvolution and curve fitting of the FTIR amide I band.

(PDF)

Acknowledgments

We thank Trent Bjorndahl for helpful critique of the work. EM was acquired with the help of Arlene Outway in the Biological Sciences Microscopy Unit.

Author Contributions

Conceived and designed the experiments: CCL DSW. Performed the experiments: CCL BJG. Analyzed the data: CCL. Wrote the paper: CCL DSW.

References

1. Collinge J, Clarke AR. (2007) A general model of prion strains and their pathogenicity. Science 316: 930–936.
2. Caughey B, Baron GS, Chesbro B, Jeffrey M (2009) Getting a Grip on Prions: Oligomers, Amyloids, and Pathological Membrane Interactions. Annu Rev Biochem 78: 177–204.
3. Stohr J, Weinmann N, Wille H, Kaimann T, Nagel-Steger L, et al. (2008) Mechanisms of prion protein assembly into amyloid. Proc Natl Acad Sci USA 105: 2409–2414.
4. Baskakov IV, Legname G, Baldwin MA, Prusiner SB, Cohen FE. (2002) Pathway complexity of prion protein assembly into amyloid. J Biol Chem 277: 21140–21148.
5. Wang F, Yang F, Hu YF, Wang X, Wang XH, et al. (2007) Lipid interaction converts prion protein to a PrP^R-like proteinase K-resistant conformation under physiological conditions. Biochemistry 46: 7045–7053.
6. Deleault NR, Harris BE, Rees JR, Supattapone S (2007) Formation of native prions from minimal components in vitro. Proc Natl Acad Sci USA 104: 9741–9746.
7. Deleault NR, Lucassen RW, Supattapone S (2003) RNA molecules stimulate prion protein conversion. Nature 425: 717–720.
8. Wong E, Thackray AM, Bujdoso R (2004) Copper induces increased beta-sheet content in the scrapie-susceptible ovine prion protein PrpVRQ, compared with the resistant allelic variant PrpMRK. Biochem J 380: 273–282.
9. Bjorndahl TC, Zhou GP, Liu XH, Perez-Pineiro R, Semenchuk V, et al. (2011) Detailed Biophysical Characterization of the Acid-Induced PrP(Sc) to PrP(beta) Conversion Process. Biochemistry 50: 1162–1173.
10. Saleem F, Bjorndahl TC, Ladner CL, Perez-Pineiro R, Ametaj BN, et al. (Accepted with revisions) Lipopolysaccharide induced conversion of recombinant prion protein. Prion.
11. Gomes MPB, Millen TA, Ferreira PS, Silva NLCE, Vieira TCRG, et al. (2008) Prion protein complexed to N2a cellular RNAs through its N-terminal domain forms aggregates and is toxic to murine neuroblastoma cells. J Biol Chem 283: 19616–19625.
12. Jucker M, Walker LC (2013) Self-propagation of pathogenic protein aggregates as a mechanism by which the α-helical PrP^C is converted into a β-sheet rich isoform. In particular, the presence of an air-water interface appears to provide a denaturing (i.e. hydrophobic) environment that causes partial unfolding and clustering of the prion protein. Several reports have recently appeared describing the importance of an air-water interface in protein denaturation, in protein aggregation and in amyloid conversion for myoglobin, Aβ and insulin [32,43,44]. These data are consistent with our results showing that conversion does not occur when PrP is shaken without the presence of a small layer of air above the solution (Fig. 3C). While air-water interfaces are easy to generate in the laboratory, they are not particularly common physiologically. However, air-water interfaces with significant levels of turbulence and shaking are found in the stomach, the large intestine and rumen of mammals. Given that prion proteins occur throughout the body (including the gut) and that prion diseases are mostly transmitted via consumption of prion-infected material, it is not hard to imagine that the initial, infectious prion seeds could be generated in the gut prior to moving to the brain.

Another mechanism explaining prion protein conversion by shaking suggests that it is due to hydrodynamic forces caused by vortexing. Vortexing leads to protein denaturation by sheering forces as well as secondary seeding effects [45]. Previously it was found that vortexing insulin solutions causes a decrease in CD ellipticity at 210 nm, which is concurrent with the formation of an insulin amyloid [45]. A similar effect is also seen in generation of denaturant-induced (urea/guanidine HCl) prion fibrils formed with shaking [30]. These prion fibrils have a 205 nm feature that may correspond to a superhelical structure [30]. The effect of hydrodynamic forces on protein aggregation suggests that shaking-induced conversion of PrP is physiologically relevant because these forces can be found in vivo with biological fluids, such as blood [46] or in the rumen, stomach, or intestine of mammals.
20. Morillas M, Yanik DL, Surewicz WK (2001) On the mechanism of alpha-helix to beta-sheet transition in the recombinant prion protein. Biochemistry 40: 6982–6987.
21. Spassov S, Beekes M, Naumann D (2006) Structural differences between TSEs strains investigated by FT-IR spectroscopy. BBA-Gen Subjects 1760: 1130–1149.
22. Ousapchenko VG, Sawaya MR, Makarava N, Savrchenko R, Nilsson KPR, et al. (2010) Two Amyloid States of the Prion Protein Display Significantly Different Folding Patterns. J Mol Biol 400: 901–921.
23. Eisenberg D, Jucker M (2012) The Amyloid State of Proteins in Human Diseases. Cell 148: 1188–1203.
24. Murray AN, Palhano FL, Bieschke J, Kelly JW (2013) Surface adsorption considerations when working with amyloid fibrils in multiwell plates and Eppendorf tubes. Protein Sci 22: 1531–1541.
25. Weissmann C (2004) The state of the prion. Nature Reviews Microbiology 2: 861–871.
26. Pospiech R (2005) Prion protein amyloid formation under folding-like conditions. Prion 1: 14–24.
27. Cobb NJ, Apetri AC, Surewicz WK (2008) Non-native Prion Protein Amyloid Formation under Native-like Conditions Involves Reverse folding of the C-terminal helical Domain. J Biol Chem 283: 3704–3711.
28. Caughey B, Raymond LD, Raymond GJ, Maxson L, Silvaira J, et al. (2003) Inhibition of protease-resistant prion protein accumulation in vitro by curcumin. J Virol 77: 5499–5502.
29. Feng BY, Toyama BH, Wille H, Colby DW, Collins SR, et al. (2008) Small molecular aggregates inhibit amyloid polymerization. Nat Chem Biol 4: 197–199.
30. Atarashi R, Satoh K, Sano K, Fuse T, Yamaguchi N, et al. (2011) Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion. Nat Med 17: 175–178.
31. McGuire LJ, Peden AH, Orru CD, Wilham JM, Appleford NE, et al. (2012) Real-time quaking-induced conversion analysis of cerebrospinal fluid in sporadic Creutzfeldt-Jakob disease. Ann Neurol 72: 278–285.
32. Orru CD, Wilham JM, Raymond LD, Kuhn F, Schroeder B, et al. (2011) Prion Disease Blood Test Using Immunoprecipitation and Improved Quaking-Induced Conversion. MBio 2.
33. Sankaranarayanan K, Bhattacharyya A, Kragel J, Miller R (2012) Interfacial Viscoelasticity of Myoglobin at Air/Water and Air/Solution Interfaces: Role of Folding and Clustering. J Phys Chem B 116: 895–902.
34. Staszek V, Tamada JA, Khilanov AM, Langer R (1991) Kinetics of Insulin Aggregation in Aqueous-Solutions Upon Agitation in the Presence of Hydrophobic Surfaces. Proc Natl Acad Sci USA 88: 9377–9381.
35. Staszek V, Tamada JA, Khilanov AM, Langer R (1991) Kinetics of Insulin Aggregation in Aqueous-Solutions Upon Agitation in the Presence of Hydrophobic Surfaces. Proc Natl Acad Sci USA 88: 9377–9381.
36. Shukla V, Tamada JA, Khilanov AM, Langer R (1991) Kinetics of Insulin Aggregation in Aqueous-Solutions Upon Agitation in the Presence of Hydrophobic Surfaces. Proc Natl Acad Sci USA 88: 9377–9381.
37. Di Stasio E, De Cristofaro R (2010) The effect of shear stress on protein conformation Physical forces operating on biochemical systems: The case of von Willebrand factor. Biophys Chem 153: 1–8.