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N-terminal Domain of Prion Protein Directs Its Oligomeric Association*

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Background: Non-fibrillar oligomers are implicated as neurotoxic species in several amyloid neurodegenerative diseases.

Results: Full-length prion protein (PrP) forms distinct non-fibrillar β-sheet-rich oligomers. Truncated protein, lacking the N terminus forms nonspecific aggregates.

Conclusion: The unstructured N terminus of PrP is key to the folding and aggregation of its structured region.

Significance: To examine the full repertoire of PrP conformers and assembly states, full-length protein should be used.

The self-association of prion protein (PrP) is a critical step in the pathology of prion diseases. It is increasingly recognized that small non-fibrillar β-sheet-rich oligomers of PrP may be of crucial importance in the prion disease process. Here, we characterize the structure of a well defined β-sheet-rich oligomer, containing ~12 PrP molecules, and often enclosing a central cavity, formed using full-length recombinant PrP. The N-terminal region of prion protein (residues 23–90) is required for the formation of this distinct oligomer; a truncated form comprising residues 91–231 forms a broad distribution of aggregated species. No infectivity or toxicity was found using cell and animal model systems. This study demonstrates that examination of the full repertoire of conformers and assembly states that can be accessed by PrP under specific experimental conditions should ideally be done using the full-length protein.

Prion diseases are a group of fatal neurodegenerative diseases that include bovine spongiform encephalopathy in cattle, scrapie in sheep and goats, and Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, fatal familial insomnia, and kuru in humans (1).

According to the “protein-only” hypothesis, the key molecular event in the pathogenesis of these diseases is the conversion of the host-encoded prion protein (PrP)8 from its normal cellular conformation (PrPC) to disease-associated isoforms often referred to as PrPSc, which may form amyloid deposits. Although the precise molecular events involved in this conversion and the precise structure of infectious prions remain ill-defined, molecular genetic and in vitro studies suggest that PrPSc acts as a template that promotes the conversion of PrPSc to PrPSc and that the difference between the two isoforms lies purely in the monomer conformation and its state of aggregation (1, 2).

PrPSc is classically defined in terms of its detergent insolubility and relative protease resistance and has a high β-sheet content (3–5). In contrast, PrPSc consists of a predominantly α-helical structured domain and an N-terminal segment, which is unstructured in solution conditions, with a single disulfide bond forming an integral part of the core of the C-terminal structured domain (6–8).

Although it is clear that prion pathology is generally associated with PrP aggregation and the deposition of abnormal protein deposits, it is increasingly recognized that there are multiple disease-related forms of PrP, including protease-sensitive species, which might comprise the majority of infectivity in some isolates (9–11). In particular, small non-fibrillar β-sheet-rich oligomers have been suggested to be the most efficient mediators of prion infectivity (12), and have been shown to exhibit more neurotoxicity both in vitro and in vivo than the fibrillar forms of PrPSc (13). Small oligomeric species have also been implicated in other amyloid-related diseases (14–20) and may provide targets for diagnostic and therapeutic treatment.

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The abbreviations used are: PrP, prion protein; SV-AUC, sedimentation velocity analytical ultracentrifugation; PrPSc, cellular PrP isoform; PrPSc, pathogenic (scrapie) PrP isoform; PrP23–231, prion protein residues 23–231; PrP91–231, prion protein residues 91–231; GdnHCl, guanidine hydrochloride; PK, proteinase K; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-propane-1,3-diol.
PrP N-terminal Domain Directs Oligomeric Association

Several non-fibrillar oligomers have been obtained through the in vitro misfolding of PrP (13, 21–31), ranging in size (10–50 nm diameter) and in the minimum number of monomer subunits required (8–10 monomers) to form the oligomer. Generally these oligomers are rather aggregation prone and appear as transient species during the conversion to larger fibrils. The transient nature of these small oligomers has made it difficult to study their properties, structure, and relationship to fibrils and their physiological role (16). Whether such oligomers represent on- or off-pathway intermediates to amyloid formation remains contentious, however many of them display increased β-sheet structure and resistance to proteolysis, despite being soluble under physiological conditions (13, 22, 23, 26, 32, 33).

One such PrP species, termed β-PrP, is formed when PrP is refolded at acidic pH in a reduced state, with the disulfide bond broken (28, 34). This form of the protein assembles into soluble oligomers that have significant β-sheet content and partial resistance to proteinase K digestion, both properties characteristic of PrP Sc, and also forms amyloid fibrils which closely resemble those isolated from diseased brains (34, 35). In addition, β-PrP is antigenically distinct from native PrP Sc and inhibits proteasome activity at nanomolar concentrations, a mechanism by which PrP Sc has been proposed to effect neuronal death (36–38). Preincubation of β-PrP with an antibody specific for oligomerized proteins relieves this inhibition, consistent with oligomeric species mediating this effect (37). These data suggest a mechanism for intracellular toxicity mediated by defined oligomers of misfolded prion protein. β-PrP-associated inhibition of proteasome activity is most potent when full-length PrP (residues 23–231) is refolded to the β-PrP conformation (β-PrP 23–231), rather than the shortened PrP molecule comprising residues 91–231 (β-PrP 91–231). This suggests that PrP is capable of adopting distinct conformational isomers that are dependent on the length of its polypeptide chain. To resolve this and also to clarify its relationship to PrPSc, and also to clarify its relationship to PrPSc, we have resolved the disulfide bond and refolding at acidic pH

as previously described (28, 34). Briefly, this was performed by denaturation of PrP in 6 M GdnHCl in the presence of 100 mM DTT to a final concentration of no more than 1 mg/ml, and subsequent refolding by dialysis against 10 mM sodium acetate, 2 mM DTT, pH 4. β-PrP 91–231 samples were subject to ultracentrifugation at 150,000 × g for 4 h. β-PrP 23–231 samples were not subjected to a similar ultracentrifugation step after establishing that the yield, CD spectrum, and sedimentation coefficient distribution of the β-PrP 23–231 preparations were not affected by the centrifugation procedure. β-PrP 23–231 was assembled into fibrils by treating 0.27 mg/ml of β-PrP 23–231 in 10 mM sodium acetate, pH 4.0, with 1/9 volume of a 5 M stock of GdnHCl or NaCl (in the same buffer) to give final protein and denaturant concentrations of 0.25 mg/ml and 0.5 M, respectively.

CD Spectroscopy—Far UV CD spectra were acquired on a JASCO J-715 spectropolarimeter using typically 0.1-mm path length cuvettes and 10 accumulations, 1 nm bandwidth, 1 s −1 integration. Near UV CD spectra were typically acquired using 2-mm path length cuvettes and 30 accumulations. β-PrP spectra were acquired in 10 mM sodium acetate, 2 mM DTT, pH 4, α-PrP in 20 mM BisTris, pH 6.5. Mean residue ellipticity was calculated as [θ]R deg cm2 dmol−1 res−1.

NMR Spectroscopy—NMR spectra were acquired at 298 K on Bruker DRX-600 and DRX-800 spectrometers equipped with 5-mm 13C/15N/1H triple-resonance probes on β-PrP 23–231 samples ranging in concentrations up to 8 mg/ml (≈330 μM). Backbone resonance assignments of β-PrP 23–231 were achieved using the standard suite of triple resonance experiments (HNO, HN(CA)CO, HNCACB, and CBCA(CO)NH) (40–43). Proton chemical shifts were referenced to 1 mM sodium 3-trimethylsilyl-2,2,3,3-(2H4)propionate, using the gyromagnetic ratios of 15N/1H (ν13b) (45). Partial specific volumes for mouse PrP Sc were acquired in 20 mM BisTris, 25 mM imidazole, pH 6.5, and elution with 1 M imidazole in the same buffer.

Protein samples were loaded into Beckman AUC sample cells with 12-mm optical path two-channel centerpieces, with matched buffer in the reference sector. Cells were spun at 50,000 rpm in an AnTi-50 rotor, and scans were acquired using both interference and absorbance optics (at 280 nm) at 10-min intervals over 16 h. The sedimentation profiles were analyzed using the software SEDFIT (v13b) (45). Partial specific volumes for mouse PrP 91–231 and PrP 23–231 were calculated from the amino acid sequence using SEDNTERP software (46). Buffer densities and viscosities were measured using an Anton Paar DMA 5000 density meter and an Anton Paar AMVn automated microviscometer, respectively. Sedimentation velocity data were analyzed using the c(s) method of distribution (45) to characterize the sedimentation coefficient distribution of all species present in solution. For the β-PrP samples, it was necessary to use a bimodal fit, to separately fit the frictional ratios for monomer and larger species. The proportions of each sample occu-
pying the main peaks in the distribution were calculated by integration of the peaks.

Asymmetric Flow Field-Flow Fractionation—Asymmetric flow field-flow fractionation (AF4) experiments were performed using the Wyatt Eclipse 3+ with delay-corrected inline monitoring of UV (275 nm), refractive index, and light scattering coupled to an Agilent Affinity 1200 HPLC. A short channel (145 mm length) containing a 350-μm trapezoidal spacer and 5-kDa molecular mass cut-off polyethersulfone membrane was used for separation. Samples were run in 10 mM sodium acetate, 2 mM DTT, pH 4. Typically, injections of 50 μl were performed with a cross-flow of 1.5 ml/min over 1 min followed by 1 min focusing time, and elution with 1.5 ml/min cross-flow. Weight-averaged molar mass (Mw), and hydrodynamic radius were calculated, where possible, from the data using ASTRA6 software.

Urea Denaturation—Samples for simultaneous CD and AUC analysis were prepared in various concentrations of urea (0.0–6.0 M) in 10 mM sodium acetate, 10 mM Tris acetate, pH 6.0. Digestion was terminated with a cross-flow of 1.5 ml/min over 1 min followed by 1 min delay, and elution with 1.5 ml/min cross-flow. Weight-averaged molar mass (Mw), and hydrodynamic radius were calculated, where possible, from the data using ASTRA6 software.

Equilibrium Denaturation Data Analysis—The amide (far UV) CD absorption of 50 μM β-PrP23–231 in 10 mM sodium acetate, 2 mM DTT, pH 4.0, was recorded in varying concentrations of urea (0.0–6.0 M) in 10 mM sodium acetate, 2 mM DTT, pH 4.0, by dilution of the β-PrP23–231 stock solution with 9 M urea in the same buffer as appropriate. Samples were then concentrated to 50 μM PrP using Vivaspin 6 spin concentrators (Sartorius), and the flow-through used as the matched buffer reference for CD and AUC experiments.

Urea Denaturation—Samples for simultaneous CD and AUC analysis were prepared in various concentrations of urea (0.0–6.0 M) in 10 mM sodium acetate, 2 mM DTT, pH 4.0, by dilution of the β-PrP23–231 stock solution with 9 M urea in the same buffer as appropriate. Samples were then concentrated to 50 μM PrP using Vivaspin 6 spin concentrators (Sartorius), and the flow-through used as the matched buffer reference for CD and AUC experiments.

Electron Tomography—Samples were prepared in the same manner as for negative stain electron microscopy. Serial EM (48) was used to acquire a single axis tilt series with a tilt range of ±60° with 2° increments. Digital images were recorded using a 1K 61K Gatan Multiscan 794 CCD camera at a nominal magnification of 42,000 with a pixel size of 4.1 Å and a typical defocus of 1.2 μm. IMOD version 4.3 (49) was used to reconstruct tomograms from the tilt series using local patch tracking for alignment.

PrP Molecular Volume Calculation—To derive estimates of the number of monomers per oligomer for the EM data for which particle dimensions were measured, two different methods to estimate the molecular volume of recombinant PrP23–231 were used. A molecular volume of 27.5 nm³ was calculated using molecular weight and the calculated partial specific volume of 0.7078 cm³/g. Molecular volume was also estimated using the program Vossvolvos (50). A modeled Protein Data Bank file of full-length PrP, comprising residues 23–231 but lacking the GPI anchor and carbohydrate moieties was used as input, giving a volume estimate of 30 nm³, in close agreement with the estimate using partial specific volume.

Cell Viability Experiments—N2a mouse neuroblastoma cells (subclone PK1) (51) chronically infected with RML prions or uninfected were cultured in OptiMEM (Invitrogen) supplemented with 10% FCS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were seeded in a 384-well plate at 6000 cells per well. Preparations of α-PrP23–231 at 6 mg/ml, β-PrP23–231 at 4.6 mg/ml, and fibrillar β-PrP23–231 (prepared as described above) in 10 mM sodium acetate, pH 4.0, and a buffer control were assessed for cytotoxicity. For each sample, a 10-fold dilution into cell media was made and then serial 3-fold dilutions were prepared. n = 3 wells were exposed to each concentration of PrP and buffer for 3 days prior to performing the CellTiter-Glo luminescent cell viability assay (Promega), which measures total viable cell number by quantification of ATP in

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whole cell lysates. Untreated cells were used as negative controls.

In Vivo Infectivity Experiments—30 μl of β-PrP23–231 at 1 mg/ml was injected into the right parietal lobes of 10 recipient CD1 mice at the age of 4–6 weeks. 10 CD1 mice were inoculated with the corresponding preparation buffer as negative controls. Mice were monitored for signs of scrapie infection throughout their normal lifetime, culled according to Home Office guidelines, and their brains taken for neuropathological examination using immunohistochemistry and Western blotting. Brain sections were checked for spongiform neurodegeneration using Harris hematoxylin and eosin staining, the proliferation of reactive astrocytes using anti-GFAP antibodies, and abnormal PrP immunoreactivity using the anti-PrP monoclonal antibody ICSM-35 (D-Gen Ltd., London) (52). Appropriate positive and negative controls were used throughout.

RESULTS

β-PrP23–231 Is Characterized by β-Sheet Structure and a Low Degree of Tertiary Organization—Both β-PrP23–231 and β-PrP91–231 display far-UV CD spectra characteristic of secondary structures dominated by a β-sheet, with a single minimum close to the characteristic β-sheet minimum at 215 nm (Fig. 1A) (53). The CD spectra are similar to those observed for a number of oligomeric and amyloid-like PrP species (54–56) and are in marked contrast to the equivalent spectra of native full-length mouse α-PrP23–231. The latter show a minimum at 208 nm and a shoulder at 222 nm, characteristic of a protein dominated by the α-helical secondary structure (Fig. 1A). The near UV (aromatic) CD spectrum of β-PrP23–231 displays markedly reduced intensity in comparison with that of natively folded α-PrP, indicating that the protein side chains of aromatic residues within β-PrP23–231 are in less asymmetric environments than in the natively folded protein (Fig. 1B).

β-PrP23–231 Populates a Discrete Oligomeric Species—The significant β-sheet content and reduced level of tertiary structure for β-PrP23–231 are features shared with β-PrP91–231. This truncated form of PrP was previously determined to be predominantly oligomerized under native conditions (28). We thus sought to confirm whether β-PrP23–231 was similarly composed primarily of oligomeric species. Intriguingly, despite displaying very similar CD spectra, the two β-PrP species, β-PrP91–231 and β-PrP23–231, were found to oligomerize in radically different manners; the former forming a broad range of soluble oligomeric species, and the latter forming a defined, distinct oligomeric state. This was shown initially using SV-AUC (Fig. 2A). Analysis at 1.0 mg/ml showed that β-PrP23–231 is comprised of two discrete species with sedimentation coefficients of 1.5 ± 0.1 and 5.6 ± 0.6 S, characterized by frictional ratios of 1.8 ± 0.3 and 3.0 ± 0.5, respectively, giving molecular masses of 23 ± 3 and 280 ± 42 kDa. These molecular masses correspond with those of the monomer and of an oligomer comprising ~12 ± 2 monomer subunits. This is markedly different to β-PrP91–231, which does not populate any dominant species larger than the monomer, but rather populates a broad distribution of oligomeric species sedimenting between 2 and 30 S, with estimated molecular masses up to 2 MDa (Fig. 2A) (28). By way of comparison, PrP23–231 and PrP91–231 refolded into the native α-PrP conformation each show a single species sedimenting at 2 and 1.7 S, with frictional ratios of 1.6 and 1.5, respectively. These values translate to molecular masses of 23 and 17 kDa, which match closely the expected molecular masses for the monomeric species (Fig. 2B).

The oligomeric species in the samples (both the discrete β-PrP23–231 oligomer and the broad distribution β-PrP91–231 oligomers) are characterized by large frictional ratios f/0 3.0 for β-PrP23–231 and f/0 3.6 for β-PrP91–231), indicating significantly non-spherical character for both oligomers. This contrasts with the α-PrP monomers, which display lower frictional ratios, consistent with the slightly elongated structured core region (residues 121–231), connected to an unstructured N-terminal tail of 100 or 30 residues (6, 39). For both full-length and truncated PrP, the β-PrP monomers are characterized by slightly larger frictional ratios (1.8) than the α-PrP monomers, which results in their slightly slower sedimentation and smaller sedimentation coefficient (Fig. 2B). This is consistent with a slightly less compact monomeric state resulting from the loss of conformational restriction imposed by the native disulfide bond (7, 8).
The presence in solution of the β-PrP23–231 monomer and a distinct, well defined β-PrP23–231 oligomeric species was confirmed by asymmetric-flow field-flow fractionation (AF4), a technique that has previously been used to separate small nonfibrillar β-sheet-rich oligomers of PrP from scrapie-infected mouse brain material (12). The molecular mass determined for the oligomer using this technique was ~226 ± 36 kDa, in agreement with the values obtained using AUC (Fig. 3), and the number of monomers in infectious prion particles (12). The monomeric species was similarly identified with a close correspondence with the AUC data (Fig. 3). The hydrodynamic radius of the oligomer was determined using in-line dynamic light scattering measurements to be ~11 nm. Notably, the AF4 data indicated a low degree of polydispersity for the oligomeric species (1.04), indicating that the β-PrP23–231 oligomer was highly homogeneous.

β-PrP23–231 β-Sheet Structure Is Associated with its Oligomerization—The relative proportions of the β-PrP23–231 monomer and oligomer could be readily changed by altering the protein concentration. The proportion of the β-PrP23–231 oligomeric species was found to increase from 24% to greater than 50% by elevating the β-PrP23–231 concentration from 0.44 to 5 mg/ml. When the protein was incubated at 5 mg/ml and then diluted back to 0.44 mg/ml this elevated oligomer/monomer ratio was retained compared with a sample refolded at 0.44 mg/ml (Fig. 4A), which indicates that the rate of equilibration between the oligomer and monomer is slow on the timescale of measurement (hours). Increasing the proportion of β-PrP23–231 oligomer was found to alter the observed far-UV CD response. The far-UV CD spectrum of a 0.44 mg/ml sample that had not been concentrated displayed a minimum at ~208 nm, consistent with a mixed population of predominantly random coil and predominantly β-sheet conformers (Fig. 4B), whereas the spectrum of a 0.44 mg/ml sample that had been previously incubated at 5 mg/ml displayed a minimum at ~213 nm indicating a shift in population in favor of β-sheet-rich conformers. This indicates that the primary β-sheet CD response is derived from the oligomers. These spectra also reflect that the monomeric form of β-PrP23–231 folds into a conformation that is characterized by a major loss of α-helical structure and increased proportion of unordered structure, and is qualitatively similar to the behavior of the monomeric form of β-PrP91–231 (57). The strong correlation between the β-sheet far-UV CD response and the aggregation state of β-PrP23–231 was further established through the equilibrium denaturation of β-PrP23–231 (Fig. 5). Comparison of the proportion of oligomeric species determined using AUC with the CD signal at 215 nm indicated that the denaturant-dependent loss of β-sheet CD signal coincided with the reduction of the oligomer population (and concomitant increase in the proportion of

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**FIGURE 2.** Oligomeric states of α-PrP, β-PrP91–231, and β-PrP23–231 as assayed by SV-AUC. Full (0–30 S) (A) and expanded (B) sedimentation coefficient distributions of α-PrP23–231 (solid black line), α-PrP91–231 (solid gray line), full-length β-PrP23–231 (dashed black line), and β-PrP91–231 (dashed gray line). Both α-PrP species sediment with derived molecular masses equivalent to the expected molecular mass of their monomers (8), whereas β-PrP23–231 is populated by a monomer and a distinct 5.6 S species, and β-PrP91–231 consists predominantly of a broad distribution of soluble species sedimenting between ~1 and 30 S. For the β-PrP23–231 SV analysis, the average and S.D. for various parameters over 13 replicate experiments on 5 different preparations are as follows: sedimentation coefficient: monomer 1.6 ± 0.1 S, oligomer 5.6 ± 0.6 S; percentage occupancy of sample: monomer 24 ± 7%, oligomer 61 ± 13%; frictional ratio: monomer 1.8 ± 0.3, oligomer 3.0 ± 0.2.

**FIGURE 3.** Asymmetric-flow field-flow fractionation (AF4) of β-PrP23–231. Two peaks are detected by UV (solid line) and light scattering (Raleigh ratio, dashed line) (C), which correspond to the monomer (elution at 7.2 min) and oligomer (elution at 10 min) species identified by AUC as shown by the derived molecular masses (23 ± 3 and 226 ± 36 kDa (B)). The hydrodynamic radius of the oligomers is 11 ± 2 nm as determined by dynamic light scattering (A). The data are representative of 10 elution runs of β-PrP23–231 at different concentrations from two different protein preparations.
monomers) (Fig. 5B). The oligomeric species was found to unfold in a smooth transition with a low dependence on denaturant (Fig. 5B), indicating that the β-oligomeric species is not associated with a large scale burial of hydrophobic groups.

β-PrP23–231 Oligomer Structure—The structural organization of the β-PrP23–231 oligomers was characterized by electron tomography (Fig. 6). Oligomers were examined by negative stain EM and reconstructed by electron tomography. The latter reveals distinct particles with a diameter of 8.4 ± 1 nm and a height of 9 ± 1.3 nm, which can associate to form extended structures composed of 2–3 oligomers. This higher-order association of oligomers may explain the extended frictional ratios required to describe the 5 S species identified using AUC (Fig. 2). In addition, a central cavity of 2.8 ± 0.7 nm diameter (Fig. 6) is observed in many of the oligomers, similar to oligomers captured along the assembly and disassembly pathways of transthyretin amyloid protofibrils (58). The size of the particles identified lies within the size estimates given the molecular weight of the oligomers determined using AUC and AF4. The size estimates for the β-PrP23–231 oligomers (7–10 nm) and minimum assembly state (11–12 monomers) are thus in keeping with other distinct non-fibrillar PrP oligomers that have been reported (see "Discussion").

NMR Characterization of β-PrP23–231—As β-PrP23–231 was found to be soluble at high concentrations (~8 mg/ml), its structural properties on a per-residue basis could be examined using solution NMR (Fig. 7), in particular allowing the determination of the involvement of the N-terminal region in the distinct oligomeric species. Intriguingly, despite oligomerizing in a radically different manner, the NMR spectrum of β-PrP23–231 was found to be very similar to that of the truncated β-PrP91–231 form. Both β-PrP species give NMR spectra displaying markedly reduced chemical shift dispersion of resonances in comparison with natively folded α-PrP. The 1H NMR spectrum of α-PrP (Fig. 7) is characteristic of a fully folded globular protein, with wide chemical shift dispersion, including a number of upfield-shifted methyl peaks that are found at <0.7 ppm, which are indicative of strong tertiary interactions between aromatic residues and methyl side chains. In contrast, the NMR spectra of the β-PrP species are dominated by narrow clusters of intense peaks, the majority of which lie within chemical shift...
PrP N-terminal Domain Directs Oligomeric Association

FIGURE 6. β-PrP23–231 oligomers imaged by electron tomography. A section of a tomographic reconstruction of negatively stained β-PrP23–231 reveals particles with dimensions of ~8 × 9 nm, and a central cavity or cleft of ~3 nm diameter (solid square), which can associate into extended structures (dotted square). Scale bar, 50 nm.

FIGURE 7. Representative one-dimensional 1H NMR spectra of α-PrP, β-PrP23–231, and β-PrP91–231. α-PrP displays a well dispersed NMR spectrum characteristic of a fully folded protein containing well defined tertiary structure. In contrast, signals observed in both β-PrP species are restricted to residues in the unstructured N terminus of the protein (up to residue 126). This is highlighted by the observed signals at ~10.2 ppm, which arise from tryptophan side chains and occur exclusively within the unstructured N terminus. Signals from the remainder of the protein are broadened beyond detection by a combination of exchange broadening from molten globule formation and the high molecular weight soluble oligomeric species. At the protein concentration used (8 mg/ml) the dominant species in the β-PrP23–231 NMR sample is the oligomer.

ranges characteristic of random coil, unfolded peptides. As found in β-PrP91–231, and a number of other partially folded and oligomeric PrP states (26, 28, 55, 59), these intense peaks observed in the β-PrP23–231 NMR spectra were assigned to residues in the N terminus of the protein, residues 23–126. These were found to have chemical shift values very similar to those observed in the unstructured N terminus of α-PrP and β-PrP91–231, consistent with this region of the protein remaining predominantly unstructured in β-PrP23–231.

The low near-UV CD signal and lack of chemical shift dispersion in the NMR spectra are indicative of little tertiary organization within β-PrP23–231. However, the strong far-UV response indicates a significant β-sheet secondary structure and thus β-PrP23–231 behaves as a partly folded, molten globule state (60). These folding intermediates are characterized by near-native secondary structure, but little tertiary structure; the fluctuating nature of this species often results in the exchange broadening of NMR signals including their complete loss (61). Indeed, the number of observable peaks in the NMR spectra of β-PrP23–231 was significantly fewer than expected, indicating that there is a loss of signal through line broadening in the β-PrP23–231 monomer and oligomer. Thus given the lack of tertiary interactions and its oligomerized state, the majority of β-PrP23–231 NMR resonances appear to be broadened due a combination of molten globule intermediate exchange kinetics and the relatively long rotational correlation time of the oligomeric species. It was not possible to differentiate signals arising from the monomeric and oligomeric species.

β-PrP23–231 Is Resistant to Proteinase K Digestion—As β-PrP23–231 forms such a distinct oligomeric state, rather than the broad distribution of large oligomers observed in β-PrP91–231, we wished to determine the relative PK resistance of β-PrP23–231 (Fig. 8). β-PrP23–231 was highly resistant to PK digestion, with undigested β-PrP23–231 still observed after digestion with 5 μg/ml of PK (1 h at 37 °C). The degree of protease resistance of β-PrP23–231 is of the same order as that observed with β-PrP91–231 (34), suggesting that resistance to PK digestion in both β-PrP conformers is likely to be primarily due to the structural reorganization from α-helical to β-sheet conformation rather than aggregation. In contrast, the predominantly α-helical α-PrP conformation was extremely sensitive to digestion with PK, and was completely digested by the addition of 0.05 μg/ml of PK after 1 h at 37 °C (Fig. 8).

β-PrP23–231 Forms Fibrils Under Increased Salt Concentrations—In common with β-PrP91–231, titration of β-PrP23–231 with the denaturant GdnHCl was found to increase its intermolecular association, and a conversion to oligomeric structures whose morphology could be studied by EM. This aggregation process was stimulated by equivalent concentrations of NaCl, indicating that this process is primarily an effect of increased ionic strength. Electron micrographs of β-PrP23–231 in 0.5 M GdnHCl and 0.5 M NaCl (Fig. 9) showed fibril-like structures with widths ranging from 10 and 16 nm, and variable lengths up to 2 μm. In addition to these, irregular amorphous aggregates of variable shape and size were also observed.

β-PrP23–231 Toxicity and Infectivity—We tested the effect of β-PrP23–231 on the viability of neuroblastoma cells (PK1) (51) by serial dilutions of the preparation into the cell growth media. No significant effect on cell viability was observed using an oligomeric β-PrP preparation at concentrations ranging from 0.002 and 460 μg/ml (Fig. 10). Fibrillar preparations of β-PrP23–231, and natively folded α-PrP23–231 over similar concentration ranges also did not affect the PK1 cell viability. This cell system thus provides no evidence for a toxic effect mediated by the β-PrP oligomers when applied externally.

The infectivity of β-PrP23–231 was also investigated. 10 CD1 mice were observed for 600 days (close to natural lifespan) for development of prion disease pathology following intracerebral injection of β-PrP23–231 (into the right parietal lobe). 10 control
CD1 mice were inoculated with the corresponding \( \beta \text{-PrP}^{23–231} \) preparation buffer. No mice developed clinical signs of murine scrapie. The brains of all mice were removed for neuropathological examination and Western blot analysis at the end of the experiment to determine whether there was evidence of subclinical prion infection. There was no prion neuropathology on histological or immunocytochemical analysis and no \( \text{PrP}^\text{Sc} \) was detected by Western blot analysis.

**DISCUSSION**

The molecular basis of prion neurotoxicity remains largely unknown. Loss of \( \text{PrP}^\text{C} \) function, as it is sequestered into aggregated forms, is not a sufficient cause because its depletion does not result in neurodegeneration (62, 63). It is increasingly recognized that multiple disease-related forms of \( \text{PrP} \), other than \( \text{PrP}^\text{Sc} \), exist and may differentially contribute to prion infectivity or toxicity, which can be uncoupled (64). Interest has focused on the role of small non-fibrillar \( \beta \)-sheet-rich oligomers in prion and other amyloid diseases (12, 14, 16–19, 21, 32). In particular, it has been proposed that soluble oligomers are common to most amyloidotic diseases and may represent the primary toxic species (65). The distinct oligomer formed by the reduced and acidified form of full-length recombinant \( \text{PrP} \) is thus of interest as a candidate toxic or infectious species.

Here, we show that full-length recombinant prion protein, comprising residues 23–231, can be refolded *in vitro* into a well defined non-fibrillar \( \beta \)-sheet-rich oligomeric form. These oligomers, formed in the folding of the full-length protein contrast markedly with similar reduction and refolding of truncated \( \text{PrP} \) residues 91–231. Our study provides a detailed structural characterization of this previously uncharacterized \( \text{PrP} \) oligomeric structure.

Electron tomograms show particles with dimensions \(~8 \times 9\) nm, often containing a cavity or cleft. Using the monomer molecular volume (see “Experimental Procedures”), and the calculated volume of the TM particles (assuming an annular structure), the number of \( \text{PrP}^{221–231} \) molecules per oligomer is estimated to be \( 16 \pm 5 \). Given the assumption that the entire

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**FIGURE 8.** Protease K resistance and cleavage patterns of full-length \( \alpha \text{-PrP}^{23–231} \) (A) and \( \beta \text{-PrP}^{23–231} \) (B). Protein samples were incubated at varying concentrations of proteinase K (0–50 \( \mu \)g/ml) in buffer at pH 6 and then analyzed by SDS-PAGE. \( \beta \text{-PrP}^{23–231} \) exhibited partial resistance to PK digestion, with undigested protein persisting at 5 \( \mu \)g/ml of PK, whereas \( \alpha \text{-PrP}^{23–231} \) is fully digested at 0.05 \( \mu \)g/ml of PK. Novex SeeBlue® Plus2 Molecular weight markers are shown on the left of each gel.

**FIGURE 9.** Assembly of \( \beta \text{-PrP}^{23–231} \) into fibrils. \( \beta \text{-PrP}^{23–231} \) is capable of associating into long unbranched fibrils, with diameters of up to 16 nm and lengths of several microns through the addition of 0.5 M GdnHCl (A) or 0.5 M NaCl (B). Both of these images are of \( \beta \text{-PrP}^{23–231} \) following 24 h incubation in either GdnHCl or NaCl, respectively. The scale bar in the images corresponds to 200 nm.
PrP N-terminal Domain Directs Oligomeric Association

FIGURE 10. Cell viability of uninfected (PK1) and chronically infected (iPK1) N2a cells with serial dilutions of α-PrP23–231, β-PrP23–231, and fibrillar β-PrP23–231 from stock solutions of 6, 4.61, and 0.25 mg/ml, respectively, with control serial dilution of buffer shown. No significant effect on cell viability was observed from any of the three preparations of PrP or buffer used. Values were normalized to average values of untreated cells; error bars are S.D. expressed as percentage of the mean for n = 3 repeats.

Attempts to reduce the PrP oligomer size, concentration, and toxicity by reducing or lowering the temperature of solution conditions have met with limited success. The lower temperature can result in large differences in biological effect as assessed by acute toxicity (77), in which only certain species were toxic (21, 32). The precise subcellular localization of PrPSc propagation, however, remains ill-defined, and late endosome-like organelles are involved in the formation of a similarly sized oligomeric PrP intermediate (21, 32).

Although we do not observe a specific cellular toxicity of this β-PrP23–231 preparation, several recombinant β-oligomers are reported to be toxic to neuronal cell cultures (13, 29, 30, 66, 74). The variety of cell types tested, the various preparations of recombinant PrP oligomers, and the considerable experimental variation may explain variability in toxicity, but it is also probable that subtle differences between the various species under investigation can result in large differences in biological effect. Indeed this has been reported for the bacterial protein HypF-N; this protein forms two oligomers that are similar according to AFM and thioflavin T reactivity, but toxicity is displayed by the one that is characterized by a lower degree of hydrophobic packing (76). The reducing/acidic conditions for the β-PrP oligomerization may be unfavorable for the production of toxic/infectious oligomers, however, it is also possible that β-PrP toxicity may occur via a more discrete mechanism, biological activity requiring perhaps other specific binding partners (for example, the PrP/Aβ interaction) (77), in which only certain transient Aβ assemblies cause PrP-dependent toxicity (20), and thus may require the application of the oligomer to more specialized models.

Notably, formation of this distinct oligomer requires residues 23–90. This region intriguingly remains unstructured and not directly incorporated into the β-sheet-rich core of the oligomer. There is precedence for the unstructured N-terminal region of PrP affecting the folding and distribution of oligomeric species formed by the remainder of the protein. For example, residues 105–120 are required for the conversion of α-PrP into a soluble β-sheet-rich oligomeric species (70).

Indeed, intrinsically disordered domains have been shown to be involved in protein-protein interactions (78), and there is also evidence that the interaction of PrPSc with β-sheet-rich con-
formers, and the induction of the pro-apoptotic signaling is dependent on the intrinsically disordered N-terminal region of PrP(Sc) (79).

Given that the N terminus of PrP contains a considerable number of charged residues (pl PrP91–231 = 7.95; pl PrP23–231 = 9.39), this suggests a principal role for electrostatic interactions in the formation of the distinct oligomeric state of β-PrP23–231. The highly charged N terminus of the protein may be involved in directing the formation of distinct oligomeric states that are not readily accessible to the truncated molecule as residues within this region transiently contact the folded region of PrP (6, 80, 81). Alternatively, charged surfaces in plasticarctic or the membrane surfaces used in dialysis may be involved, but of course this could also happen in vivo where there are a multitude of charged surfaces available to direct the assembly of discrete oligomeric species. For example, association of negatively charged lipids or detergents with PrP are required for its efficient conversion to a β-sheet-rich oligomeric form (32, 70). A role for the involvement of catalytic surfaces directing the formation of distinct assembly states thus remains a distinct possibility.

In conclusion, we have characterized a novel oligomeric PrP structure, the formation of which requires the full prion polypeptide chain. This highlights that to examine the full repertoire of conformers and assembly states that can be accessed by prion protein under specific experimental conditions ideally full-length prion protein should be used.

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Protein Structure and Folding:
N-terminal Domain of Prion Protein
Directs Its Oligomeric Association

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