Pathway Complexity of Prion Protein Assembly into Amyloid*

Received for publication, November 29, 2001, and in revised form, March 14, 2002
Published, JBC Papers in Press, March 23, 2002, DOI 10.1074/jbc.M111402200

Iliia V. Baskakov‡‡, Giuseppe Legname‡‡, Michael A. Baldwin‡‡, Stanley B. Prusiner‡**, and Fred E. Cohen‡‡‡***
From the ‡‡Institute for Neurodegenerative Diseases, the ‡Department of Neurology, the ‡Department of Pharmaceutical Chemistry, the **Department of Biochemistry and Biophysics, and the ‡‡‡Departments of Cellular and Molecular Pharmacology and of Medicine, University of California, San Francisco, California 94143

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

In vivo under pathological conditions, the normal cellular form of the prion protein, PrPc (residues 23–231), misfolds to the pathogenic isoform PrPsc, a β-rich aggregated pathogenic multimer. Proteinase K digestion of PrPSc leads to a proteolytically resistant core, PrP 27–30 (residues 90–231), that can form amyloid fibrils. To study the kinetic pathways of amyloid formation in vitro, we used unglycosylated recombinant PrP corresponding to the proteinase K-resistant core of PrPSc and found that it can adopt two non-native abnormal isoforms, a β-oligomer and an amyloid fibril. Several lines of kinetic data suggest that the β-oligomer is not on the pathway to amyloid formation. The preferences for forming either a β-oligomer or amyloid can be dictated by experimental conditions, with acidic pH similar to that seen in endocytic vesicles favoring the β-oligomer and neutral pH favoring amyloid. Although both abnormal isoforms have high β-sheet content and bind 1-anilinonaphthalene-8-sulfonate, they are dissimilar structurally. Multiple pathways of misfolding and the formation of distinct β-sheet-rich abnormal isoforms may explain the difficulties in refolding PrPSc in vitro, the need for a PrPSc template, and the significant variation in disease presentation and neuropathology.

Protein misfolding is a hallmark of the prion diseases. In vivo and recent in vitro experiments demonstrate that the misfolded pathological isoform of PrP1 (prion protein), designated PrPSc, directs the conversion of PrPc, the normal cellular isoform, into PrPSc (1). This conversion requires a substantial conformational change: PrPc is a proteinase K (PK)-sensitive, α-helical monomer, whereas PrPSc is a PK-resistant, aggregated β-sheet-rich multimer (2). Upon PK digestion, PrPSc loses residues 23 to 89, and the PK-resistant core, PrP 27–30 (residues 90–231), forms amyloid fibrils (3). PrP 27–30 remains fully infectious and retains high β-sheet content (4). The infectivity of PrPSc and PrP 27–30 presumably owes to the ability of the protein to traffic to the brain and templates the conversion of PrPc to PrPSc.

Different strains of prions have unique neurohistological and biochemical features as well as distinct clinical patterns (5, 6). Clinicopathological features include the specific incubation time, the neuroanatomic distribution, and the degree of PK resistance (7). These features, which are stable following serial transmission in a given animal, have been attributed to conformationally distinct multimeric arrangements of PrPSc. During the past several years, a considerable body of evidence has accumulated arguing that the properties of prion strains are enciphered in their conformations (8–12). Although it seems clear that prion strains are comprised of different conformers of PrPSc, glycosylation patterns and specific polymorphisms may add to strain diversity (13). Here we examine the ability of unglycosylated recombinant (rec) PrP with an intact disulfide bond and without a GPI anchor to form different non-native β-sheet-rich isoforms in vitro.

Recent studies by J. Y. Chang and co-workers (14, 15) demonstrated that the reduced form of recPrP 23–231 exists in four β-sheet-rich isoforms as judged by their distinct retention times in reverse-phase chromatography. However, other differences in the physicochemical properties of these isoforms have not been characterized. Because PrPSc and PrPc both maintain a disulfide bridge (16), we believe that it is important to focus on the ability of a nonreduced form of recPrP to adopt non-native conformations. Several recent studies have reported that nonreduced recPrP forms oligomeric β-sheet-rich isoforms (17–19). Unfortunately, a consistent picture of the differences between these distinct abnormal isoforms has yet to emerge.

By studying mouse and Syrian hamster PrP (designated Mo recPrP 89–231 and ShA recPrP 90–231, respectively), we demonstrated that the protein can adopt the α-helical native isoform, two non-native β-sheet-rich isoforms, a β-oligomer, and an amyloid fibril. These two abnormal isoforms may coexist under certain experimental conditions; however, the β-oligomer is not on the kinetic pathway to amyloid formation and is not a substructure in the assembling fibril. Instead, we have identified two novel multimeric transient intermediates in amyloid formation. The preferences for forming either a β-oligomer or amyloid can be dictated by experimental conditions, with acidic pH similar to that observed in endocytic vesicles favoring the β-oligomer and neutral pH favoring amyloid. Although both abnormal isoforms have high β-sheet content and bind 1-anilinonaphthalene-8-sulfonate (ANS), they are dissimilar structurally. In the amyloid form, residues 90–120 are buried in a manner reminiscent of PrP 27–30, whereas in the β-oligomer, this region is available for antibody binding. After PK digestion, the amyloid form is digested into a number of small fragments, whereas residues 122–221 of the β-oligomer remain intact. Analysis of the oligomerization state using elec-
trospray ionization mass spectrometry (ESI-MS) indicates that the β-oligomer is predominantly octameric. Our data demonstrate that PrP is capable of forming several abnormal isoforms and that the preference to fold into a particular abnormal isoform is influenced substantially by experimental conditions.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Refolding**—The expression and purification of Mo recPrP 89–231 and SHa recPrP 90–231 were performed as described previously (20). The purified protein was seen to be a single pure species with an intact disulfide bond as confirmed by SDS-PAGE and electron-beam mass spectrometry (Applied Biosystems Mariner; Applied Biosystems, Foster City, CA).

recPrP folds into its native α-helical conformation upon dilution from 10 mM urea (20 mM sodium acetate, pH 3.7) to 1 mM urea (20 mM sodium acetate, 0.2 mM NaCl, pH 5.5). To refold the protein into the β-oligomeric isoform, recPrP was diluted from 10 to 5 mM sodium acetate, 0.2 NaCl, pH 3.7 and incubated overnight at room temperature in 5 mM urea (18). The process of assembling to the β-oligomer was monitored by CD and size exclusion chromatography (SEC). Once recPrP was refolded into the β-oligomer, it remained stable after dilution from 5 to 1 mM urea in 20 mM sodium acetate, 0.2 mM NaCl, pH 5.5.

**Dye Binding**—The binding of Congo red (Sigma) was monitored using absorption spectroscopy. A fresh solution of Congo red was prepared by dissolving the dye in 5 mM potassium phosphate and 150 mM NaCl, filtering it five times with a 0.22-μm filter (Millipore, Bedford, MA), and adjusting the concentration to 0.3 mM. The difference spectra were obtained by subtracting the Congo red spectra obtained in the absence of protein from the Congo red spectra measured in the presence of protein. These measurements were corrected for light scattering as described by Khunk et al. (21).

Thioflavin T (ThT; Molecular Probes, Eugene, OR) fluorescence was monitored using a LS50B fluorimeter (PerkinElmer Life Sciences) at 482 nm (excitation at 450 nm; excitation slit was 5 nm; emission slit was 10 nm; 0.4-cm rectangular cuvettes) as described (23). In the time course of amyloid formation, aliquots of recPrP were diluted 20 times by phosphate buffered saline, pH 7.0, and incubated with either 5 μM ThT or 110 μM ANS for 15 min at room temperature before monitoring fluorescence.

**Negative Staining and Electron Microscopy**—Negative staining was performed on carbon-coated, 600-mesh copper grids that were glow-discharged prior to staining. The samples were adsorbed for 30 s, stained with freshly filtered 2% ammonium molybdate or 2% uranyl acetate, dried, and then viewed in a JEOL 100CX II electron microscope (Tokyo, Japan) at 80 kV at standard magnifications of 40,000 and 80,000 as described previously (24).

**Epitope Presentation**—The pattern of epitope presentation of the amyloid isoform of SHa recPrP 90–231 was assayed by enzyme-linked immunosorbent assay (ELISA) as described by Peretz et al. (25).

**Circular Dichroism**—CD spectra were recorded with a J-720 CD spectrometer (Jasco, Easton, MD) scanning at 20 nm/min, with a band width of 1 nm and data spacing of 0.5 nm using a 0.1-cm cuvette as described previously (18). Three individual scans were averaged, and the background spectra were subtracted.

**Size Exclusion Chromatography**—All of the separations were performed at 23 °C with a flow rate of 1 ml/min using a TSK-3000 HPLC gel filtration column (300 mm × 7.80 mm) equilibrated in a running buffer (pH 3.7) of 20 mM sodium acetate, 0.2 mM NaCl, and 1 mM urea. During the time course of amyloid formation, the aliquots of recPrP were diluted twice using the running buffer and immediately analyzed by SEC.

**Dynamic Light Scattering**—All of the measurements were carried out using a DynaPro-801 TC Dynamic Light Scattering Instrument (Protein Solution Inc., Lakewood, NJ). The samples (30 μl) were filtered through 100-μm membrane filters (Whatman Inc., Ann Arbor, MI), placed in the quartz cuvette of DynaPro-MSTC, and measured at a constant temperature of 23 °C (26).

**PK digestion—PK digestion was carried out with Applied Biosystems 140B syringe pump solvent delivery system using a 1 × 150-mm C-18 Yvdac column with a 300-Å pore size and a 5-μm particle size, connected to a 759A UV detector with a 35-nl capillary flow cell. Solvent A was 0.1% trifluoroacetic acid, and solvent B was acetonitrile with 0.08% trifluoroacetic acid, with a linear gradient of 5–95% over 30 min at a flow rate of 50 μl/min. The eluate was split such that ~10% was introduced into the electrospray ionization source of the orthogonal accelerated time of flight mass spectrometer (Applied Biosystems). The mass spectra were recorded continuously at 5-s intervals over the range of 600–2000 m/z. UV chromatograms recorded at 215 nm were compared with total ion current traces obtained from the mass spectrometer. Either ESI-MS spectra corresponding to peaks in the UV and total ion current traces were deconvoluted using the resulting averaged mass spectra, if deconvolved with the mass spectrometer, or spectra containing signals from multiple unresolved peptides were deconvoluted manually by visual inspection and by selection of related multiply charged ions. Deconvolution converted each multiply charged ion series into a single peak of zero charge, giving the relative molecular masses of the peptides. The experimentally determined masses were compared with calculations for all possible fragments of the Mo recPrP 89–231 sequence. The calculated values were based on monoisotopic atomic masses for the smaller peptides (<3000 Da) and on average masses for the larger peptides, for which the isotopic components could not be resolved.

For supramolecular ESI-MS, salt was removed from the protein samples by dialysis, and organic solvent (20% methanol or other solvent) was added to the buffer to enhance vaporization. The solutions were sprayed into the mass spectrometer, giving multiply charged ions of the oligomers in the gas phase with the pattern of peaks shown in Fig. 5a. This pattern was deconvoluted to give the oligomeric species shown in Fig. 5b.

**RESULTS**

In a previous study, we showed that recPrP can be folded either to its native, monomeric α-helical isoform or to a β-sheet-rich, oligomeric form (18). There is an equilibrium between the α-helical and the β-sheet-rich isoforms, where partially denaturing conditions (acidic pH and urea) and increased concentrations of PrP favor conversion to the β-oligomer. The conformational transition from the α-helical to the β-sheet-rich isoform is separated by a large energy barrier that is associated with unfolding and with a higher order kinetic process related to the oligomerization. Because of the height of the energy barrier, the conformational transition from the α-isoform to the β-isoform occurs very slowly, and PrP is kinetically trapped in its native α-helical conformation. Based on our preliminary data, we suggested that the β-oligomer could be a thermodynamically stable, on-pathway intermediate in amyloid formation.

To explore further the pathway of amyloid formation, we studied the behavior of the β-oligomer in greater detail. We incubated the β-oligomer at 37 °C under constant shaking, which led to the formation of amyloid fibrils as measured by ThT binding (Fig. 1). The kinetics of fibrillogenesis exhibit a lag phase followed by an exponential increase in fibril formation. The length of the lag phase was a function of pH, ranging from 10 h at pH 6.1 or pH 7.2 to 3 days at pH 3.7 (Fig. 1a). Additionally, the lag phase could be prolonged by increasing concentrations of urea (Fig. 1b). We found that after the kinetic trace reached a plateau, additional incubation of samples at 37 °C resulted in a decrease in the ThT binding. Under these conditions or following dialysis out of urea, fibrils tended to aggregate into large insoluble particles as seen by electron microscopy (Fig. 1c). Both Mo recPrP 89–231 and SHa recPrP 90–231 formed amyloid fibrils under similar conditions, but each had a distinct morphology. Fibrils of Mo recPrP were formed by twisted protofibrils with a diameter of ~20 nm (Fig. 1d), whereas those of SHa recPrP had a diameter of 10–15 nm (Fig. 1e). The addition of a 0.5% seed of prefolded amyloid to the fresh reaction substantially reduced the lag phase of the proc.

**Multiple Misfolding Pathways of the Prion Protein**

21141
Spectroscopic measurements were used to distinguish the /H9252-oligomer from the fibrillar form. In contrast to the /H9252-oligomer, the fibrils bind Congo red and ThT (Fig. 2), a common feature of amyloid structures. The binding of Congo red was detected as a typical red shift in its absorbance spectra (Fig. 2a) and as birefringence under polarized light (data not shown). Congo red binding was saturated at a 5:1 ratio of dye molecule to polypeptide chain. Specific binding of ThT resulted in substantial increases of fluorescence with a maximum at 482 nm (Fig. 2b).

ANS has been used to study the partially folded states of globular proteins as well as the binding pockets of a number of carrier proteins and enzymes. ANS has also been used to characterize fibrillar forms of amyloidogenic proteins and infectious
isoforms of PrP (27, 28). According to Safar et al. (23), ANS binds to PrP 27–30, as detected by increased ANS fluorescence and a blue shift of its fluorescence maxima from 540 to 490 nm. Similarly, ANS binding to fibrils of Mo recPrP 89–231 was accompanied by a substantial increase in the fluorescence yield and a blue shift of its fluorescence spectrum (Fig. 2c). However, similar changes in the fluorescence spectra were observed when ANS was added to a solution of the β-oligomer. The α-helix-rich monomeric structure did not show any evidence of ANS binding (Fig. 2c). Hence, ANS binds to both β-sheet-rich forms, amyloid fibrils, and the β-oligomer but not to the α-helical isoform.

The process of amyloid formation of recPrP can be used as a model to study the self-propagating conformational transition of PrP. However, in vitro formation of the β-oligomer and amyloid has not produced infectious prions thus far. Because these refolded molecules are not equivalent to the infectious form, we sought to characterize the structural similarities of these isoforms with PrPSc. One of the properties that distinguishes PrPSc from PrPc is the loss of antigenicity of residues 90–120. Accessibility of this epitope can be determined from a difference in the reactivities of specific antibodies to the non-denatured and denatured forms of PrP (25). To determine whether residues 90–120 are exposed or buried in the amyloid form of recPrP, we probed the binding of two antibody fragments (Fabs), D13 and 3F4, which are specific to epitopes 96–106 and 108–112, respectively. Binding of both Fabs to the non-denatured amyloid was relatively weak, whereas their reactivities were substantially greater after denaturation of the amyloid (Fig. 3). The increased reactivities of these Fabs reflect the greater exposure of epitope 96–112 to the solvent upon denaturation. In contrast to the epitope 96–112 of the N-terminal region, the C-terminal epitope 225–231 is exposed in the PrPSc isoform (25). The accessibility of this region in the amyloid form of recPrP was assayed using R1 and R2 Fabs, both of which bind to residues in the epitope 225–231. The reactivities of these Fabs were very similar regardless of whether or not the amyloid fibrils were denatured. A similar epitope presentation in the amyloid of SHa recPrP 90–231 and PrP 27–30 indicates that both forms have some common features.

**Kinetics of Amyloid Formation Probed by Multiple Methods**—To understand the basic principles involved in the conformational transition and oligomerization of PrP, we studied the kinetic pathway of amyloidogenesis in vitro. One question we wanted to address is whether the β-oligomer is on the pathway to amyloid formation. To dissect the in vitro folding pathway, the kinetics of amyloid formation were monitored in parallel by three biophysical techniques: SEC, ThT binding, and ANS binding. Employing SEC, we found four isoforms of Mo recPrP 89–231 with distinct oligomerization states (Fig. 4a). The isoform with an elution volume of 10.2 ml corresponded to the monomer, had an α-helical conformation as measured by CD, and was characterized by the absence of ThT and ANS binding. The isoform eluted at 7.2 ml corresponded to the β-oligomer and did not bind ThT but bound ANS. There were two distinct multimeric forms of recPrP with very similar elution volumes of 5.45 ml (multimer I) and 5.22 ml (multimer II) (Table I). Because of their similar elution volumes, both multimers appeared as one asymmetrical peak in most SEC experiments (Fig. 4b). However, these two multimeric isoforms could be distinguished following a 15-h incubation. Under these conditions, the multimer I and II peaks were equally populated (Fig. 4b). Both multimeric isoforms were enriched in β-sheet content and bound ANS. However, only multimer II bound ThT (data not shown). The properties of all PrP isoforms identified by SEC during the time course of amyloid formation are summarized in Table I.

To study the relationships between these four isoforms, the α-monomer, β-oligomer, and multimers I and II, we monitored the time-dependent change in the populations of these forms during the process of amyloid formation. Because of the very similar elution volumes for the peaks of multimers I and II, a single kinetic trace represents the sum of both isoforms. A decrease in the α-monomer population was accompanied by an immediate and simultaneous growth of both the β-oligomer and the combined population of the multimers (Fig. 4c). Because there was no time delay between the kinetic traces of the formation of the multimer and β-oligomer, it was difficult to judge whether the β-oligomer was an on- or off-pathway intermediate to amyloid fibril formation.

To investigate this further, we monitored the kinetics of amyloidogenesis starting from the β-oligomer of Mo recPrP 89–231 (Fig. 4d). A decrease in the β-oligomer population coincided with a growth in the α-monomer population, whereas the kinetics of accumulation of the multimer showed a lag phase (Fig. 4d). The multimer fraction started to increase only after the α-monomer concentration reached a certain level. As soon as the multimer appeared, its population grew rapidly. This coincided with a rapid decrease in the population of the α-monomer (Fig. 4d). It seems that the conversion of the β-oligomer into the α-monomer was slower than the consumption of the α-monomer during amyloidogenesis. Thus, the population of the α-monomer was exhausted after 20 h, and then tempo-

![Fig. 3. Amyloid of SHa recPrP 90–231 has an epitope presentation similar to PrP 27–30 (25). Reactivity of four different Fabs (top to bottom panels): D13, 3F4, R1, and R2, to non-denatured (○) and denatured (○) amyloid tested by enzyme-linked immunosorbent assay.](image-url)
Multiple Misfolding Pathways of the Prion Protein

... in 20 mM sodium acetate, pH 5.0, 0.2 M NaCl, and 1 M urea

FIG. 4. Kinetics of amyloid formation of Mo recPrP 89–231 (0.6 mg/ml) in 20 mM sodium acetate, pH 5.0, 0.2 M NaCl, and 1 M urea.

rarely increased again because of a slow dissociation of the β-oligomer. These data indicate that the β-oligomer may be off the pathway to amyloid formation and that the refolding and dissociation of the β-oligomer back into the α-monomer most likely precede fibril formation.

Comparison of the kinetic traces monitored by SEC and ThT binding provides important insight into the mechanism of amyloidogenesis. The kinetics of ThT binding was delayed relative to multimer formation (Fig. 4c). The initial growth of ThT fluorescence corresponded to the time point when multimer II appeared (Fig. 4c, bottom panel). This agrees with our previous result that the conversion from multimer I to multimer II is critical with respect to ThT binding (Table I). ThT binding continued to increase even after the trace for the combined population of multimers reached a plateau. Consequently, at this stage of the amyloidogenic process, the growth of ThT fluorescence cannot be explained solely by the increased multimer II population. Instead, we observed that increased ThT signals coincided with a gradual shift of the elution volume of multimer II from 5.25 to 5.0 ml (Fig. 4c). Because the SEC column has nonlinear properties in the range of 5.0–6.0 ml, even a modest change in the elution volume of multimer II corresponded to a substantial increase in its molecular mass. Hence, the kinetic trace of ThT fluorescence may represent two processes, (i) an increase in the population of multimer II and (ii) a growth in the size of multimeric species. Furthermore, the shift in the elution volume of the multimer II peak was followed by a decrease in the peak area (Fig. 4c, middle panel). It is likely that the disappearance of this peak was caused by an inability of the species to penetrate the SEC column as a result of the substantial size of multimer II. In addition, both kinetic experiments showed that the species identified by ANS binding coincided with that representing the total population of all isoforms excluding the α-monomer. This suggests that ANS binds nonselectively to all β-sheet-rich isoforms. The level of ANS fluorescence remained stable during the last stage of the fibrillogenic process, whereas the area of the multimer peak eluted from SEC decreased. This result also indicates that the last stage of amyloidogenesis monitored by SEC represents an elongation of multimer II, which affects its ability to penetrate the column. Overall, our kinetic experiments indicate that the β-oligomer is not on a direct path to amyloid formation. Furthermore, our data argue that the transition from the β-oligomer to the multimer occurs through the dissociation of the β-oligomer with refolding to form the α-monomer.

Amyloid Form and the β-Oligomer Have Distinct Conformational Properties—To probe the conformational differences between the β-oligomer and the amyloid isoform, we employed limited PK digestion combined with ESI-MS. PK digestion has been used widely to distinguish PrPSc from PrPC as well as to probe the differences between PrPSc strains (8, 11). After treatment for 1 h at 37 °C with a 1:40 ratio of PK to protein, the β-oligomer was cleaved into short peptides corresponding to the

incubated at 37 °C under continuous agitation. a, SEC profiles of (bottom to top) original sample and upon incubation for 2, 6, 9, 12, 15, 21, 31, 36, and 57 h. b, the multimer peak from SEC profiles obtained upon incubation of PrP for (bottom to top) 2, 6, 9, 12, 15, 21, 25, and 31 h. c, the kinetics of amyloid formation of recPrP that was initially refolded to the α-monomer form. Top panel, time-dependent change of ThT fluorescence (■), and the population of the α-monomer (■), the β-oligomer (▼), and the multimer (●) as monitored by SEC. Middle panel, kinetic trace of ANS fluorescence (□), the combined populations of nonmonomer isoforms (▼), and the population of multimer that does not penetrate through the SEC column (●). Bottom panel, the elution volume of multimer peak I (●) and multimer peak II (○). d, the kinetics of amyloid formation of recPrP that was initially refolded to the β-oligomer form. The symbol definitions are the same as for c.
Table I
Identification and characterization of recPrP isoforms with different oligomerization states

| Characteristic                      | α-Monomer | β-Oligomer | β Multimer I | β Multimer II | β Multimer III |
|-------------------------------------|-----------|------------|--------------|---------------|---------------|
| Secondary structure by CD           | α-helical | β-sheet    | β-sheet      | β-sheet       | β-sheet       |
| Elution volume by SEC (μl)          | 10.24 ± 0.05 | 7.20 ± 0.05 | 5.45 ± 0.05 | 5.22 ± 0.05 | 5.02 ± 0.05 |
| Oligomerization state/Mw             | monomer   | octamer    | > 1 MDa      | +             | +             |
| ANS binding                         | -         | +          | +            | +             | +             |
| ThT binding                         | -         | -          | -            | +             | +             |

N-terminal region, whereas the central and C-terminal regions (residues 122–221) remained intact (Fig. 5a). Epitope mapping confirmed that residues 122–221 comprise the proteinase-resistant core of the β-oligomer. Fab D13, which binds to the epitope including residues 96–106, does not bind the proteinase-resistant core, whereas Fabs D18 and R72, which are specific to epitopes 133–157 and 152–163, respectively, bind the PK-resistant core, as shown on Western blots (Fig. 5c). Surprisingly, the amyloid form was digested into mostly short peptides with no apparent PK-resistant core (Fig. 5c). Clearly, the amyloid form was digested into mostly short peptides with no apparent PK-resistant core, as shown on Western blots (Fig. 5c). Surprisingly, the amyloid form was digested into mostly short peptides with no apparent PK-resistant core (Fig. 5c).

Structural studies of the β-sheet-rich PrPSc isoform have been hampered by the lack of a soluble homogeneous preparation. Although the β-oligomer has not been found to be infectious, it may provide a useful surrogate for biophysical characterization by NMR spectroscopy or x-ray crystallography. Thus, we probed the stoichiometry and heterogeneity of the β-oligomer using dynamic light scattering, supramolecular ESI-MS, and SEC. The Stokes radius of the β-oligomer was 6.5 nm as measured by dynamic light scattering (data not shown). Assuming an ideal spherical shape, this translates into an average molecular mass of 300 kDa. Analysis of the squared variance of the SEC peak that corresponds to the β-oligomer indicates that it is a relatively heterogeneous population (29).

To probe the stoichiometry of the β-oligomers, we employed supramolecular ESI-MS (Fig. 6a). We observed two distinct groups of peaks: (i) narrow, well resolved peaks in the 800–2000 m/z region representing a monomeric protein with an molecular mass of 16,197 Da and (ii) broader peaks in the 2500–4000 m/z region (Fig. 5a). Deconvolution of these broad peaks suggested a maximum molecular mass of 129,867 Da (Fig. 6b), a value equivalent to the molecular mass of eight recPrP 89–231 molecules and an additional ~290 Da attributable to salt or buffer ions. Additional broad peaks in the 3500–4000 m/z region, which were difficult to deconvolute, indicated the presence of a smaller amount of species with different and possibly higher oligomerization states. A comparison of the areas under the peaks suggests that the oligomers are predominant in solution even under experimental conditions employed for ESI-MS. Changing the organic component from methanol to acetonitrile or trifluoroethanol gave a higher proportion of the monomer, showing that this isoform was formed by dissociation in solution (data not shown). Regardless of the solvent conditions, the ESI-MS spectra showed that only two species, the monomer and the oligomer (predominantly octamer), were present. This confirms that there were no significantly populated stable intermediates in the dissociation of the octamer.

We obtained a similar result by fragmenting the ions within the mass spectrometer after evaporation of the solvent by applying higher nozzle voltage. At low voltage (50 V), we observed predominantly oligomers (~99%) (Fig. 6c). By progressively increasing the velocity of the ions as they passed through residual gas molecules, we were able to break up the oligomers into monomeric products (data not shown) in a manner akin to that found by varying the solvent conditions. These results support our previous observation that the assembly of the

Fig. 5. Limited PK digestion of abnormal isoforms of Mo recPrP 89–231. HPLC-MS analysis of the peptides produced by digestion of the β-oligomer (a) and amyloid form (b) after 1 h of incubation with PK (1:40 ratio) at 37 °C. The peptides identified by MS in the course of separation by HPLC are shown on top of the peaks. The numbers correspond to the N- and C-terminal amino acids of PrP. c, immunoblots of PK-digested products of the β-oligomer treated with three Fabs: D13 (left panel), D18 (middle panel), and R72 (right panel). The fractions migrated to the molecular masses indicated (in kDa).
The oligomeric species remains assembled even in a gas phase in the absence of hydrophobic forces indicates that major contributions to the thermodynamic stability of the complex should be due to hydrogen bonds and electrostatic interactions. This exceptional stability could account for the PK resistance of the oligomer.

**DISCUSSION**

Protein misfolding appears to play a central role in prion disorders and a variety of other neurodegenerative diseases (1, 30). A unifying feature of these diseases is the existence of a native, stable monomeric isoform that is easily reached from the unfolded state and a multimeric β-sheet-rich isoform that is substantially more stable. Our recent studies indicate that the folding of PrP to its native, α-helical conformation is under kinetic rather than thermodynamic control (18). In addition, many proteins unrelated to neurodegenerative and conformational diseases are capable of adopting an alternative β-sheet-rich amyloid fold (31–34). These findings suggest that amyloidogenesis is a general phenomenon in protein folding, related to physicochemical properties of the polypeptide backbone (32, 35). This may require us to readdress many basic issues of protein folding, such as kinetic traps in the folding pathway, the complexity of the energy landscape of protein folding, and the position of the native state in this landscape. These considerations highlight the need for compartmentalization to improve the fidelity of in vivo folding (36).

Here, we demonstrate that recPrP is able to form two structurally distinct non-native isoforms: the oligomer and an amyloid isoform. Our initial assumption that the oligomer might be an intermediate on the pathway to an amyloid isoform is not supported by current results. Instead, the kinetic experiments indicate that the amyloidogenic process occurs through a pathway different from the one that leads to the oligomer (Fig. 7). The oligomer and the amyloid have different structural organizations as judged by PK digestion. Hence, the oligomer is not a substructure of the amyloid isoform. The oligomer must dissociate and refold to form the amyloid isoform. PK digestion argues that the oligomer is more like PrP 27–30, whereas epitope mapping demonstrates some structural similarities of the amyloid isoform with PrP 27–30. However, our inability to demonstrate to date that both the oligomer and the amyloid isoform are efficient infectious agents implies that the refolding protocols used in this study do not mimic the cellular misfolding process directed by PrPSc under pathological conditions.

Solution conditions play a substantial role in determining the particular route of misfolding and preferences to forming a particular abnormal isoform. The conformational transition from the α-monomer to the β-oligomer occurs at pH 5. The rate of conversion is significantly facilitated by partially denaturing concentrations of urea (4–5 M). In contrast, the optimal solution conditions for amyloidogenesis include neutral to slightly acidic pH values and the presence of a low concentration of urea (1–2 M). Both the decrease of pH from 7 to 3.7 and the increase in the concentration of urea from 2 to 4 M substantially prolonged the lag phase of amyloid formation (Fig. 1), providing an additional argument that the β-oligomer is not on the pathway to amyloid fibril formation. The conformational transition from the α-monomer to the β-oligomer was a reversible process in which the relative populations of the isoforms from +40 to +48 shown in Fig. 4e results in a peak with a maximum of 129,867 Da, which is 290 Da greater than the predicted molecular mass for the octamer. c, ESI-MS of the oligomer sprayed from 20% methanol at a nozzle voltage of 50 V.
were a complex function of pH and protein concentration. By contrast, amyloidogenesis was an essentially irreversible process. The initiation of amyloidogenesis was controlled by specific conditions, including the concentration of protein and continuous agitation at 37 °C (data not shown). Once the process of amyloidogenesis was initiated, it proceeded until the α-monomer was depleted. Hence, environmental factors, mainly pH and agitation, determine the preferences for the formation of the particular non-native isofrom.

Elucidation of the kinetic pathways and intermediate steps of amyloidogenesis in vivo are important for our understanding of prion replication. To the extent that our in vitro system captures important features of the in vivo process, this work can provide insight into the events underlying the conversion of the α-rich monomeric PrPSc form into the amyloidogenic isoform, PrPSc. Our data argue that the β-oligomer is not on the pathway to amyloid formation (Fig. 7). Instead, multimer I, a β-sheet-rich isoform that binds ANS but not ThT, appears to be a better candidate for an on-pathway role. It converts to a higher molecular mass isoform, designated multimer II, with the acquisition of ThT binding, a feature associated with amyloidogenic isoforms. We have yet to resolve the molecular events that are critical to the conversion of multimer I to multimer II and the formation of ThT-binding sites.

Although the β-oligomer of recPrP 89–231 is not on the pathway to the in vitro amyloid isoform, two other recombinant molecules, recPrP 106 and recPrP 23–231, form similar β-oligomeric isoforms (15, 26). When expressed in transgenic mice, all three proteins support PrPSc replication and lead to scrapie neuropathology (37, 38). In the case of recPrP 89–231 and recPrP 23–231, the α-rich monomer and the β-oligomer are separated by a substantial kinetic barrier. This has been attributed to the relative stability and structural complexity of the pretransition α-monomer isoform (18). For recPrP 106, however, the process of assembly of the β-oligomer occurs more rapidly and does not require the partially denaturing acidic conditions needed by recPrP 89–231 (26). Deletion of residues 141–176 destabilizes the α-helical conformation. Thus, we found that the β-oligomer is a preferred in vitro isoform of recPrP 106. However, despite differences between the pretransition states of recPrP 106 (unfolded) and recPrP 89–231 (folded α-helical), both molecules share a similar region that adopts a β-sheet-rich, PK-resistant structure upon conversion to the β-oligomer (Fig. 8) (26). Unlike PrP 27–30, the infectious PK-resistant core of PrPSc, the PK-resistant core of the β-oligomer corresponds to the α-helical folded domain of the PrPSc isoform.

At present, the biological significance of the β-oligomeric form is not clear. The formation of nonfibrillar oligomeric forms has been observed in other neurodegenerative diseases. Recent studies have suggested that the nonfibrillar, soluble oligomeric form of Aβ peptides possesses neurotoxic properties (39) and may therefore play a role in the pathogenesis of Alzheimer’s disease (39). Rochet et al. (40) proposed that nonfibrillar oligomers of α-synuclein, rather than the end product fibrils, are associated with the pathogenesis of Parkinson’s disease. It remains to be established whether either of the abnormal forms of recPrP, the β-oligomer or the amyloid form, can cause neuronal degeneration or prion disease.
of the conformational diversity of misfolded forms of PrP and the kinetic routes that they follow to the various β-sheet-rich isoforms should improve our understanding of the molecular mechanism of PrPSc formation and the specific roles that cellular compartments play in shepherding this process.

REFERENCES

1. Prusiner, S. B. (1997) Science 278, 245–251
2. Cohen, F. E., and Prusiner, S. B. (1998) Annu. Rev. Biochem. 67, 783–819
3. Prusiner, S. B., McKinney, M. P., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F., and Glenner, G. G. (1983) Cell 35, 349–358
4. Pan, K.-M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fleiterick, R. J., Cohen, F. E., and Prusiner, S. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10962–10966
5. Bessen, R. A., and Marsh, R. F. (1992) J. Virol. 66, 2109–2110
6. Hecker, R., Tarahouliou, A., Scott, M., Pan, K.-M., Torchia, M., Sendrowska, K., DeArmond, S. J., and Prusiner, S. B. (1992) Genes Dev. 6, 1213–1228
7. Scott, M. R., Groth, D., Taizel, J., Torchia, M., Tremblay, P., DeArmond, S. J., and Prusiner, S. B. (1997) J. Virol. 71, 9032–9044
8. Bessen, R. A., and Marsh, R. F. (1994) J. Virol. 68, 7859–7868
9. Telling, G. C., Haga, T., Torchia, M., Tremblay, P., DeArmond, S. J., and Prusiner, S. B. (1996) Genes Dev. 10, 1736–1750
10. Caughey, E., Raymond, G. J., and Bessen, R. A. (1998) J. Biol. Chem. 273, 32230–32235
11. Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E., and Prusiner, S. B. (1998) Nat. Med. 4, 1157–1165
12. Peretz, D., Scott, M., Groth, D., Williamon, A., Burton, D., Cohen, F. E., and Prusiner, S. B. (2001) Protein Sci. 10, 854–863
13. Somerville, R. A. (1999) J. Gen. Virol. 80, 1965–1972
14. Lu, B.-Y., Beck, P. J., and Chang, J. Y. (2001) Eur. J. Biochem. 268, 3767–3773
15. Lu, B.-Y., and Chang, J. Y. (2001) Biochemistry 40, 13390–13396
16. Turk, E., Teplow, D. B., Hood, L. E., and Prusiner, S. B. (1988) Eur. J. Biochem. 176, 21–30
17. Swietnicki, W., Morillas, M., Chen, S. G., Gambetti, P., and Szurewicz, W. K. (2000) Biochemistry 39, 424–431
18. Engh, R. V., McKinney, M. P., Prusiner, S. B., and Cohen, F. E. (2001) J. Biol. Chem. 276, 19687–19690
19. Morillas, V., Vanik, D. L., and Szurewicz, W. K. (2001) Biochemistry 40, 6982–6987
20. Mehlhorn, I., Groth, D., Stöckel, J., Mollot, B., Reilly, D., Yansura, D., Willett, W. S., Baldwin, M., Fleiterick, R., Cohen, F. E., Vandlen, R., Henner, D., and Prusiner, S. B. (1996) Biochemistry 35, 5528–5537
21. Kunk, W. E., Jacob, R. P., and Mason, R. P. (1999) Methods Enzymol. 308, 285–305
22. LeVine, H. (1993) Protein Sci. 2, 404–410
23. Safar, J., Roller, P. P., Gajdusek, D. C., and Gibbs, C. J., Jr. (1994) Biochemistry 33, 8375–8383
24. Will, H., Zhang, G.-F., Baldwin, M. A., Cohen, F. E., and Prusiner, S. B. (1996) J. Mol. Biol. 259, 668–681
25. Peretz, D., Williamson, R. A., Matsunaga, Y., Serban, H., Pinilla, C., Bastidas, R. B., Rozensteïn, R., James, T. L., Houghten, R. A., Cohen, F. E., Prusiner, S. B., and Burton, D. R. (1997) J. Mol. Biol. 273, 614–622
26. Baskakov, I. V., Aagaard, C., Mehlhorn, I., Wille, H., Groth, D., Baldwin, M. A., Prusiner, S. B., and Cohen, F. E. (2000) Biochemistry 39, 2792–2804
27. Litvintsev, S. V., Brew, S. A., Auda, S., Akiyama, S. K., Haudenschild, C., and Ingham, K. C. (1996) J. Mol. Biol. 259, 245–258
28. Kayed, R., Bernhagen, J., Greenfield, N., Sweeney, K., Brunner, H., Voelter, W., and Kapurniotu, A. (1999) J. Mol. Biol. 287, 781–796
29. Baskakov, I. V., and Bolen, D. W. (1998) Biochemistry 37, 18010–18017
30. Carrell, R. W., and Lomas, D. A. (1997) Lancet 350, 134–138
31. Guijarro, J. I., Sunde, M., Jones, J. A., Campbell, I. D., and Dobson, C. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4224–4228
32. Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3590–3594
33. Konno, T., Murata, K., and Nagayama, K. (1999) FEBS Lett. 454, 122–126
34. Fändrich, M., Fletcher, M. A., and Dobson, C. M. (2001) Nature 410, 165–166
35. Dobson, C. M. (1999) Trends Biochem. Sci. 24, 329–332
36. Sigler, P. B., Xu, Z., Rye, H. S., Burston, S. G., Fenton, W. A., and Horwich, A. L. (1998) Annu. Rev. Biochem. 67, 581–608
37. Supattapone, S., Bocquel, P., Maramoto, T., Wille, H., Aagaard, C., Peretz, D., Nguyen, H.-O. B., Heinrich, C., Torchia, M., Safar, J., Cohen, F. E., DeArmond, S. J., and Prusiner, S. B. (1999) Cell 96, 869–878
38. Fischer, M., Rulicke, T., Raeber, A., Sailer, A., Moser, M., Oesch, B., Brandner, S., Aquzzi, A., and Weisshmann, C. (1999) EMBO J. 18, 1255–1264
39. Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liatsatos, M., Morgan, T. E., Rozovskiy, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Krafft, G. A., and Klein, W. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6448–6453
40. Roche, J. C., Conway, K. A., and Lansbury, P. T., Jr. (2000) Biochemistry 39, 10619–10626
41. Jackson, G. S., Hassou, L. L. P., Power, A., Hill, A. F., Kenney, J., Suiblip, H., Craven, C. J., Walbro, J. P., Clarke, A. E., and Ellingboe, J. (1999) Science 283, 1935–1937
42. Maiti, N. R., and Szurewicz, W. K. (2001) J. Biol. Chem. 276, 2427–2431
43. Horvath, M. S., and Glickshneider, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6010–6014
44. Swietnicki, W., Petersen, R., Gambetti, P., and Szurewicz, W. K. (1997) J. Biol. Chem. 272, 27517–27520
45. Shibuya, G. P., Permanne, B., and Soto, C. (2001) Nature 411, 810–813
46. Kocisko, D. A., Come, J. H., Priola, S. A., Chesbro, B., Raymond, G. J., Lansbury, P. T., Jr., and Caughey, B. (1994) Nature 370, 471–474