Partial Unfolding and Refolding of Scrapie-Associated Prion Protein: Evidence for a Critical 16-kDa C-Terminal Domain†

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ABSTRACT: The conversion of the normal form of prion protein (PrP^C) to a disease-specific form (PrP^{Sc}) is a central event in scrapie and other transmissible spongiform encephalopathies. PrP^{Sc} is distinguished from PrP^C by its insolubility and its resistance to proteolysis. PrP^{Sc} is also capable of converting ^{35}S-PrP^C, *in vitro*, into a form which is indistinguishable from PrP^{Sc} with respect to its protease-sensitivity. Both the "converting activity" and the protease-resistance of isolated hamster PrP^{Sc} can be at least partially eliminated by denaturation and recovered by renaturation, provided that the concentration of denaturant does not exceed a threshhold. This study was undertaken in order to localize the regions of native PrP^{Sc} structure that must remain intact to allow refolding. Proteinase K was used to digest exposed, denatured PrP^{Sc} sequences, and the residual fragments were characterized using anti-PrP antibodies directed toward four PrP epitopes. A 16-kDa fragment marked by an epitope within residues 143−156 remained protease-resistant under conditions which at least partially unfolded epitopes within residues 90−115 and 217−232. However, dilution of denaturant restored protease-resistance to these epitopes. This reversible unfolding was observed with both purified PrP^{Sc} and PrP^{Sc} in crude brain homogenates. Size fractionation of partially GdnHCl-solubilized PrP^{Sc} revealed that only the insoluble aggregates retained the ability to refold, consistent with the hypothesis that native PrP^{Sc} is an ordered aggregate. When the threshold denaturant concentration was exceeded, both protease-resistance of the 16-kDa C-terminal domain and converting activity were irreversibly destroyed. These results suggest that the *in vitro* converting activity requires ordered, protease-resistant PrP^{Sc} aggregates and that a critical aspect of the PrP^{Sc} structure is the folding of a particularly stable ∼16-kDa C-terminal domain.

The transmissible spongiform encephalopathies (TSEs)[1] are a class of fatal neurodegenerative diseases which include scrapie in hamsters and sheep, Creutzfeldt−Jakob disease in humans, and bovine spongiform encephalopathy ("mad cow disease"). The TSEs can be transmitted via infected tissue, but the identity of the infectious agent remains a mystery. Although the agent has a number of biological properties which resemble conventional viruses, it is more resistant than typical viruses to treatments that degrade nucleic acids (Alper et al., 1966; Bellinger-Kawahara et al., 1988). Furthermore, no agent-specific nucleic acid has been identified in infectious preparations. These and other considerations led to the hypothesis that the infectious agent is a protein (Griffith, 1967). The determination that the accumulation of an abnormal form (PrP^{Sc}) of host-derived prion protein (PrP^C) is characteristic of the TSEs (Bolton et al., 1982; Diringer et al., 1983) led to the proposal that PrP^{Sc} is the infectious protein. This "protein-only" theory holds that the propagation of PrP^{Sc} infectivity results from the autocatalytic facilitation of the conversion of PrP^C to PrP^{Sc}. We have demonstrated that PrP^{Sc} can induce a similar, if not identical, conversion of PrP^C in a cell-free system, providing biochemical support for the feasibility of this proposal (Kocisko et al., 1994). However, it is not yet known whether the formation of PrP^{Sc} creates new infectivity; thus, proof of the protein-only theory is lacking.

Despite the uncertainty regarding the relationship between PrP^{Sc} and the TSE agents, it is apparent that structural changes in PrP play an important role in TSE pathogenesis. Structural comparisons of PrP^C and PrP^{Sc} have revealed that PrP^{Sc} has more β sheet structure, and less helical structure, than PrP^C (Caughey et al., 1991; Pan et al., 1993; Safar et al., 1993), but no general covalent differences have been identified (Hope et al., 1986; Stahl et al., 1993). Besides differing in conformation, the two forms of the prion protein have divergent physical properties. PrP^{Sc} forms protease-resistant, metabolically stable aggregates, often in the form of amyloid fibrils (Bolton et al., 1982; Diringer et al., 1983; Prusiner et al., 1983). Limited treatment of native PrP^{Sc} with the nonspecific enzyme proteinase K (PK) results in digestion of approximately 67 amino acids from its N-terminus (Oesch et al., 1985; Hope et al., 1986), the exact amount depending on the PrP^{Sc} strain (Bessen et al., 1994). The uniformity of the partial digestion suggests that PrP^{Sc} is a highly ordered aggregate. The residual, protease-resistant material, which

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[1] Abbreviations: GdnHCl, guanidine hydrochloride; GdnSCN, guanidine thiocyanate; NP-40, Nonidet P40 or ethyl phenyl poly(ethylene glycol); PK, proteinase K; PNGase, peptide *N*-glycosidase F; PrP^{Sc}, scrapie-associated form of prion protein; PrP^C, normal (cellular) form of prion protein; SB, sulfobetaine 3−14; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophesis; TSE, transmissible spongiform encephalopathy.

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retains infectivity, has been designated PrP 27−30 in reference to the 27−30 kDa apparent monomeric molecular mass of its fully-glycoslyated form. In contrast to PrP^{Sc}, PrP^{C} is soluble in mild detergents, is sensitive to proteolysis (Meyer et al., 1986; Rubenstein et al., 1986), and rapidly turned over in live cells (Caughey et al., 1989). While the normal function of PrP^{C} is not known, it appears to serve as the precursor of PrP^{Sc} in TSE-infected hosts (Borchelt et al., 1990; Caughey & Raymond, 1991).

The cell-free system, in which PrP^{Sc} induces the conversion of radiolabeled PrP^{C} to a PrP^{Sc}-like, protease-resistant state, was developed to study the PrP^{C}-to-PrP^{Sc} conversion under defined conditions (Kocisko et al., 1994). This cell-free conversion reaction exhibits striking species- and scrapie-strain specificities which mimic the *in vivo* manifestations of different scrapie strains (Kocisko et al., 1995; Bessen et al., 1995). The physical−chemical properties of the entity with "converting activity" indicate that it is associated with aggregates of PrP^{Sc} (Caughey et al., 1995). This observation is consistent with a "nucleated polymerization" mechanistic model, which states that conversion requires a preformed nucleus which seeds the polymerization of PrP.

We have also shown that treatment of PrP^{Sc} preparations with a high concentration of chaotropic denaturant [6 M guanidine hydrochloride (GdnHCl)] irreversibly destroys its converting activity and protease-resistance (Kocisko et al., 1994). However, we observed that after treatment of PrP^{Sc} with intermediate denaturant concentrations (≤3 M GdnHCl), converting activity and protease-resistance could be restored by diluting the denaturant. We postulated that converting activity and recovery of the characteristic protease-resistance required the maintenance of some native PrP^{Sc} structure. Furthermore, we proposed that by exceeding the threshold denaturant concentration, the native structure was completely destroyed, resulting in irreversible denaturation.

In the present study, we provide a detailed analysis of the effects of various concentrations of GdnHCl and GdnSCN on the PK-resistance of PrP^{Sc}. The results confirmed that protease-resistance and converting activity are linked. Furthermore, partially protease-resistant PrP^{Sc} aggregates were found to be neccessary for refolding and converting activity. Individual PrP^{Sc} preparations varied slightly with respect to their sensitivity to denaturant. The unfolding of PrP^{Sc} was monitored with four antibodies directed to epitopes spread throughout the PrP sequence. These studies showed that unfolding of certain regions of the PrP^{Sc} did not affect its ability to refold, but unfolding of a 16-kDa C-terminal domain of PrP resulted in irreversible denaturation.

MATERIALS AND METHODS

Denaturation and Renaturation of PrP^{Sc}. PrP^{Sc} was purified from scrapie- infected Syrian Golden hamster brains (Kocisko et al., 1994). PrP^{Sc} was stored in a 1% sulfobetaine 3−14 and PBS solution at −20 °C. PrP^{Sc} was treated as a 1 μg/μL solution in the desired concentration of GdnHCl. The solution was incubated for at least 1 h but not more than 24 h at 37 °C before further manipulation. A portion of the solution was then diluted as noted in the figure legends with TN (100 mM TrisHCl, 130 mM NaCl, pH 7.4) and incubated at 37 °C for up to 2 days to allow renaturation. This solution was then further diluted with 4 volumes of TN and treated with 50 μg/mL PK for 1 h at 37 °C. The final concentrations of GdnHCl and PrP^{Sc} were approximately 0.15 M and 50 μg/mL, respectively, during the PK treatment. The other portion of the original PrP^{Sc} denaturation solution was diluted with 19 volumes of the same concentration of GdnHCl and treated with PK to measure protease-resistance before any renaturation could occur. Treatment with 50 μg/mL PK proceeded in a 50 μg of PrP^{Sc}/mL solution for 1 h at 37 °C. After all PK treatments, Pefabloc to 1 mM, 20 μg of thyroglobulin as a carrier protein, and 4 volume equiv of methanol were added. After 1 h at −20 °C, samples were centrifuged in a Tomy microcentrifuge for 15 min at 15 000 rpm. The resulting pellets were boiled in sample buffer, and SDS−PAGE was performed. Immunoblots were visualized with rabbit antibodies (α) raised against one of three PrP peptides corresponding to residues 90−104, 143−156, and 217−232 (Caughey et al., 1995) or to monoclonal antibody 3F4 (Kascsak et al., 1987) which recognizes an epitope in the region of residues 106−115 (Bolton et al., 1991).

Denaturation/renaturation with GdnSCN was done in a similar manner. PrP^{Sc} was denatured with 1−4 M GdnSCN, diluted to 0.75 M GdnSCN, and incubated overnight. All other manipulations were identical to those detailed above for GdnHCl treatment. PK was not active at GdnSCN concentrations above 2 M.

PNGase F Treatment of Renatured PrP^{Sc}. PrP^{Sc} was treated with PNGase F after the final methanol precipitation, after all other manipulations of the denaturing and renaturing treatment were finished. The PrP^{Sc} methanol precipitate was taken up in 20 μL of 88% formic acid, and after 10 min at 4 °C, it was rotary-evaporated to dryness. Water (20 μL), SDS (to 0.5% w/v), and β-mercaptoethanol (to 1% w/v) were added, and the sample was boiled for 5 min. NP-40 (to 1% v/v) and 10 μL of sodium phosphate buffer were added; 2 μL of the PNGase F (New England Biolabs) solution was added and the solution incubated at 37 °C for 12 h. Sample buffer was added to the solution and SDS−PAGE performed as before.

Denaturation/Renaturation of Fractions Derived from Centrifugation of PrP^{Sc}. PrP^{Sc} at 1 μg/μL in 3 M GdnHCl was separated based on the aggregation state using a Beckman TLS 55 swinging bucket rotor (Caughey et al., 1995). Twenty microliters of the PrP^{Sc} solution/suspension was placed over 20 μL of a 5% (w/v) sucrose and 3 M GdnHCl solution in a polycarbonate centrifuge tube. The gradient was spun for 30 min at 55 000 rpm (234000*g*), and afterward a golden-brown pellet was clearly visible. The top 20 μL of the gradient was designated the supernatant fraction. The 10 μL below that was discarded. Ten microliters of 3 M GdnHCl was added to the remaining 10 μL of solution (and pellet), and the solution was sonicated for 10 s in a cuphorn sonicator (Heat System Ultrasonics) to resuspend the pellet. This was designated the pellet fraction. Aliquots of the supernatant and pellet fractions were treated with 50 μg/mL PK in the presence of 3 M GdnHCl as detailed above. Other aliquots were diluted to allow renaturation, incubated at 37 °C, and similarly treated with PK (50 μg/mL). Samples were analyzed with Novex precast 14% acrylamide SDS−PAGE.

Denaturation/Renaturation of PK-Treated PrP^{Sc}. PrP^{Sc} was treated with PK prior to denaturation (Caughey et al., 1995). After the treatment with PK, the PrP^{Sc} was washed and sonicated into 1% SB and PBS. Procedures described

above for denaturation and renaturation were followed. It is likely that some residual PK survived the washing protocol and remained associated with PrP 27−30, because with extended incubations at 37 °C following the initial PK treatment, there was an overall loss of PrP in some experiments. For this reason, renaturation incubation periods were reduced to 4 h to minimize this problem.

Cell-Free Conversion of ^{35}S-PrP^{C} to the Protease-Resistant Form. This was done by a slight modification of the original procedure (Kocisko et al., 1994). PrP^{Sc} was pretreated at a concentration of *ca*. 1 μg/mL for 1 h at 37 °C, using varied concentrations of GdnHCl or GdnSCN. Two microliters of PrP^{Sc} in 0−4 M GdnHCl was then mixed with 2 μL of ^{35}S-PrP^{C} (40 000 cpm total) in 0.1 M acetic acid; 50 mM sodium citrate at pH 6 was added to bring the final volume to 8 μL, and 0.5 μL of GdnHCl was added as needed to bring the final concentration of GdnHCl to 1 M during the reaction. All reactions contained about 2 μg of PrP^{Sc}. Solutions of PrP^{Sc} in GdnSCN (1 and 2 M) were diluted to final concentrations of 0.5 or 0.75 M GdnSCN during the conversion reaction. All mixtures were incubated at 37 °C for 2 days before diluting with TN to a final volume of 80 μL and adding PK to 50 μg/mL and incubating for 1 h at 37 °C. Pefabloc, 20 μg of thyroglobulin as a carrier protein, and 4 volume equiv of methanol were added. After 1 h at −20 °C, the precipitated protein was pelleted in a microcentrifuge. The resulting pellet was boiled in sample buffer and analyzed using Novex precast 16% SDS−PAGE gels.

Unfolding and Refolding of PrP^{Sc} in Homogenate of Scrapie-Infected Brain. Syrian Golden hamsters showing signs of scrapie were sacrificed and their brains snap-frozen in liquid nitrogen and stored at −70 °C. Brains were Dounce-homogenized in either TEN (50 mM TrisHCl, 1 mM EDTA, 150 mM NaCl, pH 7.5) or TEN + 10% (w/v) sodium *N*-lauroylsarcosinate. The resulting homogenates contained 10% (w/v) of brain. Both homogenates were treated identically in subsequent steps. Homogenate (2 μL) and 5 or 6 M GdnHCl (2 μL) were combined, producing *ca*. 0.015 μg/μL PrP^{Sc} in 2.5 or 3.0 M GdnHCl. After an hour of incubation at 37 °C, this solution was diluted with 76 μL of 2.5 or 3.0 M GdnHCl and treated with 50 μg/mL PK for 1 h at 37 °C to test for the extent of denaturation. These homogenate samples were then methanol precipitated and analyzed by SDS−PAGE and immunoblotting as detailed above. To analyze renaturation, the 2.5 or 3.0 M GdnHCl/PrP^{Sc} homogenate solutions were diluted with 3 volumes of TN and incubated 1 day at 37 °C. Four volumes of TN and PK (to a final concentration of 50 μg/mL) were then added and incubated 1 h at 37 °C. These samples were methanol-precipitated and analyzed by SDS−PAGE and immunoblotting.

RESULTS

Multiple Antibody Analysis of PrP^{Sc} Resistance to PK. In this study, we have monitored denaturant-induced changes in the folded state of PrP^{Sc} by digesting with PK, a largely nonspecific protease. We have assumed that unfolding, albeit at an undefined level, is required to expose a normally protease-resistant sequence to proteolysis by PK. An increase in PK sensitivity might be due to disruption of secondary, tertiary, and/or quaternary structures within the PrP^{Sc} aggregate. We will use the terms "unfolding" or "denaturation" to encompass any or all of these possibilities since, in most cases, we cannot distinguish between them.

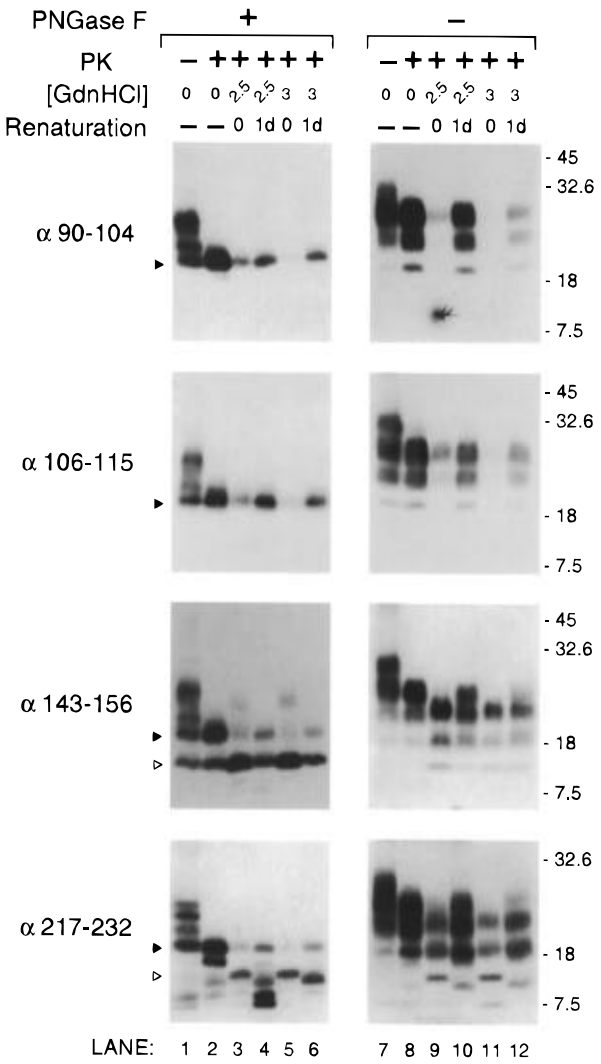

FIGURE 1: Partial reversible unfolding of PrP^{Sc} in 2.5 and 3 M GdnHCl. The panels within each column represent replicate gels analyzed with antibodies (α) recognizing epitopes within the designated PrP residues. Aliquots of PrP^{Sc} were treated with PK in the designated concentration of GdnHCl (renaturation = 0) or after 4-fold dilution and incubation for 1 day (renaturation = 1 d) and further dilution prior to PK treatment. Control samples were not treated with PK (lanes 1 and 7) or treated with PK without GdnHCl treatment (lanes 2 and 8). The two columns of panels represent identically treated sets of samples except for the use of PNGase F to deglycosylate the PrP samples immediately prior to SDS−PAGE in the left column (lanes 1−6). In lanes 3 and 5 in the α143−156 panel, the faint 27−30-kDa bands provided evidence that the PNGase F was not always completely effective. Molecular mass markers are indicated at the right in kilodaltons.

The portion of the monomeric units of PrP^{Sc} remaining after GdnHCl and PK treatment was monitored by SDS−PAGE and immunoblotting with different antibodies to epitopes throughout the PrP molecule. Also, to simplify the analysis of PrP polypeptides generated in this type of experiment, some samples were treated with PNGase F immediately prior to SDS−PAGE. PNGase F removes N-linked sugars which account for much of the band multiplicity of PrP on SDS−PAGE.

As expected, PK treatment of native PrP^{Sc} in the absence of GdnHCl resulted in the removal of 6−7 kDa of the polypeptide as indicated by downward shifts of both the glycosylated and deglycosylated species [Figure 1, lanes 1

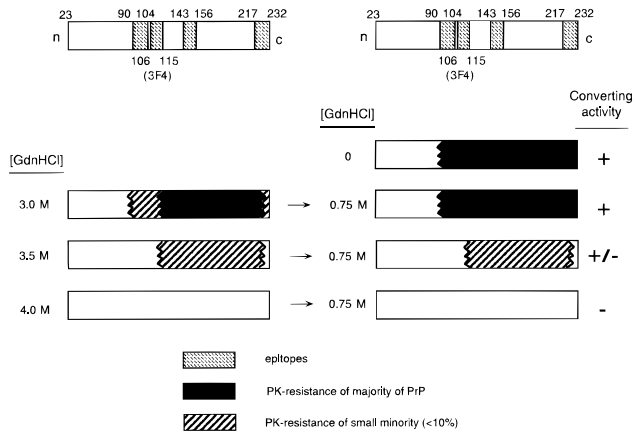

FIGURE 2: Schematic depiction of PrP sequence, indicating PK-resistance of the monomeric units comprising PrP^{Sc} under various denaturing and renatured conditions. The bars at the top represent PrP and the epitopes of the antibodies used in this study. The PK-resistant regions are approximated based on a combination of their reactivities with the antibodies and their sizes on the SDS−PAGE gels shown in Figures 1 and 5. Where the termini are approximate, the borders are shown with jagged lines. With no GdnHCl present, PK cleaves PrP^{Sc} to about residue 90 (Oesch et al., 1985; Hope et al., 1986). In 3 M GdnHCl, PK eliminated most of the signal by α90−104 and α106−115, so the majority of N-terminal cleavage was at least to 115. Based on the loss of α217−232 signal, some proteolysis into its epitope at the C-terminus also occurred. Upon treatment by PK in 3.5 M GdnHCl, and also after attempted renaturation by dilution to 0.75 M GdnHCl, a small amount of PrP was detected by α143−156 and α217−232, both reduced in mass by ∼3 kDa from normal PrP. This portion of PrP indicated by the hatched area in 3.5 M GdnHCl and the solid area in 3 M GdnHCl seemed to be the most resistant to denaturation (the 16-kDa protease-resistant peptide core). 4 M GdnHCl irreversibly eliminated all detectable PK-resistant PrP. Converting activity (the ability to convert ^{35}S-PrP^{C} to the PK-resistant form) was present in PrP^{Sc} not treated with GdnHCl (+) and that which was treated with 3 M and diluted to 0.75 or 1.0 M (+). A 3.5 M GdnHCl treatment and dilution to 0.75 or 1.0 M left only barely detectable converting activity (+/−), and 4 M GdnHCl irreversibly eliminated all detectable conversion (−). Converting activity in 3 M or higher GdnHCl (without further dilution) was not detected.

and 2, lanes 7 and 8 (glycosylated)]. Previous studies have indicated that this corresponds to the removal of a *ca*. 67 amino acid fragment (up to residue 90; numbering includes cleaved signal sequence) from the N-terminus (Oesch et al., 1985; Hope et al., 1986). The residual unglycosylated core had an apparent molecular mass of ∼19 kDa (Figure 1, solid arrowheads). The major PK-digested species were detected by all four antibodies, indicating the resistance of their epitopes to proteolysis. The PK-resistance of native PrP^{Sc} and the relative positions of the epitopes are shown schematically in Figure 2. The detection of additional lower molecular mass protease-resistant fragments by α143−156 and α217−232 and not by α90−104 and α106−115 indicated that some unfolding/digestion of the two N-terminal epitopes PrP 90−104 and PrP 106−115 occurred.

GdnHCl-Induced Unfolding of PrP^{Sc} Enhances Exposure of Its Termini to Proteolysis. Treatment of PrP^{Sc} with PK in the presence of 2.5 (Figure 1, lane 3) or 3 M GdnHCl (lane 5) resulted in nearly complete elimination of the 19-kDa fragment. A prominent protease-resistant fragment of *ca*. 16 kDa (open arrowheads) was observed using the α143−156 and α217−232 antisera, but not the α90−104 or 106−115 antisera (lanes 3 and 5). These results showed that the GdnHCl treatment made an additional 3 kDa of the PrP^{Sc} polypeptide backbone sensitive to PK. A similar 3-kDa reduction in the size of the PK-resistant portion of PrP^{Sc} was observed using fully glycosylated PrP^{Sc} (lanes 9 and 11). Because the detection by α90−104 and α106−115 was reduced much more than the detection by α217−232, we conclude that most of the additional digestion occurred at the N-terminus. The calculated molecular mass of residues 90−115 is 3210 Da, so removal of these residues alone could account for the observed increase in mobility in SDS−PAGE. However, there was also a decrease in the quantity of the fragments containing α217−232 (compare lanes 8 and 9, lower panel), indicating that part of this epitope was partially removed by PK as well (see schematic in Figure 2). Thus, the major effect of treatment with 2.5−3 M GdnHCl was to denature PrP^{Sc} from residue 90 to the vicinity of residue 115, with a less pronounced unfolding of the 217−232 epitope at the C-terminus.

Reversibility of Partial Unfolding of PrP^{Sc}. The refolding of GdnHCl-treated PrP^{Sc} after dilution of GdnHCl was monitored with four antibodies (Figure 1, lanes 4, 6, 10, and 12). Analysis of the refolded protease-resistant products with the antibodies to the two most N-terminal epitopes showed the reappearance of the 19-kDa unglycosylated material (lanes 4 and 6, top two panels). Analysis using the antibodies directed toward the central and C-terminal epitopes also indicated recovery of the 19-kDa product, concomitant with a decrease of the 16-kDa species. Analogous trends, i.e., recovery of the original PK-resistance upon dilution, were seen using fully glycosylated PrP^{Sc}. Recovery of the 19-kDa PK-resistant species was more complete after the 2.5 M GdnHCl treatment than after the 3.0 M treatment.

The Refolding Behavior of PrP^{Sc} Is Not an Artifact of Purification. The PrP^{Sc} used in the experiments discussed herein was isolated from scrapie-infected hamster brain. In order to prove that the properties of this material were native-like and were not a result of the isolation procedure, a refolding experiment similar to the one shown in Figure 1 was performed using a crude homogenate of scrapie-infected brain rather than purified PrP^{Sc} (Figure 3). As in the case of the original renaturation reaction, renaturation of protease-resistance was observed, below a threshold concentration of denaturant. The threshold concentration was slightly lower (between 2.5 and 3.0 M GdnHCl) than in the original reaction.

PrP 27−30 Exhibits Refolding Behavior Similar to That of PrP^{Sc}. Having seen a recovery of the original protease-resistance of PrP^{Sc} after the GdnHCl was diluted, we tested if residues 24−89 were required for this renaturation. Native PrP^{Sc} in purified form was treated with PK to produce PrP 27−30, which, as noted above, lacks N-terminal residues 24−89. Treatment of PrP 27−30 with 2.5 or 3.0 M GdnHCl (Figure 4, lanes 2 and 4) unfolded and exposed a substantial portion of all of the epitopes, suggesting that PrP 27−30 is somewhat more sensitive to GdnHCl than the full-length PrP^{Sc}. Nevertheless, as with PrP^{Sc}, a renaturation step greatly enhanced the PK resistance of the α90−104, α106−115, and α217−232 epitopes, indicating that partial refolding from 2.5 or 3.0 M GdnHCl was accomplished by dilution of the sample (Figure 4, lanes 3 and 5). These results showed that additional N- and C-terminal digestion of the PK-resistant region of PrP 27−30 occurred in the presence of 2.5 or 3 M GdnHCl and that refolding of the unfolded portions did not require residues 24−89.

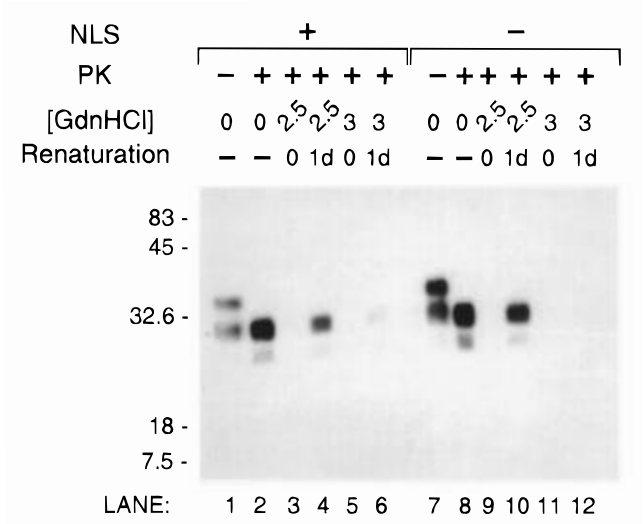

FIGURE 3: Unfolding/refolding of PrP^{Sc} in crude brain homogenate. All samples in this figure were visualized by immunoblotting with the α106−115 antibody. Scrapie-infected brain homogenates in TEN + 10% sodium *N*-lauroylsarcosinate (NLS +) or TEN alone (NLS −) were pretreated with the indicated GdnHCl concentration. Samples without GdnHCl were only treated with PK (+) or not treated (−). Homogenate samples were treated with PK in the designated concentration of GdnHCl (renaturation = 0) or diluted 4-fold and incubated 1 day before further dilution and treatment with PK (renaturation time = 1 d). Only faint renaturation was seen after 3 M GdnHCl treatment. The concentration of PrP^{Sc} in these brain homogenate samples was about 30-fold lower than in the purified samples used in other experiments. Molecular mass markers are indicated at the left in kilodaltons.

Complete Unfolding of PrP^{Sc} Occurs between 3 and 4 M GdnHCl. Treatment of PrP^{Sc} with ≥3.5 M GdnHCl had been previously observed to render the PrP 90−104 epitope protease-sensitive and to prevent refolding of PrP^{Sc} (Kocisko et al., 1994). The unfolding and refolding of other epitopes was examined. After treatment of PK with 4 M GdnHCl, no protease-resistant fragments were detected (Figure 5, lane 9), and dilution did not reveal detectable refolding after 2 days incubation (Figure 5, lanes 10 and 11). In contrast, treatment of PrP^{Sc} with 3.5 M GdnHCl left a small amount of PrP 143−156 intact (lane 6), and dilution afforded a small amount of truncated fragments which were recognized only by α143−156 and α217−232 (lane 8). The dramatic difference between the refolding from 3, 3.5, and 4 M GdnHCl suggested that a major conformational change occurred in this concentration range.

Comparable Unfolding/Refolding Behavior Was Observed Using GdnSCN as Denaturant. Oesch et al. (1994) have shown that GdnSCN can be used to decrease the protease resistance of PrP^{Sc}. To see if a reversible partial denaturation could be accomplished with GdnSCN as well as with GdnHCl, we treated PrP^{Sc} with increasing concentrations of GdnSCN (Figure 6). Consistent with the results of Oesch et al., large changes in PK-resistance were observed in 1−2 M GdnSCN. Treatment with 1 M GdnSCN had approximately the same effect on PrP^{Sc} as 3 M GdnHCl, unfolding the 90−104 epitope and part of the 217−232 epitope but leaving the 143−156 epitope intact (Figure 6, lane 3). This confirmed that the central portion of the PrP^{Sc} sequence was the most resistant to denaturation. As was the case with GdnHCl, attempts to refold PrP^{Sc} by diluting GdnSCN to a concentration of 0.75 M led to the reformation of protease-resistant PrP^{Sc} (lanes 4 and 5). Unfolding of PrP^{Sc} was presumed to be complete at ≥2 M GdnSCN, but

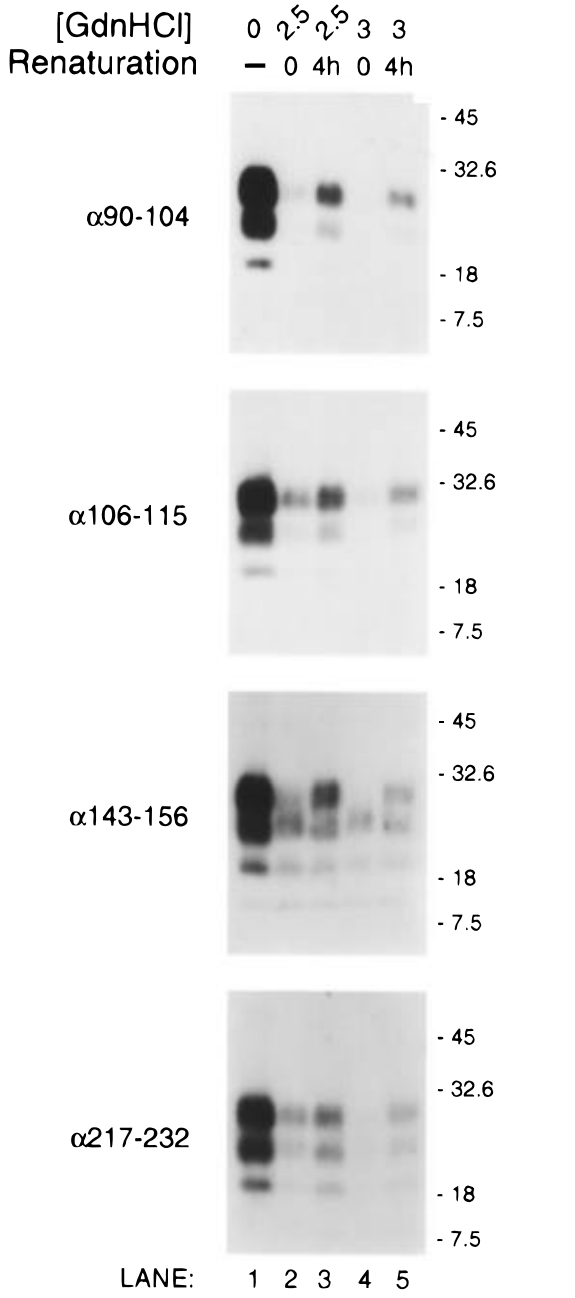

FIGURE 4: N-Terminal residues 24−89 of PrP^{Sc} were not required for reversible unfolding. The PrP^{Sc} was treated with PK in the absence of GdnHCl to generate PrP 27−30 (lacking residues 24−∼89) prior to use in GdnHCl-induced unfolding/refolding reactions like those described in Figure 1. All of the samples were subjected to a second PK treatment in the designated concentration of GdnHCl (renaturation = 0) or after dilution to 0.75 M GdnHCl, 4 h of incubation, and further dilution (renaturation = 4 h). Molecular mass markers are indicated at the right in kilodaltons.

was impossible to analyze, since PK was inactive under those conditions (Figure 6, lanes 6, 9, and 12). However, no protease-resistant material could be refolded by dilution of denaturant (lanes 7, 8, 10, 11, 13, 14), similar to the case with dilution of GdnHCl.

The PK-Resistant Species Required for Refolding Can Be Separated from Soluble PrP by Centrifugation in Denaturant. We have previously shown that aggregated PrP^{Sc} (in 2.5−3 M GdnHCl) can be separated from solubilized PrP by differential sedimentation (Caughey et al., 1995). The aggregates have *in vitro* converting activity whereas the soluble monomeric species do not (Caughey et al., 1995).

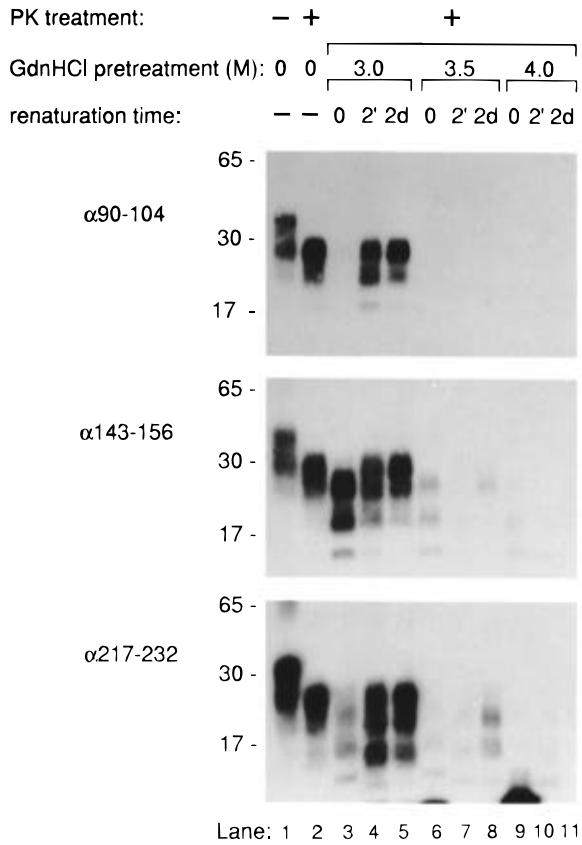

FIGURE 5: At high GdnHCl concentration, unfolding of PrP^{Sc} was not reversible. Multiple antibody analysis of GdnHCl- and PK-treated PrP^{Sc} was performed as described in Figure 1 except that the designated (higher) GdnHCl concentrations were used in the initial unfolding pretreatment. The "renatured" samples were diluted to 0.75 M GdnHCl and incubated for the designated time. The amount of PK-resistance seen after 2 min was not always as much as shown. Control PrP^{Sc} in lanes 1 and 2 was treated with PK (+) or not treated (−) without prior exposure to GdnHCl. Molecular mass markers are indicated at the left in kilodaltons.

We wondered if the PK-resistant species which are required to direct refolding could be separated from solubilized PrP^{Sc} by a similar method. This should only be possible if the partially unfolded species are aggregates, as opposed to monomers. A suspension of PrP^{Sc} at 3 M GdnHCl was centrifuged under conditions which should clear ≥90% of the particles ≥5 S (234000*g*, 30 min). Analysis of the total PrP in each fraction by immunoblotting with the four antisera (Figure 7, lanes 1−3) showed that the PrP was present to a similar extent in both fractions. Treatment of the supernatant fraction with PK resulted in complete digestion of PrP (lane 4). However, treatment of the pellet fraction with PK (lane 6) produced protease-resistant fragments. The most abundant fragment was the 16-kDa resistant core domain containing the PrP 143−156 epitope, yet a small amount of the 19-kDa band was detected by all four antisera. Refolding of PrP^{Sc} was attempted by the dilution of denaturant in both supernatant and pellet fractions. No protease-resistant fragments were derived from the supernatant fraction (lane 5). However, significant refolding of the 19-kDa PrP^{Sc} fragment was observed from the pellet fraction (lane 7). These results demonstrate that the partially folded PrP^{Sc}-derived species is an aggregate of PrP.

PK-Resistant PrP^{Sc} Has Converting Activity, but PK-Sensitive PrP Does Not. The refolded species were tested for their ability to convert ^{35}S-PrP^{C} to ^{35}S-PrP^{Sc}-like PK-resistant material (converting activity). PrP^{Sc} was pretreated with various concentrations of denaturant and then diluted into refolding conditions, in the presence of ^{35}S-PrP^{C} (Figure 8). Converting activity was detected only after pretreatment conditions which, as shown above, did not fully unfold PrP^{Sc}. Pretreatment of PrP^{Sc} with an intermediate concentration of GdnHCl (2.5 M, Figure 8, lane 3) actually enhanced the converting activity of the refolded material (*vide infra*). This effect may involve the removal of unstructured PrP molecules which adhere to the surface of the ordered PrP^{Sc} aggregate and interfere with its seeding capability.

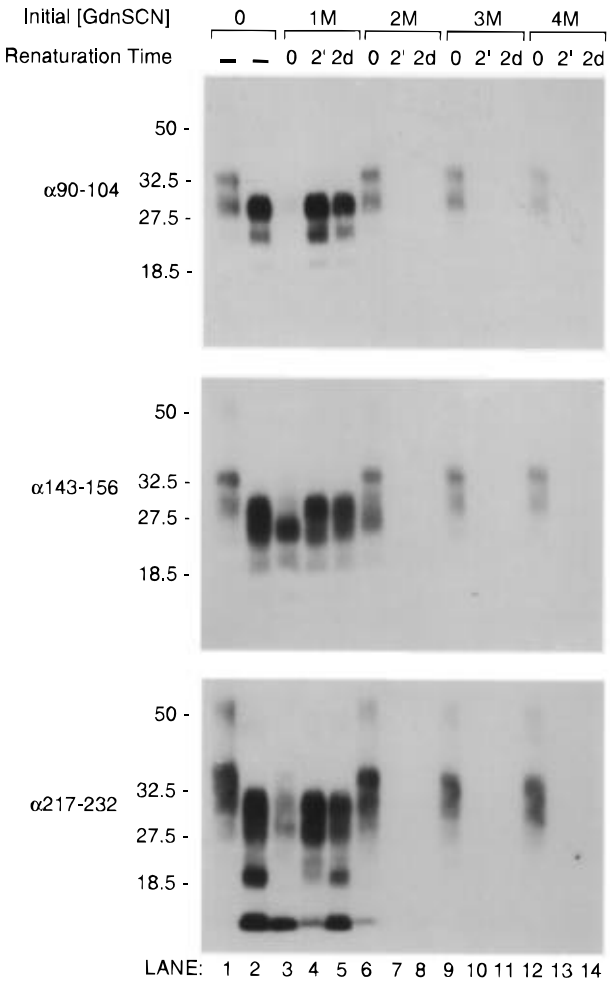

FIGURE 6: Partial unfolding of PrP^{Sc} was reversible from 1 M, but not ≥2 M, GdnSCN. GdnSCN was substituted for GdnHCl in the unfolding/refolding procedures described in Figure 1. The PK treatment was omitted in lane 1 (only). 2 M or higher GdnSCN inhibited the ability of PK to digest PrP^{Sc} (lanes 6, 9, and 12). To renature, GdnSCN was diluted to 0.75 M, and the samples were incubated for 2 min or 2 days before further dilution and treatment with PK. Note that after dilution of GdnSCN from 2, 3, or 4 M, PK was able to digest all PrP, and no resistant material was seen after 2 days of incubation. Molecular mass markers are indicated at the left in kilodaltons.

DISCUSSION

The conversion of PrP^{C} to PrP^{Sc} is a central pathological event of scrapie, and it is important to understand the mechanism of this conversion to understand the disease. Once it has begun *in vivo*, the inexorable accumulation of PrP^{Sc} appears to cause clinical disease, possibly by replication and amplification of its inherent toxicity or interference with PrP^{C}

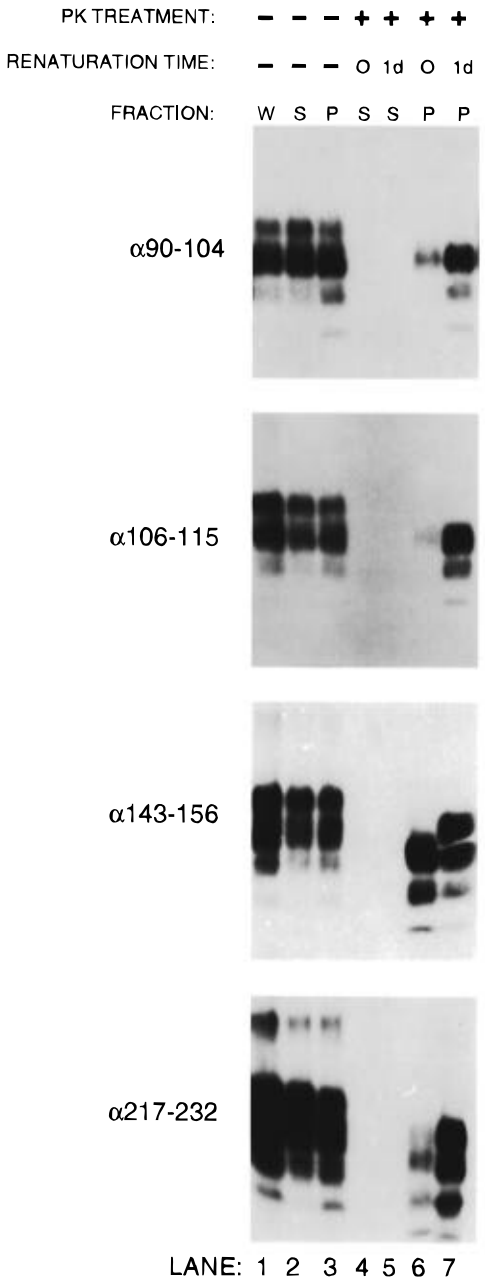

FIGURE 7: PK-resistance and refolding of soluble vs aggregated fractions of PrP^{Sc}. PrP^{Sc} in 3 M GdnHCl was fractionated by ultracentrifugation at 234000*g* as described under Materials and Methods. Fractions are designated as whole or unfractioned (W), supernatant (S), or pelleted (P). The first three lanes are to compare the total amounts of PrP in each fraction without PK treatment. The supernatant and pellet fractions were treated with PK in 3 M GdnHCl (renaturation time = 0) or diluted to 0.75 M and incubated for 1 day before further dilution and treatment with PK as described in the legend to Figure 1 and under Materials and Methods.

function. We have found using a cell-free system that PrP^{Sc} also induces the conversion of PrP^{C} to a PrP^{Sc}-like state *in vitro* (Kocisko et al., 1994). This system is the first direct evidence of the self-propagating activity of PrP^{Sc} and has allowed a more controlled study of the conversion process.

The present study was undertaken to define the extent of the native PrP^{Sc} structure which is required for refolding. Treatment of PrP^{Sc} with PK under physiological conditions produced PrP 27−30, which is truncated near residue 90 (Oesch et al., 1985; Hope et al., 1986). This protein demonstrated similar unfolding/refolding behavior as native PrP^{Sc}, indicating that the PrP 24−89 sequence did not play

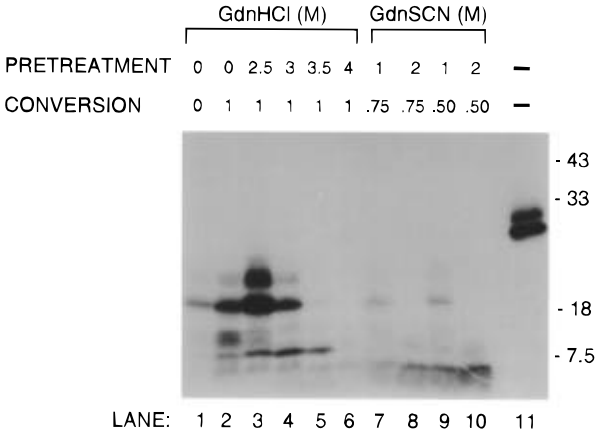

FIGURE 8: Conversion of ^{35}S-PrP^{C} to the PK-resistant form using PrP^{Sc} pretreated with GdnHCl and GdnSCN. The ^{35}S-PrP^{C} used here was a GPI^{-} construct that has been described (Kocisko et al., 1994). Lanes 1−10 contained 40 000 cpm of ^{35}S-PrP^{C} treated with PK after incubation with 2 μg of PrP^{Sc} pretreated at the indicated condition. Lane 11 contained 10 000 cpm of untreated ^{35}S-PrP^{C} that was used as substrate in the reaction. Lane 1 is the product of the conversion of ^{35}S-PrP^{C} with PrP^{Sc} in the absence of GdnHCl. Phosphorimager analysis indicated that the ratio of ^{35}S contained in the PK-resistant conversion products (≥17 kDa) in lanes 1−4 was 1:2:6:2, respectively. Molecular mass markers are indicated at the right in kilodaltons.

an essential role in refolding or in the *in vitro* conversion reaction. The analysis of PrP^{Sc} unfolding by chemical denaturants depended on the identification and analysis of protease-resistant fragments of PrP^{Sc} by antibodies to four different PrP epitopes. The 16-kDa polypeptide protease-resistant core domain, which spans approximately from residue 115 into the α217−232 epitope, was the most resistant to unfolding by denaturants. When a threshold of denaturant was reached that disrupted this region, no refolding was observed.

The 16-kDa protease-resistant core of PrP contains many residues known to affect scrapie transmission: (1) homozygosity at position 129 (M/V) seems to predispose to iatrogenic prion disease (Palmer et al., 1991; Come & Lansbury, 1994); (2) certain polymorphisms at positions 136, 154, and 171 seem to correlate with the incidence of scrapie in sheep (Goldmann et al., 1994; Belt et al., 1995); and (3) positions 139, 155, and 170 have been determined to be the critical amino acid differences which determine the mouse−hamster barrier to interspecies infection in transgenic animal models (Scott et al., 1989) and interspecies PrP^{C}-to-PrP^{Sc} conversion in scrapie-infected tissue culture cells (Priola & Chesebro, 1995) and our *in vitro* system (Kocisko et al., 1995). This overlap supports the relevance of the *in vitro* conversion to *in vivo* transmission. It is also interesting to note that the 16-kDa core of PrP^{Sc} corresponds approximately to a major protease-sensitive PrP^{C} species (C1) that has been identified in normal human tissue (Chen et al., 1995) and an autonomous folding unit that has been identified in mouse PrP^{C} (Riek et al., 1996).

Under the conditions we have established, the cell-free conversion of ^{35}S-PrP^{C} to the protease-resistant state requires preexisting PrP^{Sc}. However, as noted in a previous study (Caughey et al., 1995), the converting activity was lost if the preexisting PrP^{Sc} was pretreated with >3.5 M GdnHCl. Here we have shown with a range of GdnHCl pretreatments that the loss of converting activity correlates with a loss of PK-resistance in the 16-kDa core domain. This suggests that

this PK-resistant PrP^{Sc} structure is essential for converting activity as well as renaturation after partial unfolding.

In contrast, the ^{35}S-PrP^{C} can be pretreated with up to 7.5 M GdnHCl and still be converted to the PK-resistant state by PrP^{Sc}. This indicates that either PrP^{C} can be converted from a denatured state or it can readily refold to its native conformation after GdnHCl denaturation as suggested by Riek et al. (1996). However, conversion of native ^{35}S-PrP^{C} to the PK-resistant form does not *require* any denaturant (manuscript in preparation). This suggests that the unfolding of native PrP^{C} is not the slow step in the *in vitro* conversion reaction. While there appears to be no requirement for a specific ^{35}S-PrP^{C} conformation, there are strict structural requirements for PrP^{Sc} in the cell-free reaction.

As shown previously, the 2.5−3 M GdnHCl-solubilized fraction of PrP^{Sc} does not convert ^{35}S-PrP^{C} to the PK-resistant form in a cell-free conversion system (Caughey et al., 1995). Here we found that the solubilized fraction also had no PK-resistance in 2.5−3 M GdnHCl and no ability to become PK-resistant after dilution of the denaturant. The pelleted (and more aggregated) fraction had partial PK-resistance in 2.5−3 M GdnHCl and recovered full resistance after dilution of the GdnHCl. Thus, it appears that the polymeric nature of PrP^{Sc} is responsible for its protease-resistance and converting activity. Treatment of native PrP^{Sc} with PK not only results in N-terminal truncation to about residue 90 (formation of PrP 27−30) but also results in the loss of about half of the total PrP molecules. We suspect that the PrP^{Sc} solubilized by 2.5−3 M GdnHCl is equivalent to that which is totally digested by PK when producing PrP 27−30.

Safar et al. (1993) conclude that PrP^{Sc} is fully monomerized in 2.5−3 M GdnHCl as evidenced by a loss of turbidity and the observation of monomeric species on size-exclusion HPLC columns. In contrast, we have found by ultracentrifugation analysis that approximately half of the PrP^{Sc} and nearly all of the PrP 27−30 remain in aggregates at these GdnHCl concentrations (Caughey et al., 1995). To address this discrepancy, we suggest an alternative interpretation of the results of Safar et al. which is compatible with our own results. The GdnHCl-induced reduction in turbidity of PrP^{Sc} and PrP 27−30 may have been due to the unbundling of clusters of ordered PrP^{Sc} polymers or a reduction in their average length rather than complete dissociation into monomers. Indeed, it is known that some amyloid fibrils exhibit very low turbidity (Wood et al., 1996). The monomeric PrP 27−30 that Safar et al. (1993) observed on the size-exclusion HPLC might represent only a small fraction of the total PrP in the GdnHCl-treated sample, with the remainder being large aggregates that were lost on the column. This interpretation is consistent with the fact that they were unable to detect any eluted PrP when the samples were run as aggregates in the absence of GdnHCl. The importance of resolving this discrepancy is emphasized by our observations that PrP^{Sc} aggregates, rather than dissociated PrP monomers, are associated with converting activity and the ability of PrP^{Sc} to refold after intermediate GdnHCl treatments.

Although the basic PrP^{Sc}-induced cell-free conversion of ^{35}S-PrP^{C} to the protease-resistant form has been confirmed by Kaneko et al. (1995), these same researchers reported they were unable to renature PrP^{Sc} to a protease-resistant form after a 3 M GdnHCl treatment. However, these authors state that this was the case *only after removal of insoluble native-like* PrP^{Sc}, apparently by adsorption to the tube. They further state that both the protease-resistance and converting activity of the nonadsorbed species were lost, consistent with our proposal that these two properties are linked. Under refolding conditions identical to those originally reported by us (Kocisko et al., 1994), they report that PK-resistance *was* observed. Thus, the observations of Kaneko et al. are consistent with our original report that refolding requires the presence of a native-like seed. We want to emphasize that the precise concentration of denaturant required to completely and irreversibly unfold PrP^{Sc} (threshold concentration) may vary depending on the source and the method of isolation. However, the fact that we observed reversible unfolding of PrP^{Sc} in crude brain homogenates as well as in the purified form indicates that our purified PrP^{Sc} is representative of that in scrapie-infected hamster brain tissue.

If the infectious agent and/or neurotoxic factor of the transmissible spongiform diseases is an abnormal, disease-specific conformation and/or aggregation state of prion protein, its folding and aggregation processes are then important in the disease progression. PrP^{Sc} may be resistant to degradation because it is in an aggregate or conformation that limits exposure of cleavage sites to proteases. PrP^{C} may be converted to PrP^{Sc} and made PK-resistant by adding to a preexisting aggregate of PrP^{Sc} (seeded polymerization). The "protein only" theory of infectivity holds that this process is responsible for the spread of disease. The present study provides evidence that aggregation, PK-resistance, and PrP^{C} converting activity are inseparable.

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