During the course of the transmissible spongiform encephalopathy diseases, a protease-resistant ordered aggregate of scrapie prion protein (PrPSc) accumulates in affected animals. From mechanistic and therapeutic points of view, it is relevant to determine the extent to which PrPSc formation and aggregation are reversible. PrPSc solubilized with 5 M guanidine hydrochloride (GdnHCl) was unfolded to a predominantly random coil conformation. Upon dilution of GdnHCl, PrP refolded into a conformation that was high in α-helix as measured by CD spectroscopy, similar to the normal cellular isoform of PrP (PrPc). This provided evidence that PrPSc can be induced to revert to a PrPc-like conformation with a strong denaturant. To examine the reversibility of PrPSc formation and aggregation under more physiological conditions, PrPSc aggregates were washed and resuspended in buffers lacking GdnHCl and monitored over time for the appearance of soluble PrP. No dissociation of PrP from the PrPSc aggregates was detected in aqueous buffers at pH 6 and 7.5. The effective solubility of PrP was <0.7 mM. Treatment of PrPSc with proteinase K (PK) before the analysis did not enhance the dissociation of PrP from the PrPSc aggregates. Treatment with 2.5 M GdnHCl, which partially and reversibly unfolds PrPSc, caused only limited dissociation of PrP from the aggregates. The PrP that dissociated from the aggregates over time was entirely PK-sensitive, like PrPc, whereas all of the aggregated PrP was partially PK-resistant. PrP also dissociated from aggregates of protease-resistant PrP generated in a cell-free conversion reaction, but only if treated with GdnHCl. Overall, the results suggest that PrP aggregation is not appreciably reversible under physiological conditions, but dissociation and refolding can be enhanced by treatments with GdnHCl.

The transmissible spongiform encephalopathies (TSEs) are progressive neurodegenerative diseases of mammals. During the course of TSEs, an abnormal isoform of the prion protein (PrP) called PrPSc accumulates in the brains and some peripheral organs of affected animals (1, 2). Either PrPSc itself or the process of its formation appears to be responsible for the neuropathology associated with these diseases (3–7). Preparations of PrPSc are highly infectious, and infectivity has not been physically separated from PrPSc (8, 9). PrPSc is derived from PrPc (the normal cellular isoform of PrP), and the conversion is likely to occur either on the cell surface or in an endocytic pathway (10–12). The two isoforms of PrP do not differ in amino acid sequence or consistent covalent post-translational modifications (13, 14). PrPSc is a partially protease-resistant insoluble aggregate of PrP with a high proportion of β-sheet secondary structure (15–18). PrPc is protease-sensitive and soluble and composed predominantly of random coil and α-helical structures (19–23). Thus, the primary differences between PrPSc and PrPc appear to be conformational. According to the protein-only hypothesis, PrPSc acts as an infectious protein by inducing its own formation from PrPc (1, 24). Support for this hypothesis came from the demonstration that PrPSc could cause PrPc to convert to a PrPSc-like protease-resistant and aggregated form in the absence of intact living cells (25). However, the generation of new infectivity has not been demonstrated in these cell-free conversion reactions (26) or in any other in vitro protein-only system.

TSEs are similar in some respects to amyloid diseases, which are characterized by the deposition of insoluble ordered protein aggregates called amyloid fibrils and plaques. Fibrils composed of PrP are often found in the brains of animals infected with TSEs (2, 27–30). In addition, the staining of PrPSc with the dye Congo red and subsequent exposure to polarized light result in birefringence, an amyloid hallmark (28). Amyloid formation (32) and the assembly of other fibrillar protein structures (33, 34) often occur through a mechanism called nucleated polymerization. This mechanism has also been proposed for PrPSc formation (32, 35–38). According to this mechanistic model, a slow lag phase during which protein monomers assemble into stable oligomeric nuclei precedes the relatively rapid elongation of the nuclei into fibrillar polymers. Nucleated polymerization of at least some proteins is reversible, as demonstrated by the disassembly of actin microfilaments (39, 40) and the decrease in length of Aβ-amyloid protofibrils upon dilution (41). However, the insolubility of mature amyloid fibrils and PrPSc suggests that the protein aggregation that occurs during their formation is irreversible or only slowly reversible. Thus, the accumulation of PrPSc in TSE-infected animals may be due to an inherent irreversibility of PrP aggregation during some stage of PrPc-to-PrPSc conversion. Alternatively, aggregation may be reversible, but preparations of PrPSc may be at chemical equilibrium between soluble and aggregated PrP. The issue of reversibility of PrPSc formation and polymerization may be of significance in assessing ways of stopping and reversing PrPSc accumulation for therapeutic purposes in vivo.

To gain insight into parameters controlling PrPSc accumulation in TSE-infected animals, experiments were performed to determine if the polymerization/aggregation of PrP that occurs during PrPSc formation is a reversible process. In this study, the dissociation of PrP from PrPSc aggregates was monitored over time under a variety of conditions. Overall, the results...
suggest that PrP aggregation is not detectably reversible, but partial unfolding can induce a modest level of PrP dissociation.

**EXPERIMENTAL PROCEDURES**

**Preparation of Hamster PrPSc—PrPSc was purified from the brains of hamsters infected with the 263K strain of scrapie using a modification of the method employed by Bolton et al. (60) as previously described (15).**

**Preparation of Hamster PrP* Lack of the Glycosylphosphatidylinositol (GPI) Anchor—Hamster GPI-(1) PrP* lacks the GPI anchor due to the introduction of a stop codon in codon 231 of the gene expressing hamster PrP. PrP* was isolated from mouse p2 and PAS137 fibroblast cells overexpressing hamster GPI-(1) PrP after metabolic labeling with [35S]methionine/cysteine essentially as described previously (42). However, in this study, 4 µl of antibody 3F4 and 64 µl of a 35–40% slurry of protein A-Sepharose (Amersham Pharmacia Biotech) were used for the immunoprecipitation; a single elution of the beads in 60 µl of 0.1 M acetic acid was performed; and the eluant was not vacuum-dried. Unlabeled hamster GPI-(1) PrP secreted into the tissue culture media and purified as described previously (43) was used for spectroscopy studies.

**Fourier Transform Infrared (FTIR) Spectroscopy—**1 mg/ml PrPSc was analyzed in 1 mM NaCl and 100 mM sodium phosphate, pH 6.5, 10 mg/ml PrPSc was analyzed in 20 mM sodium phosphate, 130 mM NaCl, pH 7.5, and 0.5% sulfobetaine with or without 5 M guanidine hydrochloride (GdnHCl) for 2 h at 20 °C. 5 µl of each sample was dropped onto a Golden Gate Single Reflection Diamond attenuated total reflectance unit (Graelsey Specac, Inc.) purged with a Whatman FTIR purge system, and the sample was covered with a volatiles cover. Data was collected with a System 2000 FTIR instrument (PerkinElmer Life Sciences). Test conditions were as follows: temperature, 20 °C; resolution, 1.00 cm−1; optical path difference velocity, 5 cm/s; number of scans, 500; scan range, 1800 to 1400 cm−1; and interval, 0.5 cm−1. Data types were single for background and ratio for sample. The detector used was nbMCT cooled by liquid nitrogen. Spectra were obtained by subtracting that of the corresponding buffer or buffer with additives and water vapor, adjusting the baseline, and normalizing for comparable absorbance of different concentrations of PrP. The software used for spectrum analysis was Spectrum Version 2.00 (PerkinElmer Life Sciences).

**Circular Dichroism Spectroscopy—**Hamster GPI-(1) PrPSc (100 µg/ml) was analyzed in 10 mM sodium phosphate and 300 mM potassium fluoride, pH 6.5. PrPSc (3 mg/ml) was incubated in 20 mM sodium phosphate, 150 mM NaCl, pH 7.5, 0.5% sulfobetaine, and 2.5 M GdnHCl for 3 days at 4 °C. The mixture was pelleted and washed twice with 10 mM sodium phosphate and 300 mM potassium fluoride, pH 6.5. The resuspended pellet was incubated for 2 h at 20 °C in the wash buffer plus 6 M GdnHCl and then gradually diluted with the wash buffer alone to 0.1 M GdnHCl and incubated for another 2 h at 20 °C. The sample was pelleted, and the supernatant was subjected to CD spectroscopy as described previously (43).

**Proteinase K Digestion of PrPSc before Dissociation Analysis—**3 µg of PrPSc was either treated with 60 ng of proteinase K (PK) or mock PK-treated in 50 µl of Tris/NaCl buffer (25) at 37 °C for 1 h. The reactions were then adjusted to 20 mM EDTA, 2 mM Pefabloc (Roche Molecular Biochemicals), 1.71 M NaCl, and 1% Sarkosyl in a total volume of 500 µl. All buffers used after the PK digestion step contained 2 mM Pefabloc. The presence of a high concentration of salt was used to aid in removing bound PK from the PrPSc aggregates. Aggregates were separated from soluble PrP and PK and subjected to dissociation analysis as described below, except that 9 µg of bovine serum albumin was added to the suspensions just before dissociation analysis as a final control for PK contamination.

**Cell-free Conversion Reactions—**Guinea-free cell-free conversion reactions were performed as described previously (44), except that reactions were scaled up by 5-fold. To measure the total amount of radiolabeled PrPSc in the reactions, a 2-µl aliquot was directly methanol-precipitated, and another 2-µl aliquot was counted by liquid scintillation. The amount of converted radiolabeled PrPSc was determined by treatment of a 10-µl aliquot with 10 µl/mg PK at 37 °C for 1 h, followed by the addition of 1 µl Pefabloc and methanol precipitation. Methanol-precipitated samples were analyzed by SDS-polyacrylamide gel electrophoresis and Storm PhosphorImager (Molecular Dynamics, Inc.) analysis. The remaining portions of the conversion reactions were used for dissociation analysis after removal of soluble PrPSc as described below.

**Removal of Soluble PrP from PrPSc—**Aliquots and Cell-free Conversion Reactions—The indicated amounts of PK-treated or untreated PrPSc were incubated under various conditions for 2.5 h or overnight at 37 °C. The suspensions were layered over 1 or 2 volumes of the same buffer used for the incubation supplemented with 1 M sucrose in polycarbonate ultracentrifuge tubes. The sucrose concentration was reduced for samples containing GdnHCl such that the sucrose/GdnHCl layers approximated the density of 1 M sucrose without GdnHCl. The tubes were centrifuged at 52,000–55,000 rpm for 30–36 min at 25 °C at the slowest acceleration/deceleration setting in a Beckman TL-100 ultracentrifuge. All of the sample zone and 90–95% of the sucrose layer were discarded, and the pellets were resuspended in the original volume or half of the original volume. Aliquots of this resuspended pellet were used for dissociation analysis as described below for the experiment shown in Fig. 2. For all other experiments, the resuspended PrPSc mixtures were pipetted over a second layer of sucrose identical to the first, and the centrifugation was repeated. All but the bottom 50–100 µl was discarded; 1 ml of buffer was added; and the samples were mixed briefly. The tubes were centrifuged at 55,000 rpm for 30 min at the fastest acceleration/deceleration setting. All but the bottom 50 µl of the wash was removed, and the pellets were resuspended in a total volume of 250–364 µl. Aliquots of these final PrPSc suspensions were used for dissociation analysis as described below.

**PrP Dissociation Analysis—**The centrifugation conditions used for the dissociation analyses described throughout this study are predicted to result in >90% of particles with s20,w ≥ 17S being cleared from the sample zone (top fraction). Soluble PrP refers to protein that remained in the sample zone, the vast majority of which would have an S value of <17S.

The resuspended PrPSc aggregate mixtures were incubated (in the same buffer used for the soluble PrP aggregate separation and subsequent washings) at 37 °C for 3 days, unless otherwise noted. At the beginning and end of the incubation period, 50-µl aliquots were removed and layered over 100 µl of the same sucrose solution used during the initial separation of soluble PrP from aggregate in polycarbonate centrifuge tubes. The step gradients were spun at 52,000 rpm for 16 min (reactions without GdnHCl) or 30 min (reactions with GdnHCl) in a Beckman TLS-55 swinging bucket rotor at the slowest acceleration/deceleration setting. Two 50-µl fractions (top and middle) and one 35–50-µl fraction (bottom) were removed. The remaining liquid was brought up to a volume of ~50 µl with the same buffer used for the incubation, and the pellets were resuspended. (For the experiment shown in Fig. 2, 25-µl aliquots were layered over 50 µl of sucrose solution, and the volume of the fractions was 25 µl.) All fractions were either counted using liquid scintillation (for radiolabeled PrPSc) or methanol-precipitated and stored at −20 °C until Western blotting could be performed.

In the experiment in which the PK sensitivity of the soluble and aggregate forms of PrPSc was assessed, the PrPSc aggregate mixtures were incubated at 37 °C for 3 days. Two 25-µl aliquots were removed from the PrPSc mixtures at days 0 and 3 of the incubation period. The reactions were brought up to a volume of 77.5 µl in Tris/NaCl buffer and incubated at 37 °C for 10 min to give the region of the protein containing the PK epitope a chance to renature and reacquire protease resistance (45). 2.5 µl of PK (1 mg/ml) was added to each of one pair of duplicate samples, and the same volume of PK storage buffer was added to the other (mock PK digest). Reactions were incubated for 1 h at 37 °C, and then 8 µl of 0.1 M Pefabloc was added. The reactions were layered over 0.3 M sucrose and 2.5 M GdnHCl, and the centrifugation and fractionation steps were performed as described above, except that the top fractions collected from the gradients were 90 µl, and the middle fractions were 48 µl.

**Western Blotting—**Proteins in methanol-pelleted proteins were separated by microcentrifugation and resuspended in urea sample buffer. Proteins were electroblotted to Tris/glycine-buffered 16% or NuPAGE BisTris-buffered 10% polyacrylamide gels (Novex) and transferred to Immobilon-P membranes (Millipore Corp.) using a semidy electrophoretic apparatus (Biometra, Inc.). Antigens were detected with either a 1:10,000 dilution of the anti-PrP monoclonal antibody 3F4 or a 1:1000 dilution of an anti-bovine serum albumin monoclonal antibody (Sigma, B2901). Primary antibodies were detected with a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed Laboratories, Inc.) and Attophos substrate (Promega) and visualized with a Storm fluorescence imaging system (Molecular Dynamics, Inc.).

**RESULTS**

**Guanidine Hydrochloride-induced Reversion of PrPSc to a PrP*like Conformation—**To determine whether or not PrPSc can be forced to revert to a PrP*like state, PrPSc was incubated...
at ~40 μg/ml (1.3 μM) in 6 M GdnHCl at 37 °C overnight. The sample was diluted 10-fold and immediately subjected to centrifugation through a sucrose solution and fractionated as described under “Experimental Procedures.” 81% of the guanidine-treated PrP was found in the top (soluble) fraction, whereas 12% was pelleted (data not shown). These results demonstrate that >80% of the PrPSc was solubilized and that solubilized PrP fractionated to the top of the gradients under the centrifugation conditions used in this study. To verify that the guanidine-solubilized PrP was unfolded, PrPSc disaggregated in 5 M GdnHCl was analyzed by FTIR spectroscopy. The spectrum was clearly different from the spectra of untreated PrPSc (high β-sheet) and PrPSc (predominantly α-helix) (Fig. 1A), and its absorbance maximum of 1646 cm⁻¹ is consistent with a predominantly random coil structure (46, 47). This provides evidence that PrPSc was largely unfolded by GdnHCl. To examine the ability of solubilized and unfolded PrP to refold into a PrP⁰-like conformation, PrPSc was first washed with 2.5 M GdnHCl to remove protease-sensitive PrP from the aggregates (9, 45, 48). The washed aggregates were then disaggregated in 6 M GdnHCl, and the mixture was gradually diluted to give a final GdnHCl concentration of 0.1 M. Any remaining aggregates were pelleted by centrifugation, and the supernatant was analyzed by CD spectroscopy. The solubilized PrP generated a CD spectrum similar to that of PrP² (Fig. 1B). These results indicate that a majority of the molecules in PrPSc could be induced to refold into a PrP²-like conformation after treatment with a strong denaturant.

Reversibility of PrPSc Aggregation under Near-physiological Conditions—For the purposes of this work, we define reversibility of PrP aggregation as an increase in soluble PrP over time in a suspension of PrPSc aggregates after the initial removal of soluble protein. To determine if any PrP dissociates from PrPSc aggregates under conditions that approximate those present at the cell surface or in early endosomes in vivo, suspensions of hamster PrPSc in buffers at pH 7.5 or 6.0, respectively, were washed by ultracentrifugation through a sucrose solution to separate any preexisting soluble PrP from the PrPSc aggregates. Pellets were washed and resuspended in the same buffer, and a portion of the mixture was immediately subjected to dissociation analysis. The remainder of the mixture was incubated at 37 °C for 3 or 4 days and then assayed for the presence of soluble PrP. Under these conditions, no detectable soluble PrP was observed at either the beginning or end of the incubations (Figs. 2 and 3, T lanes). Based on PrPSc standards included on the blots, the concentration of soluble PrP in the aggregate suspensions was <0.7 nM. These results indicate that the aggregation of PrPSc was not detectably reversible at two pH levels that may be encountered by PrPSc in vivo.

To verify that small amounts of soluble PrP in a sample could be recovered after centrifugation, a portion of the above-mentioned 6 M GdnHCl-treated PrPSc sample was diluted in citrate-buffered KCl with Sarkosyl, pH 6.0 (CBKS; 0.625% Sarkosyl, 0.625% Sarkosyl, 0.625% Sarkosyl).

FIG. 2. Dissociation of PrP under nondenaturing conditions at pH 6.0. A 2-μg aliquot of PrPSc was incubated in CBKS overnight (~17 h) at 37 °C. PrPSc aggregates were washed and analyzed for dissociation of PrP immediately or after 4 days. PrP in fractions from the gradients was detected by Western blot analysis. The band marked with the single asterisk is unglycosylated PrP that is N-terminally truncated by proteolysis in vivo or during the PrPSc purification procedure (45). In this work (Figs. 2–7) and in previous studies (31, 45, 59), discrete higher molecular mass forms of PrP were detectable by Western blotting of PrPSc or PrPSc⁰ (where CJD is Creutzfeldt-Jakob disease). The two most visible high molecular mass bands (**) seen in this study may represent small oligomeric species within the PrPSc aggregate in which the subunits are covalently cross-linked to each other. These stable oligomers display similar biochemical behavior to the monomeric PrP subunits throughout this study (Figs. 2–7). For this reason and because of the inability to measure possible larger stable oligomers that do not migrate into the gels, we have excluded these forms of PrP from our quantitative assessment of PrP dissociation. T, top fraction; M, middle fraction; B, bottom fraction; P, pellet. T and M lanes contain the equivalent of one-half of each fraction.

FIG. 1. Dissociation, unfolding, and refolding of PrPSc. A, soluble protein generated by treatment of PrPSc with 5 M GdnHCl was analyzed by FTIR spectroscopy. Shown for comparison are FTIR spectra of untreated PrPSc and untreated PrPC. Amide I absorbance maxima at 1655 and 1626 cm⁻¹ are indicative of predominant α-helix and β-sheet, respectively, whereas absorbance at 1646 cm⁻¹ is consistent with high random coil or disordered secondary structure (46, 47). B, a sample of guanidine-solubilized PrP was diluted to 0.1 M GdnHCl and analyzed by CD spectroscopy. Shown for comparison is the CD spectrum of native PrPC. Both of these CD spectra are indicative of the high α-helix content that is known to exist in PrPC. The CD spectrum of PrPSc (not shown) is significantly different and indicates a much higher proportion of β-sheet structure (17).
Protease Sensitivity of Soluble and Aggregate Forms of PrP

In addition to aggregation, another process that occurs during the conversion of PrP to PrPSc is the acquisition of protease resistance. To determine if this conversion is also reversible, the PrPSc mixture was diluted to 1 μM GdnHCl before the dissociation analysis. The concentration of soluble PrP in the PrPSc mixture increased to a concentration of ~10 nM after 3 days (data not shown). The amount of PrP in the soluble fraction on day 3 was ~5% of the total PrP. These results indicate that treatment with 2.5 mM GdnHCl enhanced the ability of PrP to dissociate from the PrPSc aggregate.

To determine if PrP will dissociate from partially unfolded and refolded PrPSc aggregates, the same experiment was performed, except that the PrPSc mixture was diluted to 1 μM GdnHCl before the dissociation analysis. The concentration of soluble PrP increased from undetectable to only ~1 nM during the 3-day incubation period (data not shown). These results suggest that the refolding of partially unfolded PrPSc significantly decreased the dissociation of PrP from the aggregate.

Dissociation of PK-treated PrPSc—Treatment of hamster 263K-associated PrPSc with PK completely degrades roughly half of the total PrP molecules (48). The remaining PrP molecules are N-terminally truncated by ~67 amino acids (13, 49). These effects of PK may affect aggregate ultrastructure (50) and stability. To determine if PK treatment affects the ability of PrP to dissociate from PrPSc aggregates, PK-treated PrPSc was subjected to dissociation analysis in parallel with untreated PrPSc. Because any PK present in the suspension during the dissociation analysis could degrade any protease-sensitive PrP that may dissociate from the aggregate, multiple measures were taken to control for PK carryover as described under “Experimental Procedures.”

As in the experiments shown in Figs. 2 and 3, no detectable PrP was present in the soluble fractions from the untreated PrPSc samples at both the 0- and 3-day time points (Fig. 4, −PK). Based on the PrPSc standards on this blot, the concentration of soluble PrP in the suspension of untreated PrPSc was estimated to be <0.7 nM. When PrPSc was treated with PK, the results were identical (Fig. 4, +PK). In addition, there was no increase in soluble PrP over time regardless of whether or not the PrPSc had been digested with PK. All of the PrP in the dissociation reactions was pelleted at both time points (data not shown). The amount of bovine serum albumin in the suspensions did not change during the incubation period, demonstrating that there was little or no active PK present in the suspensions that could have degraded any dissociated PrP. These results indicate that predigestion of PrPSc with PK did not affect the reversibility of PrPSc aggregation.

Dissociation of PrP from PrPSc Aggregates after Partial Unfolding—Treatments with 2.5–3 M GdnHCl can partially and reversibly unfold hamster PrPSc (45). To determine if partial unfolding of PrPSc can increase the dissociation of PrP from PrPSc aggregates, the separation of soluble and aggregated PrP was performed using PrPSc incubated in 2.5 M GdnHCl. After washing and resuspending the pellet in 2.5 M GdnHCl, dissociation analysis was performed (Fig. 5). The concentration of PrPSc aggregate at the beginning of the incubation period was ~170 nM (data not shown). 94–100% of the PrP in the mixture was pelletable at both time points (data not shown). Without incubation, no detectable PrP partitioned to the soluble fraction of the gradients (Fig. 5, day 0). The amount of soluble PrP in the PrPSc mixture increased to a concentration of ~10 nM after 3 days (data not shown). The amount of PrP in the soluble fraction on day 3 was ~6% of the total PrP. These results indicate that treatment with 2.5 mM GdnHCl enhanced the ability of PrP to dissociate from the PrPSc aggregate.
that the PrP that dissociated from the partially unfolded aggregate did not retain PK resistance.

Dissociation of Aggregated PrP in Cell-free Conversion Reactions—When brain-derived PrPSc is incubated with 35S-labeled PrPC in vitro, the radiolabeled PrPSc is incorporated into the PrPSc aggregates (44) and converted to a partially PK-resistant form (9, 25). To determine if PrP aggregation resulting from cell-free conversion is reversible, four conversion reactions were incubated for 2, 8, and 48 h and pooled for each time point. Soluble PrP was separated from aggregates, and the washed and resuspended aggregates were subjected to dissociation analysis. At all time points of the conversion reactions, the amount of radiolabeled PrP that was converted was always less than the amount that bound to the PrPSc aggregates (Fig. 7A). For each reaction, the amount of radiolabeled PrP that bound during the conversion reaction was considered 100% for the subsequent dissociation analysis. For all time points of conversion and dissociation, >97% of the total radiolabeled PrP in each dissociation reaction was pelleted during ultracentrifugation (data not shown). In all reactions, 1–3% of the total 35S-

labeled PrP was present in the soluble fractions at the beginning and end of the dissociation period (Fig. 7B). Moreover, regardless of the length of time of the conversion reaction, the amount of soluble radiolabeled PrP did not increase during the dissociation period. These results suggest that the association of radiolabeled PrP with the PrPSc aggregates was not measurably reversible throughout the course of the conversion reaction.
To determine if the PrP that bound to the PrP$^{\text{Sc}}$ aggregates during cell-free conversion reactions can dissociate after partial unfolding, dissociation analysis was performed on pelleted and washed cell-free conversion products after treatment with 2.5 M GdnHCl for 2.5 h at 37 °C. At the beginning and end of the dissociation reactions, 87–99% of the radiolabeled PrP was pelleted during ultracentrifugation (data not shown). At the 0-day dissociation time point, very little $^{35}$S-labeled PrP (1–2% of the total $^{35}$S-labeled PrP in the dissociation reactions) was present in the soluble fraction, but the amount of soluble $^{35}$S-labeled PrP increased almost 9-fold (to 13% of the total $^{35}$S-labeled PrP in the dissociation reactions) was present in the soluble fraction (data not shown). During the dissociation period, the amount of soluble PrP increased from 1% to 8% (data not shown). During the dissociation period, the amount of soluble PrP increased from $<1\%$ to 8% ($\approx 8\%$ of the total PrP (Fig. 7C). These results indicate that a portion of the radiolabeled PrP that was incorporated into the aggregate during the conversion reaction dissociated over time from the partially unfolded PrP$^{\text{Sc}}$ aggregates.

In addition to determining the amount of radiolabeled PrP, portions of the same gradient fractions that were subjected to scintillation counting were also analyzed by Western blotting to determine the total amount of soluble PrP. The concentration of PrP$^{\text{Sc}}$ aggregate after removal of soluble PrP at the beginning of the incubation period was $\approx 290$ nM (data not shown). As with the radiolabeled PrP, 92–99% of the total PrP in the reaction at both dissociation time points was pelleted during ultracentrifugation (data not shown). During the dissociation period, the amount of soluble PrP increased from $<1\%$ to 8% (data not shown). These results demonstrate that the dissociation of PrP from PK-resistant PrP generated in vitro and PrP$^{\text{Sc}}$ from the brains of scrapie-infected animals can be induced to roughly similar extents by treatment with 2.5 M GdnHCl.

DISCUSSION

Measuring the reversibility of PrP aggregation may help elucidate aspects of the mechanism of PrP$^{\text{Sc}}$ formation and may aid in the testing of potential therapeutic agents that could enhance the dissociation of PrP from PrP$^{\text{Sc}}$ aggregates. Our data indicate that PrP aggregation is not detectably reversible in the absence of GdnHCl. No detectable PrP dissociated from brain-derived PrP$^{\text{Sc}}$ aggregates under guanidine-free conditions over periods of 3–4 days (Figs. 2–4), despite the fact that PrP$^{\text{Sc}}$ aggregates were found to contain a substantial proportion of protease-sensitive PrP (48). In addition, dissociation of radiolabeled PrP was not detected from aggregated cell-free conversion products, regardless of the duration of the conversion reaction (Fig. 7B). Moreover, throughout cell-free conversion reactions, a significant proportion of the aggregate-associated radiolabeled PrP was not converted to the protease-resistant state (Fig. 7A) (44, 51). These combined observations suggest that PrP aggregation becomes practically irreversible even without the acquisition of protease resistance.

One explanation for these results is that PrP aggregation is governed by a reversible nucleated polymerization mechanism, but the equilibrium between soluble protein and aggregate is highly skewed toward the aggregate state. The critical concentration may be so low that the amount of PrP that dissociates from the aggregates is below the limits of detection by Western blotting. Based on the lowest standard used on the blots (Figs. 2 and 3), the critical concentration of PrP aggregation would be $<0.7$ nM.

Alternatively, the results could be explained by a multistep mechanism of PrP$^{\text{Sc}}$ formation in which one or more of the steps are irreversible. In this scenario, a reversible process such as nucleated polymerization could govern an early step. However, because a subsequent step is irreversible, the overall process is effectively irreversible when the final product (PrP$^{\text{Sc}}$) is examined. The irreversible step follows soon after aggregation, but may precede the acquisition of protease resistance and would lock PrP into the aggregate state. This is analogous to recent findings regarding the polymerization of Aβ peptide into Alzheimer’s disease amyloid in vitro. This process is controlled by nucleated polymerization (36, 52). Recent studies have identified a kinetic intermediate between Aβ monomer and fibril called the protofibril (53). Dilution of a mixture of protofibrils results in a decrease in protofibril length over time, indicating that the process of protofibril formation is reversible. However, one or more of the steps in the transition from protofibrils to fibrils are irreversible (32). Moreover, studies of β-amloid fibril elongation have revealed that the binding of monomeric Aβ to fibrils is initially reversible, but becomes irreversible over time, presumably due to conformational changes in the peptide after incorporation into the fibril (54, 55). This so-called “dock-lock” mechanism may also govern PrP$^{\text{Sc}}$ formation.

The detection of high molecular mass forms of PrP on Western blots may be the result of some PrP molecules within the PrP$^{\text{Sc}}$ aggregates becoming covalently cross-linked in vivo (56). Although the dissociability of the detectable high molecular mass species of PrP appeared to parallel the dissociation behavior of the monomeric PrP subunits (Figs. 2–6), larger cross-linked PrP species that do not migrate into the gel may behave differently. Covalently linked PrP multimers would act as single subunits with multivalent noncovalent contacts with the rest of the aggregate, resulting in a higher affinity interaction. Even if some individual PrP molecules near the ends of the aggregates are able to dissociate, cross-linked species with a lower tendency to dissociate will eventually be exposed. The high affinity interaction of such forms of PrP with the aggregates could effectively trap both covalent multimers and non-covalently associated PrP molecules within the aggregate. Thus, cross-linking of aggregated PrP molecules in vivo may contribute to the insolubility of PrP$^{\text{Sc}}$.

Treatment with 2.5–3 M GdnHCl reduces the size of the fragment remaining after PK digestion of both PrP$^{\text{Sc}}$ (45) and the cell-free conversion product by reversibly unfolding a 3–4-kDa region near the N terminus of the protease-resistant core of PrP$^{\text{Sc}}$. A large proportion of the structure of amyloids consists of intermolecular β-sheet (32), and this structure probably makes up some part of the PK-resistant core of PrP$^{\text{Sc}}$. If so, then intermolecular β-sheet may mediate many of the physical contacts between monomers within the aggregate. The GdnHCl-induced unfolding of a portion of the PK-resistant core of PrP$^{\text{Sc}}$ may result in the disruption of contacts between monomers within the 3–4-kDa unfolded region. With fewer contacts between monomers, the aggregates will be destabilized, and PrP will more readily dissociate.

The experiments conducted in this work were performed in vitro using PrP$^{\text{Sc}}$ from scrapie-infected hamsters to investigate the reversibility of aggregation of this infectivity-associated, biochemically active, TSE-related molecule. However, the PrP$^{\text{Sc}}$ purification procedure may remove molecules present in intact hamster brain. Certain factors that are normally present in the brain may influence the reversibility of PrP aggregation during the course of TSE diseases. Also, the purification procedure might alter the structure or biochemical properties of PrP$^{\text{Sc}}$. Hence, it remains possible that PrP dissociates more readily from the PrP$^{\text{Sc}}$ aggregates in vivo. Assays such as those used in this study could aid in the identification of exogenous molecules capable of stimulating the dissociation of PrP. Such molecules, perhaps in cooperation with endogenous factors in the brain, may be able to dissolve PrP$^{\text{Sc}}$ deposits in vivo, reverse disease pathology, and alleviate symptoms. Along these lines, peptides called β-breakers can help reverse in vitro the

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2 M. A. Callahan, unpublished observations.
conformational changes and aggregation associated with PrPSc and partially neutralize scrapie infectivity (57). Also, branched polyanines can disaggregate and reduce the β-sheet content and protease resistance of PrPSc in vitro and eliminate scrapie infectivity in infected neuroblastoma cells (58).

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