Protease-resistant and Detergent-insoluble Prion Protein Is Not Necessarily Associated with Prion Infectivity*

(Received for publication, December 29, 1998, and in revised form, April 8, 1999)

Gideon M. Shaked‡, Gilgi Fridlander§, Zeev Meiner‡, Albert Taraboulos§, and Ruth Gabizon‡¶

From the ‡Department of Neurology, Hadassah University Hospital, Jerusalem 91120, Israel and the §Department of Molecular Biology, Hebrew University Medical School, Jerusalem 91120, Israel

PrPSc, an abnormal isoform of PrPC, is the only known component of the prion, an agent causing fatal neurodegenerative disorders such as bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD). It has been postulated that prion diseases propagate by the conversion of detergent-soluble and protease-sensitive PrPC molecules into protease-resistant and insoluble PrPSc molecules by a mechanism in which PrPSc serves as a template. We show here that the chemical chaperone dimethyl sulfoxide (Me2SO) can partially inhibit the aggregation of either PrPSc or that of its protease-resistant core PrP27–30. Following Me2SO removal by methanol precipitation, solubilized PrP27–30 molecules aggregated into small and amorphous structures that did not resemble the rod configuration observed when scrapie brain membranes were extracted with Sarkosyl and digested with proteinase K. Interestingly, aggregates derived from Me2SO-solubilized PrP27–30 presented less than 1% of the prion infectivity obtained when the same amount of PrP27–30 in rods was inoculated into hamsters. These results suggest that the conversion of PrPC into protease-resistant and detergent-insoluble PrP molecules is not the only crucial step in prion replication. Whether an additional requirement is the aggregation of newly formed proteinase K-resistant PrP molecules into uniquely structured aggregates remains to be established.

PrPSc, an abnormal isoform of PrPC (1), is the only known component of the prion, an agent causing fatal neurodegenerative disorders such as BSE and CJD (2). It has been postulated that prion diseases propagate by the conversion of PrPSc molecules into protease-resistant and insoluble PrPSc molecules by a mechanism in which PrPSc serves as a template (3). The pathway for PrPSc synthesis may feature the formation of PrPSc-PrPSc heterodimers (4). Alternatively, the nucleation-dependent protein polymerization model argues that the formation of new PrPSc molecules depends on the presence of a seed composed of aggregated PrPSc molecules and that new PrPSc molecules join previously assembled prion polymers (5). Although many lines of evidence suggest that PrPSc is the crucial and even the only prion component, up till today infectivity could not be associated with PrPSc like PrP molecules produced by an array of in vitro conversion protocols (6–8).

The organic solvent dimethyl sulfoxide (Me2SO) was shown to block the formation of amyloid fibrils by Aβ peptide in vitro (9). After a single dose of Me2SO, the urine of human amyloidotic patients contained fibrils with the tinctorial properties of amyloids, suggesting that Me2SO can either break large amyloid fibrils or inhibit their formation, resulting in smaller structures that can be mobilized from the connective tissue and eliminated by the kidneys (10). Me2SO was also shown to inhibit the accumulation of PrPSc in scrapie-infected neuroblastoma cells (11), suggesting that Me2SO, in its function as a “chemical chaperone,” stabilized the conformation of PrPC molecules, thereby preventing them from undergoing the conformational changes required for the conversion of PrPC to PrPSc.

In this work, we investigated whether the hallmark properties of PrPSc, i.e., resistance to proteases and insolubility in detergents, are affected by in vitro treatment with Me2SO. These biochemical properties of PrP have been traditionally linked to the presence of prion infectivity (12), although in some experimental setups, protease-resistant PrP could not be found in samples that contain prion infectivity (13–15). Interestingly, it was shown lately that in prion strains with long incubation times, PrPSc is considerably less resistant to proteases than in short incubation time strains (16).

Our results show that when membranes prepared from brains of hamsters terminally ill with scrapie were incubated in the presence of Me2SO and detergents, as opposed to detergent only, part of the PK-resistant PrP molecules could neither be precipitated by high speed centrifugation nor did they aggregate into very large structures. Me2SO, although it can inhibit the aggregation of protease-resistant PrP molecules, could not solubilize previously aggregated PrPSc. These soluble PrP27–30 molecules will aggregate upon the removal of Me2SO, albeit not to the characteristic rod structure obtained when scrapie brain membranes are extracted with Sarkosyl and digested with proteinase K (PK) (17). When inoculated into hamster brains, only traces of scrapie infectivity were associated with this prion-specific isoform.

EXPERIMENTAL PROCEDURES

Sucrose Gradients—Three hundred microliters of 10, 15, 20, 25, 30, and 60% sucrose in phosphate-buffered saline were loaded into TLS-55 ultracentrifuge tubes (Beckman Instruments) to form a zonal gradient. Microsomes from brains of scrapie-infected hamsters (60–80 μg containing about 15 μg/ml protein) were diluted with STE buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) to 240 μl. Sarkosyl (2%) and when appropriate Me2SO (10%) were added to the mixture and incubated for 16 h at 4 °C before loading on top of the gradient and centrifuged at 100,000 × g for 1 h at 20 °C. After the centrifugation, gradient fractions of equal volume were collected and immunoblotted with anti-PrP mAb 3F4.

In Vivo Infectivity Experiments—Top and bottom fractions from sucrose gradients (after PK digestion) with and without Me2SO were...
**RESULTS**

10-μl microsomes (20) (15 μg/ml protein) prepared from the brains of Syrian hamsters infected with experimental scrapie 263K (21) were diluted to 100 μl in STE buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) and incubated with 2% sodium sarcosinate (Sarkosyl) in the presence or absence of 10% Me2SO at 4 °C. Following the incubation, all samples were digested with 40 μg/ml PK for 1 h at 37 °C and centrifuged at 100,000 x g. Pellets and supernatants (sup) were tested for the presence of PK-resistant PrP by immunoblotting with aPrP mAb 3F4. Lane 1, incubation with Sarkosyl and Me2SO for 16 h. Lane 2, Me2SO was added 2 h after Sarkosyl, and the sample was incubated for 14 additional hours. Lane 3, incubation with Sarkosyl alone for 16 h. Lane 4, Sarkosyl was added to this sample 2 h before the end of the incubation.

precipitated by 4 volumes of methanol (to discard the Me2SO) and resuspended in 100 μl of saline, 10% bovine serum albumin. 10 μl of each sample was serially diluted in STE (3×10), and all samples were immunoblotted with mAb 3F4. After comparing the protease-resistant PrP signal in control microsomes, bottom fractions from sucrose gradients with and without Me2SO, as well as top fractions of the Me2SO gradient, the top samples were diluted 10 times and the rest 100 times to produce solutions with similar concentrations of PrP27–30. Four-week-old male Syrian hamsters were inoculated intracerebrally with samples to be tested for prion infectivity (50 μl). All experiments were performed in duplicates. The hamsters were tested daily. Prion titers were measured by monitoring the incubation period until the appearance of symptoms (18).

**Fig. 1.** Me2SO inhibits the aggregation of PrPSc molecules. Membranes from scrapie-infected brains were incubated with 2% Sarkosyl in the presence or absence of 10% Me2SO at 4 °C. Following the incubation, all samples were digested with 40 μg/ml PK for 1 h at 37 °C and centrifuged at 100,000 x g. Pellets and supernatants (sup) were tested for the presence of PK-resistant PrP by immunoblotting with aPrP mAb 3F4. Lane 1, incubation with Sarkosyl and Me2SO for 16 h. Lane 2, Me2SO was added 2 h after Sarkosyl, and the sample was incubated for 14 additional hours. Lane 3, incubation with Sarkosyl alone for 16 h. Lane 4, Sarkosyl was added to this sample 2 h before the end of the incubation.

*Cross-linking by DSS—*Disuccinimidyl suberate, an N-hydroxy succinimide ester homobifunctional cross-linker (reacting with NH₂ groups), was dissolved in Me2SO (to 1 μl) and subsequently diluted into double-distilled water to 500 μl. Samples of sucrose gradient fractions were incubated with DSS at a final concentration of 125 μM DSS (30 min, room temperature). Following the incubation, the reaction was terminated by the addition of 1 × Tris. The samples were precipitated by methanol and immunoblotted with mAb 3F4.

**HistobLOTS—**Histoblots were carried out as described by Taraboulos et al. (19). Shortly, glass slides carrying 8-μm thick cryostat sections were quickly thawed and immediately pressed onto nitrocellulose membrane saturated with lysis buffer. The membranes were thoroughly air-dried, rehydrated for 1 h in TBST (10 mM Tris, pH 8, 100 mM NaCl, 1.5% Tween 20), and then subjected to limited proteolysis in digestion buffer containing 40 μg/ml PK for 1 h at 37 °C, followed by incubation of the blots in 3 M guanidine thiocyanate/10 mM Tris-HCl, pH 7.8. Subsequently, the blots were processed as for immunoblotting.

**RESULTS**

10-μl microsomes (20) (15 μg/ml protein) prepared from the brains of Syrian hamsters infected with experimental scrapie 263K (21) were diluted to 100 μl in STE buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) and incubated with 2% sodium sarcosinate (Sarkosyl) in the presence or absence of 10% Me2SO at 4 °C for 16 h before centrifugation for 1 h at 100,000 × g. Me2SO was removed from the supernatants by methanol precipitation, and pellets were rinsed once with 70% methanol. Ethanol or methanol precipitation are established methods to precipitate PrPSc and prepare for infectivity assays as well as for biochemical manipulations (22–25). All samples were resuspended in STE with 2% Sarkosyl, incubated with proteinase K (40 μg/ml, 1 h, 37 °C), and then analyzed by SDS-PAGE followed by immunoblotting with aPrP mAb 3F4 (26). Proteinase K completely digests PrPSc and concomitantly converts PrPSc into PrP27–30 (1). In the presence of Me2SO (Fig. 1, lane 1), a considerable part of the protease-resistant PrP remained in the supernatant; otherwise almost all of it precipitated in the high speed spin (lane 3). Me2SO was also ineffective in solubilizing PrPSc if added 2 h after the detergent, when most PrPSc molecules were already aggregated (compare lanes 2 and 4). This suggests that although Me2SO can inhibit PrPSc aggregation, it does not solubilize previously aggregated PrPSc. Me2SO was also unable to solubilize purified PrP27–30 or PrPSc 33–35, which are already aggregated (not shown).

To test whether the results obtained in Fig. 1 resulted from the *in vitro* conversion of PrPSc to a protease-resistant species by the Me2SO treatment or whether traces of Me2SO inhibit the activity of PK, microsomes from scrapie-infected hamster brains were pretreated with PK before the incubation in the presence or absence of Me2SO. The effect of Me2SO on PrPSc aggregation was identical regardless of whether Me2SO was applied before or after digestion with PK (Fig. 2). We conclude therefore that Me2SO does not confer protease resistance to otherwise PK-sensitive PrP molecules but rather inhibits the aggregation of PrPSc molecules. Whether Me2SO just reveals and amplifies a pre-existing difference between two populations of PrPSc molecules (such as preaggregation) or actively partitions PrPSc molecules into two distinct populations remains to be established.

To estimate the degree of aggregation of Me2SO-solubilized PrPSc, normal and scrapie-infected hamster brain microsomes were incubated for 16 h in Sarkosyl in the presence or absence of Me2SO, and the resulting lysates were sedimented through 10–60% sucrose gradients containing 2% Sarkosyl. Molecules migrating to the bottom of such gradients are either in very large aggregates or of very large molecular weight, and detergent-soluble proteins of small size are expected to remain in the upper gradient fractions. As can be seen in Fig. 3, PrPSc from normal microsomes, which solubilizes readily in the presence of detergents (20), remained in the upper fractions of the gradient in the presence or absence of Me2SO. In addition, the incubation of PrPSc with Me2SO treatment did not convert the normal prion isoform into protease-resistant PrP (Fig. 3, a–d).

Microsomes from scrapie-infected brains, which are believed to contain both PrPSc and PrPfv (20), yielded a bimodal PrP distribution in the absence of Me2SO; about half of the PrP (presumably PrPfv) was found in the top of the gradient, whereas the rest migrated to the bottom (Fig. 3e). However, only the PrP at the bottom of the gradient resisted the action of proteinase K, as expected from PrPSc (Fig. 3f).

When Me2SO was present during the lysis of scrapie microsomes, a profound change in the distribution of protease-resistant PrP was observed (Fig. 3b). A considerable portion of the PrP molecules at the top of the gradient resisted proteinase K-catalyzed proteolysis, which instead produced PrP27–30, the protease-resistant core of PrPSc. Thus, the action of Me2SO on scrapie microsomes yielded protease-resistant, prion-specific...
Prion Protein Not Necessarily Associated with Infectivity

PrP structures that are contained in low degree oligomers.

To reinforce the conclusion that the Me2SO-soluble PrP27–30 is the protease-resistant core of PrPSc and not a partially resistant PrPCs promoted either by the Me2SO incubation or by methanol precipitation, the three lightest fractions from sucrose gradients c and g, respectively, in Fig. 3 were combined, precipitated with methanol, resuspended in Sarkosyl, and subsequently digested with low PK concentrations for short periods. It has been shown lately that under such mild conditions of PK digestion, PrPC reveals a PK-resistant core of a lower Mr than PrP27–30, since the 3F4 epitope (residues 108–111) is absent from this peptide (27). These PK-digested samples were immunoblotted either with mAb 3F4 or with mAb 13A5, which reacts with residue 138 of hamster PrP. As can be seen in Fig. 4, while in the protease-resistant core of Me2SO-treated PrP in the scrapie fractions, both epitopes were present after 1 h PK digestion, and this was not the case for PrP from normal brain. These results show that Me2SO-solubilized PrP from scrapie brains was indeed PrPSc which, unlike PrPC, produced upon mild or harsh PK digestion a protease-resistant core of the same molecular weight as aggregated PrPSc (see also Figs. 3 and 8). In addition, these results also show that methanol precipitation does not confer or reduce protease resistance.

To investigate the degree of oligomerization of soluble PrP27–30 in Fig. 3, PK-digested Me2SO-treated scrapie microsomes were resolved on a sucrose gradient as described above, and the diverse gradient fractions were subsequently cross-linked by DSS. The rationale of this experiment is that although monomeric PrPSc will not cross-link, aggregated PrPSc will cross-link heavily and thereby not enter the polyacrylamide gel. Indeed, as can be seen in Fig. 5, while treatment of the heavy fractions from the Me2SO gradient with DSS resulted in a reduction in the PrP27–30 band seen by 3F4 immunoblotting, PrP27–30 in the light fractions was resistant to cross-linking.

To test whether Me2SO-solubilized PrPSc molecules will either aggregate or remain soluble following the removal of Me2SO, fractions from the top and from the bottom of sucrose gradients were incubated with PK and then precipitated with methanol. Me2SO is very soluble in this alcohol. Methanol pellets of top and of bottom fractions were resuspended in 2% Sarkosyl, and subsequently digested with low PK concentrations.

When the aggregates produced by Me2SO-solubilized PrP27–30 were looked upon by electron microscopy and compared with those in the original heavy fractions, a profound difference in structure was observed. Although PrP27–30 from the heavy fractions aggregated into the familiar rod-like structure (29), the new aggregates were amorphous (Fig. 7).
Prion infectivity is mostly associated with rods like PrPSc. PK-resistant control scrapie microsomes and fractions from sucrose gradients of scrapie-infected membranes with and without Me2SO as in Fig. 3 were precipitated with 4 volumes of methanol and resuspended so that they contain identical concentrations of PrP27–30. Light fractions prepared without Me2SO, which do not contain PrP27–30, were diluted similarly as the Me2SO light fractions. 50 µl of sample C and bars 1–4 in the immunoblot, shown in the figure, were inoculated into Syrian hamster brains and monitored for signs of prion infection. Infectivity titers were calculated from incubation time (days) according to Prusiner et al. (29). C, control scrapie microsomes. Bar 1, light fractions of gradient without Me2SO. Bar 2, light fraction of gradient with Me2SO. Bar 3, heavy fraction of bar 1. Bar 4, heavy fraction of bar 2.

A possible explanation for the infectivity results presented above is that Me2SO produced a profound change in the conformation of PrPSc, therefore generating a new prion strain (30, 31). To test this possibility, we looked for evidence of strain-specific parameters in the hamsters that were inoculated with samples from the top and the bottom fractions of Me2SO-treated microsomes. Clinical signs observed in all groups were similar and were characteristic of the parent Sc263K strain (32). PrP27–30 banding (Fig. 9a) and PrPSc histoblot patterns (Fig. 9b) (33–35) were also similar in all the four groups. However, since there is a residual infectivity in the light fractions of untreated microsomes, these results do not rule out the presence, in the Me2SO-treated samples, of a new prion strain with a much longer incubation time than that of the parent Sc263 strain.

**DISCUSSION**

Although there is little doubt that PrPSc plays a crucial role in prion diseases (2), the mechanism by which PrPSc converts...
Prion Protein Not Necessarily Associated with Infectivity

specific prion titers. Surprisingly, this was not the case. The light samples in the gradients shown in Fig. 3, with and without Me2SO, presented the same low prion titer, even though the control sample had no apparent PrPSc in contrast to the Me2SO-treated sample, which contains large quantities of the protease-resistant PrP isoform. It should be noted that the DLPC dispersion experiments were performed mostly on pre-aggregated rods, which as described above contain a priori all the infectivity. In addition, it is possible that the inoculation of PrPSc in DLPC, although dispersed into monomers or small oligomers, represents a favorable pharmacological pathway to infect cells with prions. Me2SO-solubilized PrPSc may not present such a biological advantage.

Our results show that prion-specific PrP molecules can be differentiated into two distinct species of disparate physicochemical properties: “classical” PrPSc and Me2SO-soluble PrPSc. This demonstrates that prion-specific PrP molecules can exist as a soluble species and yet possess the protease-resistant core PrP27–30. Apart from being resistant to proteases, these soluble molecules differ from PrPSc in that they have not lost their propensity to aggregate (albeit to amorphous, non-rod structures) when Me2SO is removed. However, this species is not associated with prion infectivity.

We have also shown here that soluble PrPSc can be dissociated from prion infectivity only when aggregation is inhibited during membrane extraction and not by dissociation from previously aggregated PrPSc. This is the fundamental difference between the experiments presented here and other approaches which failed to show non-infectious protease-resistant PrPSc (44).

As shown above, non-infectious and infectious PrPSc have similar aggregation properties, and therefore, once aggregated in the presence of detergent they are biochemically indistinguishable. Me2SO-solubilized PrPSc may be a metabolic intermediate in the formation of infectivity-associated PrPSc. If so, then the process of prion replication may be composed of more than one irreversible step, the conversion of PrPSc to a protease-resistant species being only one of them. In vitro converted PrPSc may also be such an intermediate, since although presenting the biochemical properties of PrPSc, it has not been shown to be infectious (7, 8). Prion infectivity may only be associated with the final step, which would involve the specific aggregation of PrPSc into a structure with the pharmacological properties required for the biology of the infectious process.

Although it is not impossible that Me2SO caused a profound change in the structure of part of the PrPSc molecules, the fact that Me2SO-soluble PrPSc could only be generated from aggregates and not from preformed rods is more consistent with the possibility of more than one prion-specific PrPSc existing before the addition of Me2SO and the extraction of scrapie brain membranes with detergents. Regardless of the mechanism, our results show that not all PK-resistant PrP molecules are associated with prion infectivity.

Me2SO-soluble PrPSc species, although not infectious, could still play an important role in the neuropathology of prion diseases. At the last stages of the disease, when the load of total PrPSc molecules in the brain is large, non-infectious PrPSc molecules may replace most of the PrPSc molecules or otherwise inhibit their normal function. A large load of non-infectious PrPSc molecules may also be neurotoxic or contribute to brain degeneration in as yet unknown mechanisms.

REFERENCES
1. Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., Barry, R. A., Tempst, P., Teglov, D. B., Hood, L. E., Prusiner, S. B., and Weismann, C. (1985) Cell 40, 735–746
2. Prusiner, S. B. (1998) Brain Pathol. 8, 499–513
3. Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E., and Prusiner, S. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10962–10966
