Purified prion proteins and scrapie infectivity copartition into liposomes

(slow infections/phospholipid vesicles/amyloid/light scattering/brain degeneration)

RUTH GABIZON*, MICHAEL P. MCKINLEY*, AND STANLEY B. PRUSINER*†‡

Departments of *Neurology and †Biochemistry and Biophysics, University of California, San Francisco, CA 94143

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ABSTRACT Considerable evidence indicates that the scrapie prion protein (PrP 27-30) is required for infectivity. Aggregates of PrP 27-30 form insoluble amyloid rods that resist dissociation by nondenaturing detergents. Mixtures of the detergent cholate and phospholipids were found to solubilize purified PrP 27-30 in the form of detergent-lipid-protein complexes. Removal of the cholate by dialysis resulted in the formation of closed liposomes. Both the detergent-lipid-protein complexes and the liposomes often but not always exhibited a 10-fold increase in scrapie infectivity compared to that observed with the rods. No evidence for a prion-associated nucleic acid could be found when the phospholipid vesicles containing PrP 27-30 were digested with nucleases and Zn^{2+} under conditions that allowed hydrolysis of exogenously added nucleic acids. No filamentous or rod-shaped particles were found amongst prion liposomes by electron microscopy in our search for a putative filamentous "scrapie virus." The partitioning of PrP 27-30 and scrapie infectivity into phospholipid vesicles contends that PrP 27-30 has a central role in scrapie pathogenesis, establishes that the prion amyloid rods are not essential for infectivity, and argues that prions are fundamentally different from viruses.

The integral membrane sialoglycoprotein, prion protein (PrP) 27-30, is the only identifiable macromolecule in purified preparations of infectious particles or prions causing scrapie (1-3). PrP 27-30 is derived from a larger protein, designated PrP^{Sc}, by proteinase K digestion (4-7). The primary structure of PrP deduced from a cDNA suggested (5), and cell-free translation studies established (8), that PrP is a transmembrane protein. Recent studies showed that rod-shaped particles are produced by detergent extraction of scrapie-infected brain membranes (4). This observation links our studies (2, 9) and those of others (10) with earlier investigations in which scrapie infectivity was reported to be intimately associated with membranes (11, 12).

Morphologic investigations of the prion rods established that they are ultrastructurally and histochemically indistinguishable from amyloid (2, 13). PrP antibodies were used to demonstrate that PrP^{Sc} molecules aggregate into filamentous polymers during scrapie infection, and these extracellular filaments coalesce to form amyloid plaques (6, 13, 14). Some investigators have suggested that the prion amyloid rods found in purified preparations are the same as scrapie-associated fibrils (10); however, the fibrils have been distinguished repeatedly from amyloids based on their ultrastructure and histochemical properties (15-17).

PrP 27-30 aggregation into prion amyloid rods and its protease resistance are the basis of a purification procedure which yields highly purified prions (2). However, the formation of the rods created another problem; they could not be

solubilized in nondenaturing detergent. Discovering conditions for functional solubilization of PrP^{Sc} with retention of associated scrapie prion infectivity would greatly facilitate studies defining the molecular structure of the infectious particle. We now report a procedure whereby the prion rods are dissociated by using a mixture of phospholipids and nondenaturing detergent with full retention of scrapie infectivity.

MATERIALS AND METHODS

Source, Bioassay, and Purification of Scrapie Prions. Hamster-adapted scrapie prions (18) were assayed by the incubation-time-interval procedure (19). Purification of scrapie prions from infected hamster brains was accomplished as described (2). Purified samples contain primarily one protein, PrP 27-30, as judged by NaDodSO₄/polyacrylamide gel electrophoresis (20).

Production of Liposomes. Sodium cholate was purchased from Sigma. Egg phosphatidylcholine (PtdCho) was obtained from Avanti Biochemicals. Purified prions from sucrose gradient fractions (30 µg/ml) were precipitated with methanol and resuspended to a final protein concentration of 50 µg/ml in a buffer containing 10 mM Hepes sodium salt (pH 7.4) and 100 mM NaCl. The only protein identifiable by silver staining of NaDodSO₄/polyacrylamide gel electrophoresis was PrP 27-30, which was polymerized into rods. The resuspended rods (100 µg of protein) were mixed with 2% (wt/vol) sodium cholate (pH 7.4) and then added to a Corex glass 30-ml test tube containing dried PtdCho (10 mg). The sample was mixed, sonicated, and centrifuged at 31,000 × g for 25 min. This sample was called D-L-P complex (detergent-lipid-protein complex). Liposomes were formed from the D-L-P complexes by dialysis against detergent-free buffer.

Polyacrylamide Gel Electrophoresis. Samples were precipitated upon chloroform/methanol, 1:2 (vol/vol), extraction and resuspended in a buffer containing 1% NaDodSO₄, 5% 2-mercaptoethanol, and bromphenol blue. Samples were boiled for 3 min and electrophoresed into NaDodSO₄/15% polyacrylamide gels (20). Protein was detected by silver staining (21).

Nucleic Acid Digestion and Hybridization. Exogenous nucleic acids were added to D-L-P complexes to measure the efficiency of various digestion methods. To obtain sufficient sensitivity, a slot-blot-hybridization assay was used. For DNA, a cloned PrP cDNA open reading frame insert (22) was added; for RNA, brain poly(A)⁺ RNA was used. After digestion, the remaining PrP DNA or RNA sequences were hybridized to a ³²P-labeled PrP cDNA probe (22). For a prion

titer of $\approx 10^{8.5}$ ID₅₀ per ml in the D-L-P complex preparation, we added 10 pg of PrP cDNA to 50 μ l of the D-L-P complexes to give one DNA molecule per ID₅₀ unit. For poly(A)⁺ RNA, 10^4 times more RNA was added because PrP mRNA comprises only $\approx 0.01\%$ of poly(A)⁺ RNA. After addition of control DNA or RNA to the D-L-P complexes, the samples were mixed briefly in a bath sonicator, digested for 24 hr, and blotted onto nitrocellulose. For DNA analysis, these samples were dissolved in 400 μ l of 10 mM Tris-HCl, pH 7.4/1 mM EDTA. After the addition of 0.1 volume of 3 M NaOH, the samples were incubated at 65°C for 30 min. The samples were chilled and, after the addition of 400 μ l of 2 M ammonium acetate, were loaded under vacuum onto nitrocellulose prewetted with 1 M ammonium acetate (23). For RNA analysis, ethanol-precipitated samples were dissolved in 1.5 M NaCl/0.15 M sodium citrate/6.1 M formaldehyde, incubated at 65°C for 15 min, chilled, and loaded under vacuum onto nitrocellulose prewetted with 1.5 M NaCl/0.15 M sodium citrate (24). In both cases, a slot manifold was used for application of samples. Autoradiograms were scanned with a densitometer, and the values were expressed as a percentage of the undigested control.

Electron Microscopy and Light Scattering. Grids were coated with polylysine (25), and samples were negatively stained with uranyl formate as described (2). Photomicrographs were taken using a JEOL 100B electron microscope set at 80 keV. Tobacco mosaic virus (TMV) was a gift from Robley Williams. The concentration of virus was determined spectrophotometrically: one $A_{260} = 0.6 \times 10^{13}$ virions per ml.

The sizes of the particles (liposomes and rods) were measured by dynamic light scattering in a model NY, Coulter Electronics, laser particle analyzer. The results are expressed by differential weight.

RESULTS

To solubilize PrP 27-30, a hydrophobic environment is needed to replace that provided by the rod structure. Phospholipids are the natural environment for a membrane protein and were used as the needed "detergent" in order to solubilize PrP 27-30. PrP 27-30, which had polymerized into rods, was sedimented completely by low-speed centrifugation even in the presence of detergent (Fig. 1). In contrast, most of the PrP 27-30 did not sediment in the presence of PtdCho, even with ultracentrifugation ($170,000 \times g$ for 30 min). The microheterogeneity of PrP 27-30 shown in Fig. 1 appears to arise, at least in part, from N-linked oligosaccharides attached to the protein (1, 5, 26, 27). The lightly stained proteins of 23- to 26-kDa molecular mass are probably smaller PrP molecules generated during proteinase K digestion as judged by immunoblotting studies (2, 6, 7, 26, 28, 29).

The partitioning of PrP 27-30 between the pellet and the supernatant depends on several factors. Prolonged sonication during formation of detergent-lipid-protein (D-L-P) complexes resulted in smaller-sized aggregates of lipids and more protein in the supernatant. The ratio of lipid to protein needed to solubilize PrP 27-30 is an important factor. When the molar ratio of lipid molecules to protein molecules was more than 4000:1, most of the PrP 27-30 was solubilized. At lower molar ratios, the solubilization diminished in proportion to the decrease of the molar ratio. At a molar ratio of 4000:1, no rods were found in the $170,000 \times g$ pellet by electron microscopy. We attribute the small amount of PrP 27-30 found in this fraction to large lipid-protein aggregates, which were sedimentable even though the rods were fully disrupted (Fig. 1). When we removed much of the detergent by dialysis, we obtained liposomes containing PrP 27-30.

Electron micrographs of prion rods and PrP 27-30 liposomes revealed striking differences in structure and size; yet both were associated with high levels of infectivity (Fig. 2).

FIG. 1. Incorporation of PrP 27-30 into phospholipid vesicles. Analysis by sedimentation and NaDodSO₄/polyacrylamide gel electrophoresis. Purified PrP 27-30 (10 ml; 30 μ g/ml) in 50% (wt/vol) sucrose was concentrated by precipitation with methanol. The precipitate was dried and resuspended in 4 ml of buffer containing 10 mM Hepes sodium salt (pH 7.4) and 100 mM NaCl. The suspension was divided into four equal aliquots, and the sedimentation behavior of PrP 27-30 was analyzed by subjecting these aliquots to differential centrifugation. The concentration of PrP 27-30 in the supernatant and pellet fractions was assessed by NaDodSO₄/polyacrylamide gel electrophoresis. The gels were stained with silver. Aliquots A, B, and C were centrifuged at $21,000 \times g$ for 25 min; pellet fractions are in odd-numbered lanes (1, 3 and 5) and supernatant fractions are in even-numbered lanes (2, 4 and 6). Aliquot A was the control and received no further treatment. Aliquot B received sodium cholate to a final concentration of 2% (pH 7.4). Aliquot C received sodium cholate and then was added to a glass test tube containing 30 mg of PtdCho. The tube was mixed for 10 sec (or until no more lipid was observed on the test tube wall) and then sonicated in a cylindrical bath sonicator until the solution was transparent (around 15 min). The fourth aliquot (D) was prepared in the same manner as aliquot C; the $21,000 \times g$ pellet fraction is shown in lane 7. The supernatant fraction of aliquot D was subjected to an additional centrifugation at $170,000 \times g$ for 30 min. The pellet fraction from the $170,000 \times g$ centrifugation is in lane 8, while the supernatant fraction is in lane 9. Molecular masses are shown in kDa.

The rods measured 10–20 nm in diameter and 100–200 nm in length, while the average diameter of the liposomes was about 20 nm. Laser light-scattering measurements of particle sizes (30) confirmed those determined by electron microscopy. Thus, the rods cannot be hidden inside the liposomes. To determine the average number of PrP 27-30 molecules in a liposome, we calculated the number of PtdCho molecules per 20-nm liposome. Based on published data (31), there are ≈ 4000 PtdCho molecules per liposome. Phospholipids were measured by the ammonium ferrothiocyanate method (32), and protein, by the bicinchoninic acid (BCA) procedure (33). We estimated the phospholipid/protein ratio to be between 1000:1 and 2000:1; thus, the average number of PrP 27-30 molecules per liposome is between two and four. This estimate of between two and four PrP 27-30 molecules per liposome is in accord with earlier ionizing radiation and size-exclusion chromatography studies suggesting that the smallest infectious unit of the scrapie agent has a molecular weight of $<10^5$ (34, 35).

Bioassays in hamsters showed that scrapie prion infectivity is retained when amyloid rods composed of PrP 27-30 undergo dissociation, and the protein is incorporated into phospholipid vesicles (Table 1). We observed often, but not

FIG. 2. Electron microscopy and dynamic light scattering of scrapie prion rods and liposomes. (A) Typical prion rods negatively stained with uranyl formate. (Bar = 100 nm.) (B) Light-scattering profile of rods determined with laser particle analyzer (model NY, Coulter Electronics). (C) Negatively stained liposomes containing PrP 27-30. (D) Light-scattering profile of liposomes.

always, that prion titers rose ≈ 10 -fold when PrP 27-30 was dispersed into liposomes.

The transfer of scrapie prion infectivity from rods to liposomes provided a new method by which we could search for a hidden or cryptic nucleic acid within the prion. Treatment of D-L-P complexes as well as liposomes with nucleases or Zn^{2+} failed to alter scrapie infectivity (Table 2). In control experiments, nucleic acids were added to D-L-P complexes at a concentration of one molecule per infectious unit. Under the conditions of our experiments, nucleases or Zn^{2+} degraded the exogenously added DNA or RNA molecules to $<1\%$ of their initial concentration (Table 3). In contrast to the hydrophobic PrP, polynucleotides are water soluble and would be expected to be excluded, largely if not entirely, from the phospholipid bilayer; thus, they should be accessible to nuclease or Zn^{2+} -catalyzed hydrolysis.

We have considered the possibility that a small virus having a particle-to-infectivity ratio of unity is hiding in our preparations (5) and that most or even all of the PrP 27-30 is unassociated with the virus. Let us assume, as some inves-

tigators have done, that a filamentous virus is responsible for scrapie infectivity (15) and it is hidden amongst the PrP 27-30 amyloid rods. Then, upon dissolving the rods into liposomes, we would expect to visualize the putative filamentous "scrapie virus"; however, no elongated particles were found. As a control, TMV at a concentration of $10^{6.7}$ virions per ml was added to prion liposomes having infectivity titers of $\approx 10^{7.5}$ or $\approx 10^{8.5}$ ID₅₀ per ml. While the TMV was observed by electron microscopy, no other elongated structures were seen (Fig. 3A). The efficacy of TMV binding to electron microscopy grids was the same whether it was performed in the presence or absence of liposomes containing PrP 27-30

Table 2. Resistance of scrapie prion infectivity in phospholipid vesicles to inactivation by nucleases or Zn^{2+}

Treatment	Log titer, ID ₅₀ per ml $\pm$ SEM		
	Rods	D-L-P complex	Liposomes
Control	7.7 $\pm$ 0.3	8.7 $\pm$ 0.1	9.0 $\pm$ 0.8
DNase I (100 μ g/ml)	7.2 $\pm$ 0.2	8.8 $\pm$ 0.2	8.9 $\pm$ 0.2
RNase A (100 μ g/ml)	7.4 $\pm$ 0.3	9.5 $\pm$ 0.2	8.7 $\pm$ 0.2
MN (12.5 units/ml)	7.8 $\pm$ 0.3	8.8 $\pm$ 0.3	8.7 $\pm$ 0.1
Zn^{2+} (2 mM)	7.8 $\pm$ 0.2	8.6 $\pm$ 0.2	8.5 $\pm$ 0.2

Fractions were digested with nucleases (MN = micrococcal nuclease) for 24 hr at 37°C or incubated with $ZnSO_4$ for 24 hr at 65°C. In control experiments, exogenous nucleic acids were added at concentrations of one molecule per ID₅₀ to establish that the nucleases and Zn^{2+} hydrolyzed these molecules in the presence of D-L-P complexes. For convenience, ^{32}P -labeled PrP cDNA probes were used to assay the degradation of exogenously added PrP cDNA or poly(A)⁺ RNA by using slot blots as described (5). After nuclease or Zn^{2+} -catalyzed hydrolysis of the nucleic acids, $<1\%$ of the exogenously added nucleic acid could be detected by hybridization.

Table 1. Scrapie prion infectivity in phospholipid vesicles

	Log titer*, ID ₅₀ per ml $\pm$ SEM		
	Exp. 1	Exp. 2†	Exp. 3
Rods	7.7 $\pm$ 0.3	6.6 $\pm$ 0.3	7.9 $\pm$ 0.2
D-L-P complex	8.7 $\pm$ 0.1	7.8 $\pm$ 0.1	8.9 $\pm$ 0.2
Liposomes	9.0 $\pm$ 0.8	7.1 $\pm$ 0.2	8.7 $\pm$ 0.1

*Inocula for bioassay were prepared by diluting 10 μ l of the above preparations into 0.99 ml of phosphate-buffered saline containing 9.5% (vol/vol) bovine serum albumin.

†The protein concentrations for the rods, D-L-P complexes, and liposomes were 25, 12, and 18 μ g/ml, respectively.

Table 3. Degradation of control nucleic acids added to D-L-P complexes

Digestion method	% of control nucleic acid	
	DNA	RNA
DNase I	0.8	
RNase A		<0.1
Zn ²⁺		<0.1
Micrococcal nuclease	0.9	<0.1

DNA or RNA was added after precipitation of the rods, and D-L-P complexes containing PrP 27-30 were produced as described. For DNA, we used a cDNA clone of PrP, and for RNA, we used total poly(A)⁺ RNA. The concentration of DNA or RNA was adjusted to obtain one PrP nucleic acid per ID₅₀ unit. Digestion conditions were the same as those described in Table 2. The amount of nucleic acid was measured by hybridization with a ³²P-labeled PrP cDNA probe.

molecules. If a filamentous or rod-shaped "scrapie virus" were hidden amongst the prion amyloid rods, then at least one of these putative virions would be present for each ID₅₀ unit—i.e., a particle-to-infectivity ratio of unity. Numerous TMV were seen amongst the liposomes when added at a concentration of 10^{8.5} virions per ml—i.e., one virion per prion ID₅₀ unit (Fig. 3B). Our experimental results show that scrapie infectivity does not require a filamentous or rod-shaped particle, a finding in accord with earlier studies (36). However, the possibility remains that the putative scrapie virus did not bind to the grid under the conditions of the experiment even though the PrP 27-30 liposomes and TMV did bind.

DISCUSSION

The incorporation of PrP 27-30 and scrapie prion infectivity into phospholipid vesicles provides compelling evidence for

PrP 27-30 as a component of the infectious scrapie prion. The results from six different experimental approaches contend that PrP 27-30 is required for scrapie infectivity. First, PrP 27-30 and scrapie prions copurify, indicating that their physical properties are similar; PrP 27-30 is the most abundant macromolecule in purified prion preparations (2). Second, the concentration of PrP 27-30 is proportional to scrapie prion titer (2, 9, 37). Third, hydrolysis or denaturation of PrP 27-30 results in a diminution of prion titer (28, 37). Fourth, the murine PrP gene (*Prn-p*) on chromosome 2 (38) is linked to a gene (*Prn-i*) controlling the length of the scrapie incubation time (39). Prolonged incubation periods are a cardinal feature of scrapie infection. Fifth, PrP 27-30 is specific for prion diseases. It has been found only in transmissible degenerative neurologic disorders (28, 40–42). Sixth, the studies reported here show that nondenatured PrP 27-30 and scrapie prion infectivity partition together—both may exist as rods, D-L-P complexes, and liposomes.

Recent molecular cloning studies have shown that the prion protein is encoded by a cellular gene and not by a nucleic acid within the infectious particle (5, 43). Dissociation of the rods with retention of infectivity allowed us to extend our search for a prion nucleic acid; however, no evidence for a polynucleotide could be found by digesting phospholipid vesicles containing PrP 27-30 with nucleases or Zn²⁺ (Table 2). Although liposomes have been used to protect nucleic acids by entrapping them within vesicles, this is not the case in the presence of detergent where D-L-P complexes or fragments of vesicles are formed. The results described here (Table 2) are in accord with earlier studies, all of which have failed to provide evidence for a nucleic acid moiety within the infectious prion particle (35, 44–47); however, it can be argued that the putative prion nucleic acid remains protected from nucleases and Zn²⁺ even during the transfer of PrP 27-30 and infectivity from rods to phospholipid vesicles.

FIG. 3. Search for filamentous or rod-shaped viruses in scrapie prion preparations. TMV was added to prion liposomes. (A) Prion titer, $\approx 10^{7.5}$ ID₅₀ per ml; TMV concentration, $10^{6.7}$ virions per ml. (B) Prion titer, $\approx 10^{8.5}$ ID₅₀ per ml; TMV concentration, $10^{8.5}$ virions per ml. All preparations were spread onto polylysine-coated grids and stained with uranyl formate for electron microscopy. (Bars = 100 nm.)

The copartitioning of PrP 27-30 and scrapie infectivity into many different molecular forms argues that PrP 27-30 is a component of the infectious prion. Presumably, hydrophobic interactions are the driving force for the partitioning of PrP 27-30, which is an integral membrane protein, into lipid bilayers or into rods when lipids are removed either by detergent or organic solvent extraction. This copartitioning of PrP 27-30 and scrapie prion infectivity is clearly reversible, is independent of exogenous energy sources such as ATP, and does not require divalent cations. The extreme variation in the size of the scrapie agent has presented a puzzling dilemma for many years (2, 34, 35), and while hydrophobic interactions were suggested as the mechanism (48), strong experimental evidence to support this hypothesis has been lacking until now. The changes in both size and morphology of particles, reported here (Fig. 2), for purified prion preparations are uncharacteristic of viruses.

Our studies clearly demonstrate the existence and inter-conversion of multiple molecular forms of scrapie prions. The functional solubilization of PrP 27-30 and scrapie infectivity into phospholipid vesicles should facilitate analysis of the molecular components of the prion as well as the mechanism of prion replication.

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