Structural Studies of the Scrapie Prion Protein Using Mass Spectrometry and Amino Acid Sequencing†

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ABSTRACT: The only component of the infectious scrapie prion identified to date is a protein designated PrP^{Sc}. A posttranslational process converts the cellular PrP isoform (PrP^{C}) into PrP^{Sc}. Denatured PrP^{Sc} was digested with endoproteases, and the resulting fragments were isolated by HPLC. By both mass spectrometry and Edman sequencing, the primary structure of PrP^{Sc} was found to be the same as that deduced from the PrP gene sequence, arguing that neither RNA editing nor protein splicing feature in the synthesis of PrP^{Sc}. Mass spectrometry also was used to search for posttranslational chemical modifications other than the glycosylinositol phospholipid anchor attached to the C-terminus and two Asn-linked oligosaccharides already known to occur on both PrP^{Sc} and PrP^{C}. These results contend that PrP^{Sc} molecules do not differ from PrP^{C} at the level of an amino acid substitution or a posttranslational chemical modification; however, we cannot eliminate the possibility that a small fraction of PrP^{Sc} is modified by an as yet unidentified posttranslational process or that PrP^{C} carries a modification that is removed in the formation of PrP^{Sc}. It seems likely that PrP^{Sc} differs from PrP^{C} in its secondary and tertiary structure, but the possibility of a tightly bound, disease-specific molecule which purifies with PrP^{Sc} must also be considered.

Studies designed to assess the molecular composition of the transmissible pathogen causing scrapie by UV and ionizing radiation gave results which led to the unorthodox suggestion that scrapie agent infectivity might replicate in the absence of a nucleic acid (Alper et al., 1966, 1967, 1978). Reaction to this proposal was mixed, leading, on one hand, to a variety of structural hypotheses about the composition of the infectious scrapie particle (Hunter, 1972; Pattison, 1988) and, on the other, to considerable skepticism about the interpretation of the irradiation results (Rohwer, 1986). Most investigators argued that the scrapie agent ought to have a nucleic acid genome because (i) this pathogen behaved like a virus or possibly a viroid and (ii) "strains" or distinct isolates of the pathogen exhibited different biological properties such as incubation times and patterns of CNS vacuolar lesions (Bruce & Dickinson, 1987; Dickinson et al., 1968, 1984). Despite continuing attempts to modify, identify, or clone scrapie-specific nucleic acids from infectious fractions (Kellings et al., 1992; Meyer et al., 1991; Oesch et al., 1988; Prusiner, 1991), no candidate polynucleotide has emerged.

To distinguish the infectious pathogen causing scrapie from viruses, viroids, and microorganisms, the term "prion" was proposed (Prusiner, 1982). To date, the only component of the scrapie prion that has been identified is the scrapie isoform of the prion protein designated PrP^{Sc}. Both PrP^{Sc} and the cellular isoform of the prion protein designated PrP^{C} are encoded by a chromosomal gene (Basler et al., 1986). PrP^{Sc} is specific for scrapie and related neurodegenerative disease of animals and humans (Bockman et al., 1985; Bolton et al., 1982; Brown et al., 1986; Hope et al., 1988b; Prusiner et al., 1984). The physical properties of PrP^{Sc} that distinguish it from PrP^{C} have been described previously; for example, PrP^{C} is soluble in detergents and disappears upon digestion with proteinase K (Meyer et al., 1986; Oesch et al., 1985). While limited proteolysis of PrP^{Sc} yields a protease-resistant core designated PrP 27–30, this polypeptide aggregates into amyloid rods in the presence of detergents (McKinley et al., 1991a).

Related prion diseases of humans are kuru, Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), and fatal familial insomnia (FFI) (Gajdusek, 1977; Gajdusek et al., 1966; Gibbs et al., 1968; Masters et al., 1981; Medori et al., 1992). Familial CJD, GSS, and FFI are autosomal dominant disorders caused by mutations in the PrP gene, yet extracts of brain and other tissues from afflicted individuals transmit disease to experimental animals (Hsiao et al., 1989, 1991a,b, 1992; Masters et al., 1981; Medori et al., 1992). At least 12 mutations of the prion protein gene (PRNP) on chromosome 20 (Sparkes et al., 1986) have been found to segregate with the inherited human prion diseases (Collinge et al., 1989, 1992; Doh-ura et al., 1989; Goldfarb et al., 1989, 1990, 1991a,b, 1992; Owen et al., 1989, 1992). Some investigators have chosen to explain these findings by claiming that a ubiquitous scrapie "virus" exists which rarely infects animals and humans except if they have a mutation in their prion protein which functions as a receptor for the putative "virus" (Chesebro, 1992; Kimberlin, 1990). A more likely scenario seems to involve the spontaneous conversion

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of mutant PrP^C into PrP^{Sc} which then catalyzes the conversion of more PrP^C into PrP^{Sc}. Genetic linkage between PRNP mutations and development of inherited prion diseases as well as a wealth of experimental data derived from studies with laboratory rodents argues in favor of the latter hypothesis (Prusiner, 1991).

Two findings suggested that PrP^{Sc} might arise from PrP^C or a precursor through a posttranslational process: (i) only one PrP mRNA was found in normal and scrapie-infected brains, and its level did not change throughout the course of scrapie infection (Oesch et al., 1985), and (ii) the entire open reading frame of the PrP gene was found within a single exon, allowing no possibility for alternatively spliced species of PrP (Basler et al., 1986). These observations led to studies designed to identify and characterize the posttranslational modifications of PrP^C and PrP^{Sc}. Studies with scrapie-infected cultured cells have provided evidence that the conversion of PrP^C or a precursor into PrP^{Sc} is a posttranslational process (Borchelt et al., 1990) that is likely to occur in the endocytic pathway (Borchelt et al., 1992; Caughey & Raymond, 1991; Caughey et al., 1991a; McKinley et al., 1991b; Taraboulos et al., 1990b, 1992). It is currently unknown whether the synthesis of PrP^{Sc} through a posttranslational process represents a chemical modification, a conformational change, or the binding of an as yet unidentified second component of the infectious prion.

Since considerable evidence argues that infectious prions are composed largely, if not entirely, of PrP^{Sc} molecules and a central event in prion replication is the synthesis of PrP^{Sc}, we set out to identify all of the posttranslational modifications of PrP^{Sc}. The study reported here was designed to determine whether a posttranslational chemical modification might distinguish PrP^{Sc} from PrP^C, and thus feature in prion replication and pathogenicity. Every peptide expected to arise by endoproteinase Lys-C digestion of PrP^{Sc} was identified after purification by reverse-phase HPLC and then analyzed by Edman sequencing and mass spectrometry. Quantitative amino acid analysis of the purified fractions provided an estimate of recovery, which was 65–90% for most peptides. We conclude from these analyses that the amino acid sequence of PrP^{Sc} is identical to that anticipated from the translated PrP gene sequence. Although it is conceivable that a small fraction of the PrP^{Sc} that purifies with scrapie prion infectivity is chemically modified in a unique fashion, the findings of this study and data on the secondary structure of PrP 27-30 (Gasset et al., 1993) make it seem likely that PrP^{Sc} and PrP^C differ by conformation or by the presence of some tightly bound molecules.

MATERIALS AND METHODS

All procedures for the purification of PrP 27–30 and PrP^{Sc} from the brains of Syrian golden hamsters (Prusiner et al., 1983; Turk et al., 1988) as well as the reduction, alkylation, digestion with endoproteinase Lys-C, treatment with phosphatidylinositol-specific phospholipase C (PIPLC), precipitation of sodium dodecyl sulfate (SDS) with 6 M guanidine hydrochloride, reverse-phase high-performance liquid chromatographic (HPLC) separation of the peptides, capillary electrophoresis (CE), amino acid analysis (AAA), and mass spectrometric analysis by liquid secondary ion mass spectrometry (LSIMS) and electrospray mass spectrometry (ESMS) have been described previously (Baldwin et al., 1990b,c; Stahl et al., 1990c, 1992). LSIMS was carried out using a Kratos MS50 double-focusing sector mass spectrometer, and ESMS was carried out using a VG Biotech Bio-Q quadrupole instrument. All HPLC separations in this study used the standard conditions and gradient described before: buffer A, 0.06% trifluoroacetic acid (TFA); buffer B, 80% acetonitrile/0.052% TFA, program 100% A for 10 min and then 0–50% B at 1%/min, 2-mL injection loop, flow rate 0.75 mL/min, column 25 × 4.6 cm Vydac RP-18, UV detection at 214 and 280 nm (Stahl et al., 1990c). AAA used 6 N HCl hydrolysis in vacuo in the presence of phenol at 110 °C for 18 h followed by *o*-phthalaldehyde (OPA) (Fluoroaldehyde, Pierce) derivatization and HPLC analysis (Stahl et al., 1990c). CE was carried out using a Beckman P/ACE instrument with 100 mM phosphate buffer, pH 2.5, in a 50 cm × 50 μm open tubular column. Detection was by UV absorbance at 200 nm. Edman sequencing was carried out by procedures that have been described before (Turk et al., 1988). Amino-terminal derivatization of K7 used a modification of a previous method (Stultz et al., 1989) which was applied to the unretained fraction from the HPLC separation of the endoproteinase Lys-C digest. Unretained fractions (0.45 mL) containing guanidine hydrochloride (GdnHCl) were brought to pH 6 by the addition of 10 mM 2-(*N*-morpholino)-ethanesulfonic acid and incubated with 2 μL of freshly prepared 300 mM iodoacetic anhydride for 12 min at room temperature. The solution was brought to pH 8 by the addition of 8.3 μL of 3 M Tris-HCl, pH 8.5, and incubated with 10 μL of 400 mM thiocholine iodide for 2 h, which displaced the iodide to form an *N*-(thiocholinyl)acetyl derivative. The reaction was terminated by injection into the HPLC which gave a peak that eluted at 10% acetonitrile and was analyzed by AAA and LSIMS. Purified K8 was subdigested with sequencing-grade trypsin (Boehringer Mannheim) in 50 mM Tris, pH 8.0, for 2.5 h at 37 °C, and the products were separated by HPLC. The C-terminal fragment formed by limited digestion eluting at 15% acetonitrile was further digested with endoproteinase Asp-N (Boehringer Mannheim) in 50 mM Tris, pH 8.0, for 20 h at 37 °C, and the products were separated by HPLC. In separate experiments, K8 or the C-terminal peptide from limited tryptic digestion of K8 was treated with peptide *N*-glycosidase-F (PNGase) in 50 mM Tris, pH 8.6, and 1% octyl β-glucoside for 20 h at 37 °C and repurified by HPLC. Purification and analysis of K12 and the removal of the glycosylinositol phospholipid (GPI) anchor by incubation with 50% aqueous HF have been described previously (Baldwin et al., 1990b,c; Stahl et al., 1990c). Radioiodination of prion rods was carried out by derivatizing with 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID) by partitioning into the hydrophobic component and irradiating with ultraviolet light to form a highly reactive carbene (Rosenberry et al., 1986).

RESULTS

Endoproteinase Lys-C Digestion of PrP 27–30. Digestion with endoproteinase Lys-C was used to generate peptides from PrP 27–30 and PrP^{Sc} for analysis. The nucleotide sequence for PrP predicts 11 lysines which in theory could result in 12 peptides, referred to here as K1 through K12 (Figure 1). There were several goals for this analysis: to examine as large a fraction of the PrP molecules as possible; to perform few separations once the scrapie infectivity was lost by denaturation to prevent losing a modified fraction of the protein; to achieve as complete a digest as possible so as to simplify the chromatogram and the quantitation of the peptide recovery, as well as to limit the number of large peptides to be analyzed; and to establish procedures for the proteolytic digestion of 2–10 nmol (<300 μg) of PrP 27–30 or PrP^{Sc} in volumes of <2 mL. Both PrP^{Sc} and PrP 27–30 are relatively insoluble and resistant to protease digestion; therefore, several different sets of conditions were examined for denaturation and

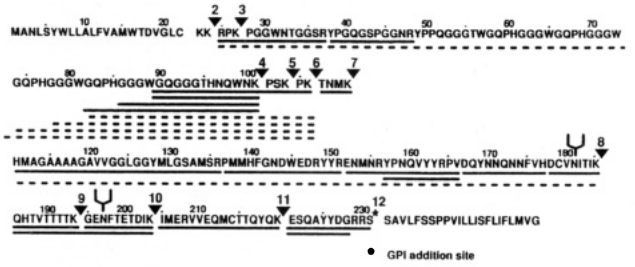

FIGURE 1: Peptides from PrP^{Sc} and PrP 27–30 confirmed by mass spectrometry. Underlined portions signify peptides that have been identified by LSIMS (solid line) or ESMS (dashed line). The arrows indicate the potential cleavage sites for endo Lys-C, which in theory can give rise to the peptides K1–K12. N-Terminal and C-terminal signal sequences of 22 and 23 amino acids, respectively, are not found in the mature protein.

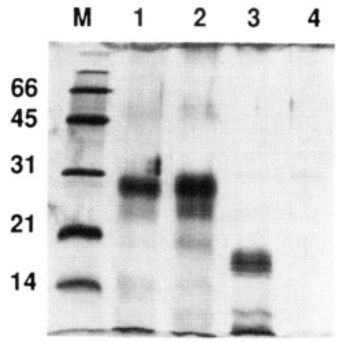

FIGURE 2: Silver-stained 12% gel from PAGE (Laemmli, 1970) of endoproteinase Lys-C digest of PrP 27–30. M signifies molecular weight markers of the indicated size ($\times 10^{-3}$) (Bio-Rad). Lane 1, infectious prions boiled in sample buffer. Lane 2, sample after denaturation in GdnHCl and carboxymethylation. Lane 3, sample following overnight digestion with endoproteinase Lys-C. Lane 4, endoproteinase Lys-C alone. The prominent triplet in lane 3 represents the glycosylated K8 peptide.

solubilization of the preparation. The most effective procedure involved denaturation (with concomitant loss of scrapie infectivity) in 6 M GdnHCl, cleavage of disulfide bonds with dithiothreitol, alkylation of cysteines with iodoacetate, precipitation from the GdnHCl with 20 volumes of ethanol, and solubilization of the pellet in 0.1% SDS. Following this procedure, endo Lys-C digestion in 0.1% SDS routinely gave complete disappearance of PrP 27–30 as determined by SDS–PAGE with the accompanying appearance of the glycosylated triplet of the K8 peptide migrating with $M_r \sim 16K$ (Figure 2). Alternative procedures that omitted carboxymethylation, or simply employed boiling SDS or urea denaturation, resulted in only partial disappearance of PrP 27–30 along with the accumulation of larger, partially digested intermediates (not shown). This is consistent with the observation that boiling SDS is only partially effective at reducing scrapie infectivity, leaving a fraction of aggregated PrP 27–30 in the SDS–PAGE stacking gel (Brown et al., 1990; Prusiner et al., 1980). Similarly, incomplete digestion or poor solubilization of PrP 27–30 was observed if SDS was substituted with other detergents such as deoxycholate, *N*-laurylsarcosine, Nonidet P-40 (NP-40), or octyl β-glucoside (not shown). Fortunately, PIPLC proved to be completely effective at cleaving the diradylglycerol from the GPI anchor in a solution containing 0.1% SDS (Stahl et al., 1990c), thereby making the C-terminal peptide GPI amenable to separation by HPLC.

Peptides generated by endo Lys-C were separated from SDS by precipitation of the latter with excess GdnHCl (Shivley, 1986) and separated by reverse-phase HPLC to give chromatograms such as those shown in panels A and B of

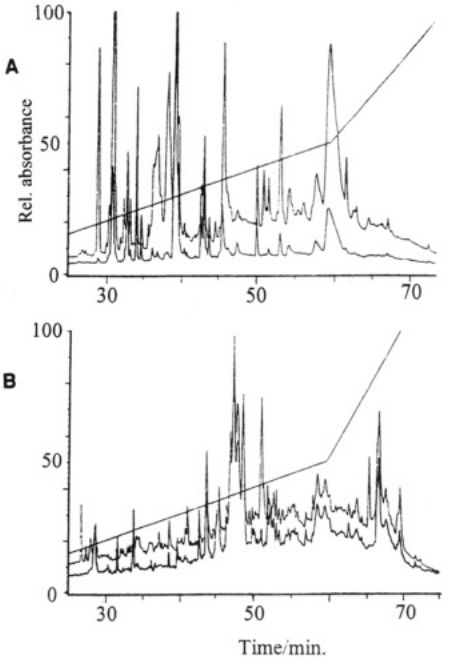

FIGURE 3: Reverse-phase HPLC of endoproteinase Lys-C digested prion proteins: (A) PrP 27–30 or (B) PrP^{Sc}. The gradient lines indicate % B (80% acetonitrile). Each chromatogram shows the absorbance at 214 nm (upper trace, sensitivity normalized to 100% = FSD) and 280 nm (lower trace, sensitivity 5× greater).

Figure 3 for PrP 27-30 and PrP^{Sc}, respectively. SDS is well-known to be incompatible with reverse-phase HPLC purification of peptides, probably by coating the stationary phase with negative charge to give a mixed-phase separation. Although the preparations of PrP^{Sc} were somewhat less pure than those for PrP 27–30 as the former could not be purified by selective proteolysis, differences in the chromatograms should reveal the elution positions of the respective N-terminal peptides, which differ after truncation of PrP 27–30 with proteinase K. Accordingly, several peaks migrating between 30 and 35 min in Figure 3A corresponding to N-terminal fragments of PrP 27–30 are missing in Figure 3B, while a tight group of prominent new peaks from the N-terminus of PrP^{Sc} is observed at 45 min (Figure 3B). These peaks have intense absorbance at 280 nm, which would be expected for the tryptophan-rich K4 peptide (Figure 1). An indication of the difference in the purity of PrP 27–30 and PrP^{Sc} is given by the large broad peak centered at 59–60 min, the signal: noise ratio being considerably better for the digest of PrP 27–30 which contains the K8 peptide (see below). Variability in the recovery of this peak probably resulted from trapping of the relatively hydrophobic peptide in the guanidinium dodecyl sulfate (GdnDS) pellet that forms upon precipitation of the dodecyl sulfate. In one experiment, K8 was recovered from the pellet after removal of the GdnDS by Konigsburg extraction (Henderson et al., 1979).

Edman Sequencing of PrP 27–30 and PrP^{Sc} Peptides. Edman sequencing was used to confirm the amino acid sequence of each PrP 27–30 peptide purified by reverse-phase HPLC after digestion with endo Lys-C, resulting in nearly complete coverage of the N-terminally truncated molecule. The carboxy-terminal half of the K8 peptide was repurified and sequenced after a limited digest of K8 with trypsin as discussed in a subsequent section. Figure 4 shows the recovery of each residue for most of the peptides. Residues printed in lower case letters indicate missing signals for a particular sequencing determination. Every residue observed matches that expected from the genomic and cDNA sequences for

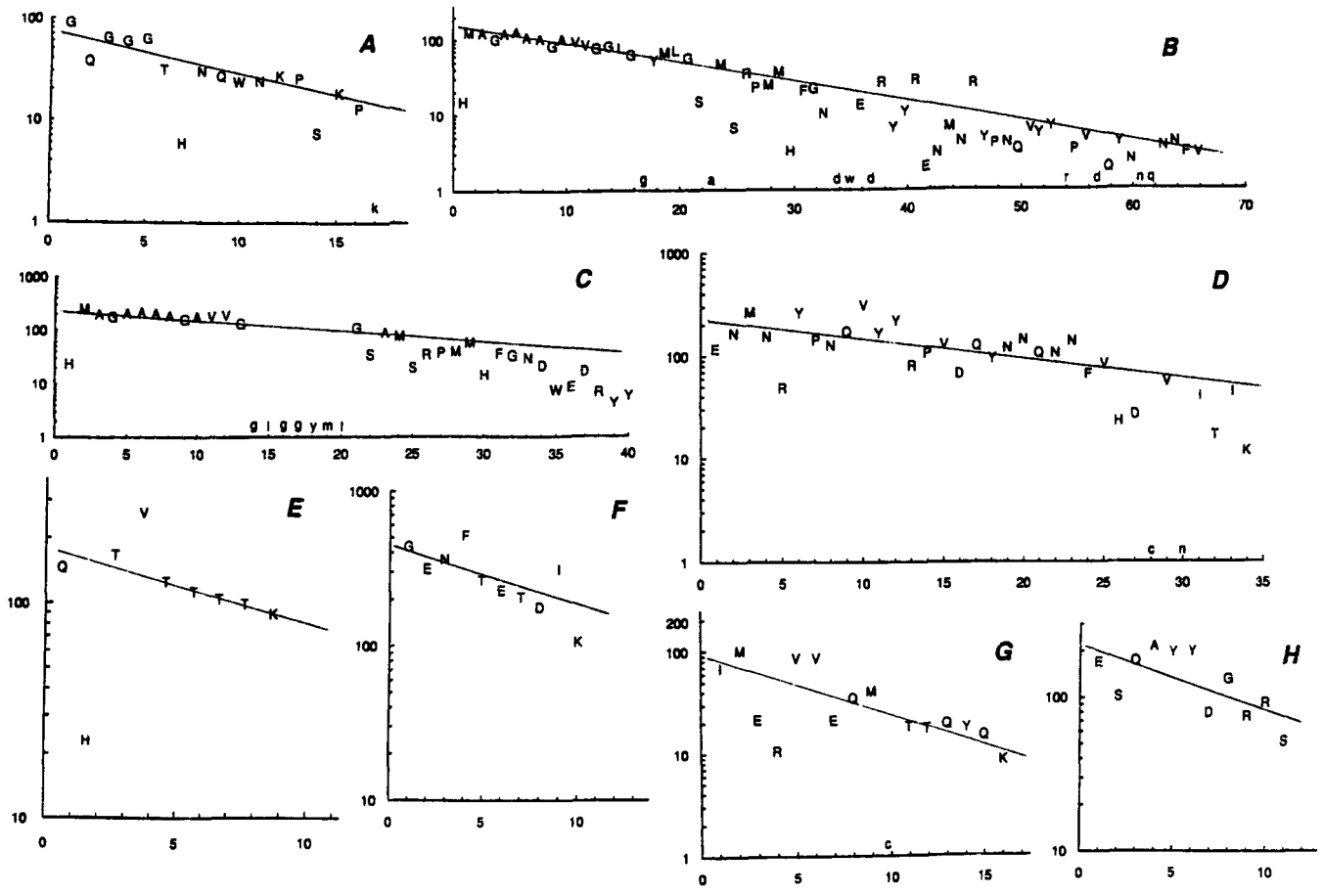

FIGURE 4: Data from Edman sequencing of PrP 27–30 peptides showing recoveries per cycle (in picomoles): (A) PrP 27–30 N-terminus; (B) K8; (C) N-terminus of K8 from a limited tryptic digest; (D) C-terminus of K8 from a limited tryptic digest; (E) K9; (F) unglycosylated fraction of K10; (G) K11; (H) K12 after release of GPI with 50% aqueous HF.

Syrian hamster PrP. The tetrapeptide K7 was not retained on the HPLC column and was only isolated after N-terminal derivatization and, thus, was not sequenced, but its identity was confirmed by mass spectrometry as described below. Virtually every residue not identified in the experiments illustrated in Figure 4 was confirmed in subdigests of larger peptides. Only four amino acids were not directly identified by sequencing: Gly_{127} (cycle 17, panel B); Asn_{181}, which because it is glycosylated would not be expected to produce a signal (panel D); and Cys_{179} and Cys_{214} (panels D and G, respectively). However, all of these missing residues were unequivocally assigned by mass spectrometric analysis.

The N-terminus of PrP^{Sc} has been sequenced previously, residues Lys_{23}–Pro_{61} being found to be identical with the predicted sequence, except for missing signals at Arg_{25} and Arg_{37} (Turk et al., 1988). In the present study, peptides from a tryptic digest of the large N-terminal peptides of PrP^{Sc} were sequenced, and a fragment was identified giving the sequence YPPQGGGTWGQPHGGGXGQP (data not shown). Assuming tryptophan for the missing cycle at residue 65, this corresponds to residues 49–68 containing the first octapeptide repeat WGQPHGGG. The initial yield was 10–12 pmol, limiting our ability to obtain further sequence information. Other portions of the octapeptide repeat region were sequenced as ragged N-terminal fragments of PrP 27–30, with peptides starting at Gly_{74}, Gly_{78}, Gly_{82}, and Gly_{86} in addition to the major start site at Gly_{90} (data not shown). Thus, the only N-terminal portion of PrP^{Sc} not covered by Edman sequencing is a short section of the octarepeat region, residues 69–73.

Mass Spectrometry of PrP 27–30 and PrP^{Sc} Peptides. Two different mass spectrometric techniques, LSIMS and ESMS, were employed to measure the masses of the various PrP fragments. In the former method, the analyte dissolved in a relatively involatile matrix solvent such as an acidified mixture of glycerol and thioglycerol is bombarded by high-energy cesium ions within the high-vacuum system. The major species detected are peptide $M+H^+$ ions. By reference to a suitable standard, the molecular masses of peptides in the mass range 500–2000 Da are routinely measured with an accuracy of ±0.3 Da. The molecular masses measured by this technique are based on monoisotopic atomic masses because the high-resolution mass spectrometer separates the different naturally occurring stable isotopes such as ^1H and ^2H; ^{12}C and ^{13}C; ^{16}O, ^{17}O, and ^{18}O; and ^{14}N and ^{15}N. By contrast, the peptides studied by ESMS were of higher mass, and the analyses were carried out using a low-resolution quadrupole mass analyzer; thus, chemical average rather than monoisotopic atomic masses were used. The ionization process involves injection of a solution of the peptide into a flowing stream of 1:1 water/acetonitrile acidified with 1% acetic acid which is sprayed as a series of small droplets from a fine needle held at a positive potential of ~4 kV into a chamber at atmospheric pressure. A stream of nitrogen gas causes the solvent to evaporate and leave isolated ions that are sampled through a small orifice into the mass spectrometer. This technique is capable of analyzing completely involatile samples including large peptides and proteins with a mass accuracy approaching 0.01%, i.e., 1 Da for a peptide of mass 10 000 Da. Table I summarizes the recoveries and mass spectrometry of peptides purified by HPLC following digestion of PrP 27–30 and PrP^{Sc} with endo Lys-C.

Table I: Peptide HPLC Elution Times, Recoveries, and Molecular Masses from Endo Lys-C Digestion of PrP 27–30

peptide	residues	% acetonitrile	recovery (%) GP[a]	recovery (%) rods[b]	mass[c]	predicted mass[d]
K4a[e]	90–101	17.0	92	92	1283.5	1283.6
K4-K5-K6	90–106	21.2	~50	~50	1821.0	1820.9
K7	107–110	nr[f]			652.6	652.3[g]
K8	111–185	37.2		10–80	8607.8[h]	8608.6
K9	186–194	12.9	92	67	1016.6	1016.5
K10(CHO)	195–204	19.2	95	101	1154.5[h]	1154.5
K11	205–220	26.4	95	64	2044.9	2045.0
K12	221–231	14.6	92[i]	98[i]	1374.4[j]	1374.6
K12a	221–228	21.2	~15	~15	932.3	932.4

[a] Recovery percent from gel-purified PrP 27–30. [b] Recovery percent from PrP 27–30 in prion rods. [c] Masses measured by LSIMS, except for K8 which was measured by ESMS. In most cases, the quoted masses are the means of several measurements made on different occasions. [d] Based on monoisotopic atomic masses except for K8 which is based on average atomic masses. [e] K4a is N-terminally truncated, predominantly at Gly_{90}. Quantitation takes account of all N-terminal species. [f] Not retained. [g] Mass of derivative (Stahl et al., 1992; Stultz et al., 1989). [h] After digestion with PNGase, which converts a glycosylated asparagine into an aspartic acid. [i] Quantitation includes K12a which varied from 10 to 20%. [j] After incubation in 50% aqueous hydrofluoric acid, which leaves an ethanolamine attached to the peptide.

K1 and K2. A hydrophobic 22-residue signal peptide is posttranslationally cleaved from the N-terminus of PrP, leaving the first 2 residues of mature PrP^{Sc} (Hope et al., 1988a; Turk et al., 1988) and PrP^{C} (Turk et al., 1988) as Lys_{23} and Lys_{24}, which have been designated K1 and K2 by our nomenclature. These two amino acids were not recovered after endo Lys-C digestion of PrP^{Sc} and reverse-phase HPLC. It is unclear whether endo Lys-C can cleave an N-terminal lysine, but it is unlikely that either the free amino acid lysine or the dipeptide KK would be retained on the C18 column when coinjected with 1 M GdnHCl. Edman sequencing of PrP^{Sc} has always been reported to give lysine in the first two cycles. Furthermore, recovery of K3 (see below) would require that endo Lys-C accurately cleave after the preceding lysine, consistent with no modification at K2.

K3-K4-K5-K6. It is well-known that proteolytic cleavage between lysine and proline is variable and does not always proceed to completion. K3 was not recovered as an isolated peptide from the endo Lys-C digest of PrP^{Sc}, but it is unlikely that this hydrophilic tripeptide, RPK, would be retained on the reverse-phase HPLC column. However, we did find peptides corresponding to uncleaved K3-K4. Furthermore, both K5 and K6 begin with proline, and are only found as incompletely cleaved products attached to K4. Thus, the large N-terminal peptide of PrP^{Sc} is recovered after endo Lys-C digestion as a heterogeneous product with partial cleavages at both the amino and carboxy termini. The group of large peaks eluting near 45 min in the chromatogram of Figure 3B contains these peptides. Fractions taken across these peaks were analyzed by both CE and ESMS, revealing a general correspondence between the number of peaks observed by CE with that obtained by ESMS. The majority of the peptides were contained in three HPLC fractions, and comparison of the ESMS and CE peaks confirmed the CE migration order predicted on the basis of the size and charge of the peptides. Figure 5 shows the CE and ESMS data for the fraction containing the largest peptide, K3-K4-K5-K6, as the major species. The masses measured by ESMS are listed in Table II and match those predicted by the gene sequence to within <2 mass units.

Although both R_{25} and R_{37} had been implicated as the site of uncharacterized modifications (Hope et al., 1988a; Turk et al., 1988), we found no evidence for any alterations on the majority of PrP^{Sc} molecules. Known modifications of arginine include phosphorylation, ADP-ribosylation, or the addition of 1–3 methyl groups (Wold, 1981). ESMS analysis would have detected the presence of any of these on the K3-K4 peptide although arginine phosphate is very acid-labile and would be destroyed by the acidic conditions employed in the HPLC purification (Fujitaka & Smith, 1984). Furthermore, a tryptic subdigest of K3-K4 gave a smaller peptide whose mass measured by high-resolution LSIMS was consistent with residues 25–37 (RPKPGGWNTGGSR) and which gave no indication of modified arginines (measured $M+H^+ = 1369.4$, calculated = 1369.7). It is also unlikely that trypsin would cleave at Arg_{37} if this residue was modified. We cannot rule out, however, the presence of a labile modification of arginine such as an acid-sensitive phosphorylation or the modification of a small fraction of molecules.

K5 and K6, which both begin with proline, were never found as independent peptides in digests of either PrP^{Sc} or PrP 27–

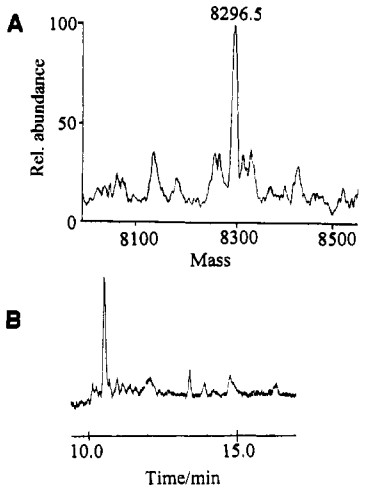

FIGURE 5: (A) ESMS and (B) CE analysis of the fraction eluting at 27.8% acetonitrile in the HPLC chromatogram of PrP^{Sc} digested with endo Lys-C (Figure 3B). The ESMS data were transformed to show a scale of molecular mass rather than mass/charge. Both the electrospray spectrum and the electrophoretogram show one predominant peak, the mass of which matches that calculated for K3-K4-K5-K6, the largest predicted N-terminal peptide. Other shorter fragments were observed in adjacent fractions (see text and Table II).

Table II: HPLC Elution Data and Molecular Masses Determined by ESMS of N-Terminal Peptides from PrP^{Sc}

peptide	residues	% acetonitrile	observed mass	predicted mass
K4	P_{28}–K_{101}	29.9	7375.6	7375.7
K3-K4	R_{25}–K_{101}	29.0	7758.4	7757.2
K4-K5-K6	P_{28}–K_{106}	28.2	7915.3	7913.4
K3-K4-K5-K6	R_{25}–K_{106}	27.8	8296.5	8294.8

30. They were only observed after partial cleavage by endo Lys-C left them attached to K4 or to the N-terminus of PrP 27–30. K5 begins with Pro_{102}, which when substituted by leucine in ataxic GSS can give rise to a neurodegenerative disease (Hsiao et al., 1989; Kitamoto et al., 1991). We estimate that from PrP 27–30, ~50% of the K5-K6 peptide was recovered attached to K4. While it is conceivable that the fraction of K5 that is cleaved by endo Lys-C does not contain a Pro at position 102, there is no evidence to support this contention. Endo Lys-C cleavage of synthetic peptides allowed us to establish that synthetic K5-K6 is unretained on a C_{18} RP-HPLC column under the conditions we employed whereas the analogous pentapeptide having Leu_{102} rather than Pro_{102} is retained. We also never observed peptides that were cleaved between K5 and K6; these two were either both found attached to K4 or both missing. Whether this results from the exact sequence, the secondary structure of the peptide, or the presence of a cis proline at position 105 is unknown. Endo Lys-C digestion of synthetic peptides confirmed that limited cleavage occurred at this particular Lys-Pro site only in the absence of SDS.

PrP 27–30 N-Termini. As mentioned above, analysis of various HPLC fractions from the endo Lys-C digest of PrP 27–30 revealed a ragged N-terminus consisting of five different start sites (summarized in Figure 1). The shorter of these extended peptides were identified by LSIMS whereas ESMS was used to confirm the longer ones. Heterogeneity was also noted in the initial N-terminal sequencing of PrP 27–30 following purification in the presence of proteinase K (Prusiner et al., 1984). We observed peptides starting at Gly_{90}, Gly_{86}, Gly_{82}, Gly_{78}, and Gly_{74}. This is consistent with the preference displayed by proteinase K for hydrolysis at aromatic residues; this array of N-termini would arise by cleavage after every tryptophan and histidine in this portion of the protein. There is no evidence that this ragged N-terminus plays a role in scrapie infectivity since fractions enriched for infectivity in the absence of proteolysis contain full-length PrP^{Sc} (Bolton et al., 1987; Hope et al., 1986; Turk et al., 1988). While polymerization of PrP^{Sc} into amyloid rods requires limited proteolysis, proteases other than proteinase K such as Pronase or trypsin also produce rods (McKinley et al., 1991a), arguing that ragged N-termini are not a prerequisite. Like the K4 peptide discussed above, there are also peptides containing the K5-6 extension at their C-termini resulting from the partial resistance of the Lys–Pro bonds to endo Lys-C. In fact, only 50% of the PrP 27–30 N-terminal peptides were recovered without K5-K6 attached. On occasion, we observed minor mass spectrometric components 16 Da higher than predicted for K4-K5-K6. This mass difference is consistent with a proline to leucine mutation but could arise through oxidation or be due to an unrelated coeluting impurity. Chemical cleavage of PrP 27–30 by cyanogen bromide gave a peptide terminating at Met_{109} (converted to homoserine). Immediately after separation, ESMS gave only the anticipated mass, whereas a +16 component was observed after drying the sample and analyzing it 24 h later, implicating oxidation as the most probable cause.

K7. Differences in the amino acid sequence encompassing the tetrapeptide TNMK distinguish PrP from Syrian, Chinese, and Armenian hamsters which display variations in scrapie incubation times, neuropathology, and varying behavior with different prion strains (Kimberlin et al., 1986; Lowenstein et al., 1990). Furthermore, PrP from mice with long and short scrapie incubation times exhibits one of two amino acid differences in this region (Westaway et al., 1987). K7 was not recovered in any HPLC fraction that was retained on the column, and a synthetic peptide of the predicted sequence eluted in the unretained fractions when coinjected with 1 M GdnHCl. We thus carried out an N-(thiocholinyl)acetylation procedure that could be applied to the unretained fraction in the presence of GdnHCl, thereby increasing the retention of the peptide on the C18 column. This gave a positively charged quaternary nitrogen on the N-terminus which also increased the sensitivity of detection by mass spectrometry. After derivatization and HPLC, a fraction was identified that possessed the correct amino acid composition, and the mass measured by LSIMS (652.6 Da) matched the predicted mass (652.3 Da). Although the recovery of the peptide was only ~50%, it is possible that this resulted from incomplete derivatization. Alternatively, some molecules may have received a second *N*-(thiocholinyl)acetyl group on the lysine ϵ-amino group, thereby adding a second quaternary nitrogen and either changing the elution position in RP-HPLC or affecting the detection of the doubly charged ion by LSIMS.

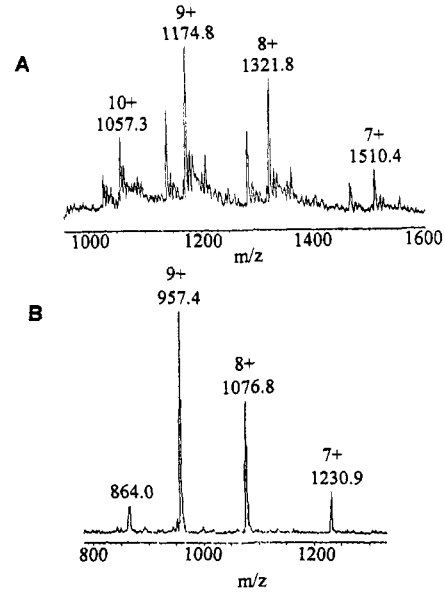

FIGURE 6: ESMS analysis of K8 (A) before and (B) after removal of the N-linked oligosaccharides by treatment with PNGase (see text). The measured molecular mass in (B) was calculated from the ions having 7, 8, and 9 charges.

K8. More than half of the sequence of PrP 27–30 is a region bounded by Lys_{110} and Lys_{185} with no intervening lysines. Thus, K8 is a 75 amino acid endo Lys-C product containing the highly conserved hydrophobic region, the first N-linked glycosylation site, and one of the cysteine residues involved in the disulfide bond. Analysis of HPLC fractions by SDS–PAGE reveals the glycosylation, resulting in a large degree of heterogeneity that gives an ill-defined triplet upon staining with silver. Incubation of the fraction with PNGase removes the carbohydrate to give a single band (data not shown). Inspection of the gel reveals that <10% of K8 is present in unglycosylated form, and mass spectrometric analysis was unable to detect any unglycosylated material, in contrast to K10 which carries the second glycosylation site and is 20% unglycosylated (see below). A peptide of the size of K8 is not amenable to LSIMS at the nanomole level, even without the N-linked glycosylation. Analysis of the untreated peptide by ESMS (Figure 6A) shows an array of peaks whose masses can be rationalized by the mass of K8 augmented by masses corresponding to N-linked sugar structures known for PrP 27–30 (Endo et al., 1989). This analysis together with data from CE reveals that a high proportion of the oligosac-

Table III: HPLC Elution Data and Molecular Masses Measured by LSIMS of Peptides from Digests of K8

peptide	% acetonitrile	observed mass	predicted mass
H_{111}–R_{136}	40.0	2363.6	2363.2
P_{137}–R_{148}	32.0	1534.5	1534.6
E_{152}–V_{166}	23.4	1943.0	1942.9
Y_{157}–V_{166}	22.8	1298.8	1298.7
D_{167}–H_{177}	18.2	1392.6	1392.6
D_{178}–K_{185} (N181 → D)	22.5	964.6	964.5

charides attached to this site are neutral. ESMS analysis of the PNGase-treated peptide shows a peak whose mass exactly matches that predicted by the nucleotide sequence (Figure 6B).

To confirm the structure of K8, we performed proteolytic subdigests, followed by HPLC repurification and mass determination by MS (Table III). Although K8 contains five arginine residues, incubation of the peptide with 3 μg/mL trypsin for 2.5 h at 37 °C resulted in limited hydrolysis, primarily at Arg_{148} and Arg_{151}, dividing the molecule approximately in half, although limited cleavage was also observed at Arg_{156}. Edman sequencing was carried out on these large fragments after HPLC purification, and the masses of the two major N-terminal and PNGase-treated C-terminal halves were confirmed by ESMS (data not shown). The deglycosylated C-terminal half was further digested with endoproteinase Asp-N for LSIMS analysis. Alternatively, overnight incubation of K8 with trypsin gave more complete cleavages allowing isolation of shorter N-terminal peptide fragments. Table III shows that peptides of the expected masses were recovered from the tryptic and Asp-N digests and provided complete coverage of K8, with the exception of the tripeptide Tyr_{149}–Arg_{151}. Although a peak eluting early in the chromatogram gave an amino acid composition corresponding to YYR, we were unable to confirm this by LSIMS analysis of this fraction. However, these three residues were confirmed by Edman sequencing, and mass spectrometry indicates that this tripeptide is unmodified on the basis of a good match between the calculated and observed mass of the intact K8 peptide shown in Figure 6B.

K9. The K9 peptide is the location of the second amino acid substitution found in murine PrP associated with long scrapie incubation times (Westaway et al., 1987), and contains a run of threonines that constitute potential sites for O-glycosylation (Wold & Moldave, 1984). However, the unmodified peptide is recovered in good yield, suggesting that this region is not glycosylated in PrP 27–30. As reported previously, a variable portion of K9 was recovered with an N-terminal pyroglutamic acid that probably formed after cleavage by endo Lys-C (Baldwin et al., 1990a). A synthetic peptide of the same sequence also displayed pyroglutamic acid at the N terminus upon storage in HPLC buffer (not shown). We frequently found that endo Lys-C cleavage between K9 and K10 was incomplete, but the purified K9-10 fragment could be digested to completion after removal of the carbohydrate with PNGase (not shown).

K10. The K10 peptide contains the second N-linked oligosaccharide acceptor site and the sites of two mutations associated with familial disease, Phe_{198}-Ser and Glu_{200}-Lys (Hsiao et al., 1991a, 1992). Approximately 20% of the recovered peptide was unglycosylated as was confirmed by the observation of Asn_{197} by Edman sequencing. This gave a separate later-eluting peak in HPLC, while the other 80% contained heterogeneous N-linked carbohydrate and gave a broad peak by HPLC. Analysis of the glycosylated peptide by LSIMS gave no signal unless the sample was first deglycosylated with PNGase (not shown). Edman sequencing of the glycosylated peptide gave a missing signal at the asparagine, but incubation with PNGase converted this to aspartic acid, increasing the mass of the peptide by 1 Da and allowing confirmation of the exact location of glycosylation by tandem mass spectrometry (Kaur et al., 1992).

K11. The K11 peptide contains the second cysteine involved in the disulfide bond, as well as the site of a point mutation (Q_{217}-R) that is found in human PrP from a family that suffers from GSS, members of which interestingly also accumulate neurofibrillary tangles of the type observed in Alzheimer's disease (Hsiao et al., 1992). Most of K11 was recovered in a single peak with the predicted mass, but we occasionally found a portion with an extra 16 or 32 mass units associated with oxidation of one or both methionines to form methionine sulfoxide or sulfone. It is unclear whether PrP^{Sc} in the brain is oxidized at these sites, or whether oxidation occurs after purification. The treatment of PrP with DTT preceding carboxymethylation could potentially reduce the sulfoxide to give methionine. We also observed that a significant fraction of K11 tended to slowly bleed off the column trailing the mean peak, which could explain the relatively low recovery of the peptide. The structure of the trailing K11 was identical to that eluting in the main peak as judged by Edman sequencing, migration on CE, and LSIMS analysis. Experiments with synthetic peptides have shown that a peptide corresponding to PrP residues 202–218 tends to aggregate into amyloid polymers, possibly explaining the observed HPLC behavior (Gasset et al., 1992). Early digestions of K11 with non-sequencing-grade endo Lys-C gave nonspecific cleavage after the N-terminal isoleucine and occasionally after the fourth residue (Arg_{208}).

K12. The C-terminal peptide carries the GPI anchor and is also the site of an apparent posttranslational cleavage (Stahl et al., 1990c). The characterization of K12 has been described before (Stahl et al., 1990c). Incubation of the GPI-linked peptide in 50% aqueous hydrofluoric acid removed the phosphodiester-linked GPI, and allowed determination of the attachment site as Ser_{231} by LSIMS. Trypsin digestion of the original GPI-linked peptide and HPLC separation gave a single product of the expected mass for cleavage between Arg_{229} and Arg_{230} ($M+H^+ = 1088.3$) (Stahl et al., 1990c). Electrospray mass spectrometry of K12–GPI gave masses corresponding to those for the unmodified peptide linked to the six structures of the GPI glycan that were independently determined (Stahl et al., 1992). This indicates that there are no other modifications of the peptide.

One apparent posttranslational modification assigned to this region originated from the observation that ~15% of the C-terminus was recovered in a truncated form ending at Gly_{228} (Stahl et al., 1990c). We postulated that this might occur through cleavage after the two arginines at position 230 by a dibasic-specific protease, followed by removal of the C-terminal arginines by a carboxypeptidase B-like enzyme. Whether this is the actual mechanism of its formation remains to be determined.

Other Molecules That Copurify with PrP 27–30 in the Scrapie Prion. Non-PrP Peptides. Every endo Lys-C digest of prion rods was found to contain peptides that were not predicted from the PrP gene or cDNA sequence. Two peptides eluting in a single HPLC fraction or in adjacent fractions together with the N-terminal peptide Gly_{90}–Lys_{101} in every PrP 27–30 digest were structurally characterized by tandem mass spectrometry high-energy collision-induced dissociations

and assigned the sequences DGPRLSK and REIVDRK. The former sequence was confirmed by Edman degradation once a purified fraction was obtained. These peptides were absent in an endo Lys-C digest of PrP 27–30 obtained by purification by SDS–PAGE. Instead, they were found in extracts from regions of the gel below PrP 27–30 and at the solvent front of the gel. The fact that both peptides end in lysine suggests that they are derived from larger precursors by endo Lys-C.

A band of the appropriate molecular weight for actin was observed by SDS–PAGE in many, but not all, prion preparations. Endo Lys-C digestion and analysis revealed several actin peptides; e.g., an HPLC peak at 23% acetonitrile gave an $M+H^+$ peak by LSIMS of m/z 1161.6. Edman sequencing and tandem mass spectrometry showed this to be due to the peptide EITALAPSTMK, completely identical to residues 318–328 of mouse skeletal actin. Mouse actin also contains the sequence REIVRDRIK, showing substantial homology with one of the other peptides referred to above.

Labeling of Prion Rods by 3-(Trifluoromethyl)-3-(m-iodophenyl)diazirine (TID). Although preparations of prions often show minimal staining on SDS–PAGE other than that arising from PrP 27–30, our previous analysis of the lipid content of these preparations revealed >4 mol of fatty acid per mole of PrP 27–30 (Stahl et al., 1987). In contrast, PrP 27–30 extracted from SDS–PAGE gels contained only 1 mol of stearic acid per mole of protein (Stahl et al., 1987). Thus, it appeared that additional molecules with very high or low molecular weight might copurify with PrP 27–30 in the prion. We therefore radiolabeled prion rods with the hydrophobic derivative TID. Surprisingly, the major product identified after TID-labeling of prion rods either before or after denaturation in GdnHCl is not PrP 27–30, but is instead a molecule that remains in the SDS–PAGE sample well, and at the interface of the stacking and resolving gels (called TID-X; data not shown). This molecule is not an aggregated form of PrP 27–30, since radioiodination on tyrosines gives very little signal at sites in the gel other than that corresponding to PrP (gels not shown). TID-X is resistant to hydrolysis by proteinase K, although the PrP band disappears after digestion. In contrast, the intensity of TID-X was diminished by incubation with sodium hydroxide and alkaline hydroxylamine, consistent with the labeling by TID of fatty acyl groups. Thin-layer chromatography after alkaline hydrolysis gave a spot that comigrated with TID-labeled fatty acids (not shown). Furthermore, a portion of TID-X appeared to be sensitive to treatment with nitrous acid, which is a characteristic of the unacetylated hexosamine found in heparan sulfate and GPI anchors. Gel filtration of the GdnHCl-denatured rods after derivatization with TID gave a large peak eluting in the void volume of a Sepharose CL-4B column in buffers containing detergents or 4 M GdnHCl, suggesting a molecular weight $>10^6$ (Figure 7). However, chromatography in a buffer containing both GdnHCl and 1% NP-40 shifted the migration of the peak to be included volume, implying a molecular weight under 60K. Precipitation of these fractions with ethanol and subsequent analysis of the pellet by SDS–PAGE indicated that the TID-X once again migrated in the stacking gel. These results indicated that TID-X was an aggregating component that could be reversibly dissociated in buffers containing NP-40 and GdnHCl. This behavior is characteristic for a glycosaminoglycan containing a hydrophobic membrane anchor.

Subsequent experiments suggested that this molecule could be observed in the SDS–PAGE stacking gel by silver staining. A purification procedure was developed based on the properties

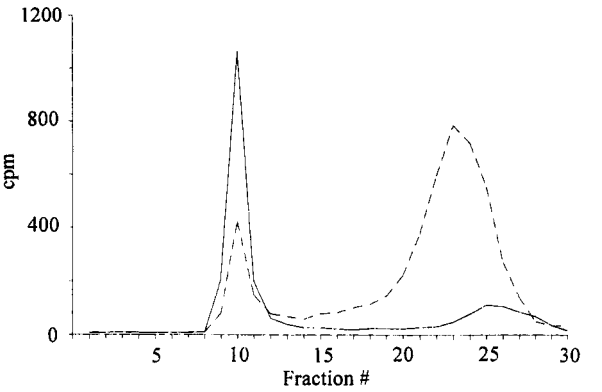

FIGURE 7: Gel filtration chromatography of TID-X [radioactivity (cpm) as a function of fraction number] showing that the sharp peak eluting in the unretained fraction in deoxycholate buffer (solid line) is retained in 4 M GdnHCl/1% NP-40 (dashed line).

observed for TID-X. Prion rods were denatured in GdnHCl, then reduced and carboxymethylated, and spun in a centrifuge at 100000*g* for 1 h. Most of the PrP 27–30 was found in the supernatant fraction, while the pellet contained a portion of the PrP 27–30 and a molecule that remained in the stacking gel. Sonication of this pellet in a buffer containing 4 M GdnHCl and 1% NP-40 released into the supernatant fraction the residual PrP and the compound migrating in the stack by SDS–PAGE. Precipitation with ethanol and resolubilization in SDS followed by digestion with proteinase K destroyed the PrP and left the unknown compound in a purified form that could be recovered in a pellet after centrifugation at 100000*g*. Amino acid analysis of this fraction gave only trace amounts of any amino acids (not shown).

GdnHCl/NP-40-Insoluble Fibrils. During the course of this purification, we observed the appearance of fibrils during the sonication of the GdnHCl-insoluble material in GdnHCl/NP-40. Sonication of buffer alone gave no fibrils. Some fibrils were visible without magnification, while negative-stained specimens viewed by electron microscopy showed many fibrillar structures that were ~100 nm long and ~10 nm in diameter. Analysis by SDS–PAGE showed no visible bands, and the fibrils did not disappear upon boiling in sample buffer (not shown). Attempts to dissociate the fibrils with guanidine thiocyanate, formic acid, hot formic acid, hexafluoroisopropyl alcohol, trifluoroethanol, and trifluoroacetic acid were unsuccessful. Prion rod preparations isolated by discontinuous sucrose gradients can contain fibers of poly(ethylene terephthalate) (polyester) and cellulose or starch as determined by Raman spectroscopy (L. Tensmeyer, M. A. Baldwin, and S. B. Prusiner, unpublished work). However, amino acid analysis of the insoluble fibrils described above showed an amino acid profile; this did not match that for PrP or any contiguous portion of PrP.

DISCUSSION

That the amino acid sequence of PrP^{Sc} is the same as that of the translated PrP gene sequence was established by two independent approaches: mass spectrometry and Edman sequencing (Figure 1, Tables I–III). Assuming that the sequence of PrP^{C} is the same as that deduced from translation of the PrP genomic DNA sequence, then our observations eliminate mechanisms such as RNA editing (Blum et al., 1990; Sommer et al., 1991) and protein splicing (Kane et al., 1990) as explanations for the two isoforms. Earlier studies showed that RNA splicing could not explain the differences in properties between PrP^{C} and PrP^{Sc} since the entire open reading frame is encoded in a single exon (Basler et al., 1986).

Although no unexpected posttranslational chemical modification of PrP^{Sc} was found, both PrP^{C} and PrP^{Sc} have been shown to have multiple modifications. The two consensus acceptor sites for Asn-linked carbohydrates of most PrP^{C} and PrP^{Sc} molecules are glycosylated (Bolton et al., 1985; Haraguchi et al., 1989). Partial characterization of the Asn-linked oligosaccharides of PrP 27–30 revealed heterogeneous, complex-type oligosaccharides some of which possessed terminal sialic acids and branched fucose residues (Endo et al., 1989). Although detailed analysis of the Asn-linked oligosaccharides has not been performed for PrP^{C}, two-dimensional gel electrophoresis indicated the presence of multiply sialylated species (Haraguchi et al., 1989). However, the Asn-linked carbohydrates do not appear to be essential for PrP^{Sc} synthesis since neither tunicamycin nor site-directed mutagenesis of the consensus sites prevented PrP^{Sc} formation (Taraboulos et al., 1990a). Both PrP^{Sc} and PrP^{C} are modified by GPI anchors (Stahl et al., 1987, 1990b). Although both the PrP^{C} and PrP^{Sc} GPI anchors are sensitive to cleavage by PIPLC after denaturation (Stahl et al., 1990c), only PrP^{C} can be released from cellular membranes by PIPLC under nondenaturing conditions (Safar et al., 1990a; Stahl et al., 1990a). The differential release of PrP^{C} and PrP^{Sc} by PIPLC digestion is possibly attributable to differences in the conformations of the two isoforms. The GPI anchors of both PrP^{Sc} and PrP^{C} have sialic acid residues which have not been identified previously in GPI anchors of other mammalian proteins (Baldwin et al., 1990b,c; Stahl et al., 1992). A C-terminal peptide is removed from Syrian hamster PrP^{Sc} upon addition of the GPI anchor to Ser_{231} (Stahl et al., 1990c). The similar migration on reverse-phase HPLC and CE of the C-terminal peptide derived from PrP^{C} and PrP^{Sc} suggests that the attachment point of the GPI anchor to PrP^{C} is the same as that for PrP^{Sc} (Stahl et al., 1992). Approximately 15% of purified Syrian hamster PrP 27–30 molecules are truncated at Gly_{228} and thus do not contain the GPI anchor (Stahl et al., 1990c). An N-terminal signal sequence of 22 amino acids that targets the proteins to the endoplasmic reticulum is removed from both PrP^{Sc} (Hope et al., 1986; Turk et al., 1988) and PrP^{C} (Turk et al., 1988). Both PrP^{C} and PrP^{Sc} contain only two cysteines that form a single disulfide bond (Turk et al., 1988). Two arginine residues at the N-terminus of PrP^{Sc} have sometimes been reported to carry unknown modifications giving either new peaks (Hope et al., 1988a) or missing cycles (Turk et al., 1988) during Edman degradation. Similar analysis of PrP^{C} also showed a missing residue at the corresponding cycle (Turk et al., 1988). The arginine modification of PrP^{Sc} is either variable or labile since arginine has also been observed at reasonable recoveries during Edman sequencing (Bolton et al., 1987; Hope et al., 1986; Safar et al., 1990b).

LSIMS analysis with a double-focusing sector mass spectrometer was performed on all the peptides encompassing PrP 27–30, which gives a mass accuracy within ±0.3 mass unit. Thus, any posttranslational modification or amino acid substitution that resulted in a difference of a single mass unit would have been detected. Long N-terminal peptides of PrP^{Sc} containing the octapeptide repeats, which were generated by endoprotease Lys-C digestion, were evaluated by ESMS and gave measured masses that differed from the theoretical by only 1–2 Da, which is within the error of the technique at this mass range. A similar detailed analysis of PrP^{C} has not yet been performed due to difficulties with the purification of sufficient quantities of PrP^{C} (Pan et al., 1992).

Three caveats pertain to our analysis of PrP^{Sc}. First, a chemical difference existing within a subpopulation of the PrP^{Sc} molecules could have gone undetected. Since the particle:infectivity ratio of scrapie prions is 10^4–10^5 molecules of PrP^{Sc} per ID_{50} (Prusiner et al., 1982, 1983), it is possible that a small fraction of PrP 27–30 exhibits an undetected chemical modification that induces transmissibility and disease. Arguing against this hypothesis is that molecules without the properties of PrP^{Sc} would be expected to be eliminated during purification of PrP 27–30 which utilizes limited proteolysis and aggregation (Meyer et al., 1986; Oesch et al., 1985). Second, a labile modification such as an arginine phosphate might have been removed upon purification of the peptides by HPLC in a solution containing 0.06% trifluoroacetic acid. An argument against this possibility is that the infectivity of prion rods is not reduced in acidic conditions (Prusiner et al., 1981), including incubation in 10 mM HCl. Furthermore, it has been established that perturbations to the secondary structure of PrP 27–30 on treatment with acid are reversible (Gasset et al., 1993). Third, the possibility exists that PrP^{C} contains an as yet unrecognized posttranslational modification that upon removal gives rise to PrP^{Sc}. If this were the case, then structural studies of PrP^{Sc} alone would fail to elucidate the process.

Although we have used mass spectrometry extensively in this study, it has only been applied to fragments of the protein produced by enzyme digestion and purified by HPLC. The techniques of ESMS and matrix-assisted laser desorption time of flight mass spectrometry (MALD–TOFMS) are capable of analyzing intact proteins with minimal preparative procedures which minimizes the possibility of losing a labile posttranslational modification. Both of these methods are limited by sample solubility in suitable buffers and by the heterogeneity associated with the GPI glycan and the Asn-linked sugar chains. Preliminary results with MALD-TOFMS comparing PrP^{Sc} and PrP^{C} indicate that the two isoforms differ by <100 Da and they may in fact be identical (M. Baldwin, K.-M. Pan, N. Stahl, R. Wang, R. Beavis, B. Chait, and S. B. Prusiner, unpublished results). As yet, it has not proved possible to obtain an ESMS spectrum of PrP^{Sc}, largely due to difficulties of solubilizing this membrane protein in a solvent compatible for ESMS ionization.

Our investigations have identified several molecules that purify with PrP 27–30 and scrapie infectivity. It is unknown whether these molecules simply represent contaminants with physical properties similar to PrP 27–30, or they participate as components of the prion. These molecules include peptides with the sequences DGPRLSK and REIVDRK that were found in every endo Lys-C digest of prions but were separable from PrP 27–30 or PrP^{Sc} by SDS–PAGE and were recovered from the low molecular weight region of the polyacrylamide gel. The source of these peptides it not yet known, although DGPRLSK is similar to the sequence DGPKLSA which is part of the leucine zipper from the *Drosophila* protein *daughterless* (Candy et al., 1988). It is curious that *daughterless* is required for the development and maintenance of neuronal cells in the fly (Candy et al., 1988). REIVDRK shows homology to mouse skeletal actin, but the corresponding hamster sequence is unknown. Besides the two peptides, a compound was identified that reacts with the hydrophobic probe TID (Rosenberry et al., 1986) and remains in the sample well and stacking gel after SDS–PAGE. This compound contains alkali-sensitive fatty acids, is resistant to proteinase K, and is at least partially susceptible to nitrous acid deamination. Furthermore, it can be disaggregated in buffers

containing GdnHCl and NP-40. This is the behavior expected for a glycosaminoglycan containing covalently bound lipid, and the susceptibility to nitrous acid is a characteristic of heparan sulfate. Rigorous identification of this compound has not been completed, and attempts to digest it with heparinase and chondroitinases have failed. A third entity found in prion preparations were large fibrils that remained after removal of the PrP 27–30 and TID-labeled compound and resisted disruption by sonication in buffers containing GdnHCl and NP-40. Attempts to obtain the amino acid sequence in fractions enriched for the fibers were unsuccessful. These fibrils may be contaminants of no relevance since polyester and cellulose or starch have been found in purified prion preparations (L. Tensmeyer, M. Baldwin, and S. B. Prusiner, unpublished data).

No posttranslational chemical modifications have been identified that differentiate PrP^{Sc} from PrP^{C}, suggesting the possibility that conformation alone distinguishes these PrP isoforms. By comparison of the amino acid sequences of 11 mammalian and 1 avian prion proteins (PrP), structural analyses predicted 4 α-helical regions (J.-M. Gabriel, F. Cohen, R. A. Fletterick, and S. B Prusiner, unpublished results) (Cohen et al., 1986). Peptides corresponding to these regions of the Syrian hamster PrP were synthesized, and contrary to predictions, three of the four spontaneously formed amyloids as shown by electron microscopy and Congo red dye staining (Gasset et al., 1992). These findings suggest that the conversion of PrP^{C} to PrP^{Sc} may involve the transition of one or more putative PrP α-helices into β-sheets. Infrared spectroscopy of PrP 27–30 showed a high β-sheet content (Caughey et al., 1991b) which decreased when PrP 27–30 was denatured, as did scrapie infectivity (Gasset et al., 1993).

The diversity of scrapie prions (Bruce & Dickinson, 1987; Dickinson & Fraser, 1979; Dickinson & Outram, 1988; Kimberlin et al., 1987) poses an interesting conundrum with respect to hypotheses about prion structure in which PrP^{Sc} is a conformer of PrP^{C} (Prusiner, 1991; Weissmann, 1991). Until recently, virtually all suggestions about the structure of the scrapie prions have demanded a polynucleotide to explain "strains" or distinct isolates. The finding that the pattern of PrP^{Sc} accumulation in the CNS is characteristic for a particular "strain" offers a novel mechanism for the propagation of distinct prion isolates (Hecker et al., 1992). In this model, a different set of cells would propagate each isolate. Whether or not the isolates are targeted to specific sets of cells by the Asn-linked oligosaccharides of PrP^{Sc} remains to be established. It is noteworthy that acquisition of proteinase K resistance and presumably PrP^{Sc} synthesis can occur in the absence of Asn-linked glycosylation in scrapie-infected cultured cells (Taraboulos et al., 1990a); further studies are needed to determine the molecular basis of the cellular tropism exhibited by prions. Alternatively, explaining the problem of multiple distinct prion isolates might be accommodated by multiple PrP^{Sc} conformers that act as templates for the folding of de novo synthesized PrP^{Sc} molecules during prion "replication" (Prusiner, 1991). Although both these proposals are rather unorthodox, they are consistent with observations generated from transgenic mouse studies contending that PrP^{Sc} in the inoculum binds to homologous PrP^{C} or a precursor to form a heterodimeric intermediate in the replication process (Prusiner et al., 1990). Whether foldases, chaperonins, or other types of molecules are involved in the conversion of the PrP^{C}/PrP^{Sc} heterodimer to two molecules of PrP^{Sc} is unknown. The molecular weight of a PrP^{Sc} homodimer is consistent with the ionizing radiation target size of 55 000 ± 9000 as determined for infectious prion particles independent of their polymeric form (Bellinger-Kawahara et al., 1988).

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