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Scrapie PrP 27-30 Is a Sialoglycoprotein

DAVID C. BOLTON, RUDOLF K. MEYER, AND STANLEY B. PRUSINER*

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

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The major scrapie prion protein, designated PrP 27-30, exhibited both charge and size heterogeneity after purification from infected hamster brains. Eight or more discrete charge isomers of PrP 27-30 with isoelectric points ranging from approximately pH 4.6 to 7.9 were found by using non-equilibrium pH gradient electrophoresis in the first dimension followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the second dimension. The charge isomers were detected by silver staining as well as by radioiodination. The procedures used to disaggregate PrP 27-30 before electrophoresis in the first dimension do not appear to be responsible for the charge heterogeneity. However, heating PrP 27-30 to 100°C for 15 min in 0.1 N NaOH or 0.1 N HCl resulted in modification of the protein and alteration of its electrophoretic pattern. A PrP 27-30 fragment (molecular weight, 17,100 to 21,900) obtained by cyanogen bromide cleavage also exhibited charge and size heterogeneity. Periodic acid-Schiff staining of PrP 27-30 electrophoresed into sodium dodecyl sulfate-polyacrylamide gels demonstrated that carbohydrate residues are attached to the protein. Digestion of PrP 27-30 with neuraminidase and endo-β-*N*-acetylglucosaminidase H resulted in significant changes in the isoelectric pH of PrP 27-30 isomers, whereas digestion with alkaline phosphatase had no effect. Our results demonstrate that PrP 27-30 is a sialoglycoprotein; this is consistent with several properties of this protein and of the scrapie prion.

The scrapie agent appears to differ from viruses in its apparent small size and resistance to procedures that inactivate nucleic acids (32, 36). Some investigators continue to classify the scrapie agent as an unconventional virus (42, 43) despite a lack of definitive data demonstrating that it contains a genomic nucleic acid and thus fulfills commonly accepted criteria for a virus. The only macromolecule that has been identified as unique to tissues infected with the scrapie agent is a protein that purifies with the infectious particle (4, 37). Several lines of investigation have shown this protein to be a structural component of the scrapie agent which is required for expression of biological activity (1, 4, 13, 31, 37, 38, 40; D. C. Bolton, M. P. McKinley, and S. B. Prusiner, Biochemistry, in press). Because the scrapie agent has not been demonstrated to be a virus, but its infectivity depends at least in part on a protein, the term "prion" was suggested to denote this class of infectious particles (36). From this terminology, the protein that is a component of the scrapie prion has been designated PrP 27-30, for prion protein with an M_r of 27,000 to 30,000 (31, 38; Bolton et al., in press). Although this nomenclature is subject to change based on new information, we use it in this report for clarity and convenience.

The size heterogeneity of PrP 27-30 and its resistance to protease digestion greatly facilitated the identification of the protein in fractions enriched for scrapie prions (4, 31, 37; Bolton et al., in press). We began investigating the molecular basis for the size heterogeneity of PrP 27-30 by using a NEPHGE-SDS-PAGE two-dimensional electrophoresis technique developed by O'Farrell et al. (34). Our initial studies indicated that PrP 27-30 was heterogenous with respect to both size and charge. One explanation for this result was that the apparent charge isomers actually represented individual unique proteins. Alternatively, the charge heterogeneity we observed could have been caused by the radioiodination methods required for detecting low concentrations of PrP 27-30 present in partially purified fractions.

* Corresponding author.

Improvements in the method for purifying and concentrating scrapie prions led to further characterization of PrP 27-30. Studies with peptide mapping in one dimension provided evidence that PrP 27-30 behaved as a single protein species (Bolton et al., in press). Further data supporting this hypothesis were obtained by N-terminal amino acid sequence analysis of PrP 27-30 purified to near homogeneity by high-pressure liquid chromatography (38). The sequence data demonstrated that PrP 27-30 possessed a single amino terminal sequence, although it remained heterogeneous with respect to size. Minor signals obtained during the sequence analysis indicated some variation within the N terminus due to "ragged ends" (38). In addition, amino acid sequence studies of zonal sucrose gradient fractions containing high titers of scrapie prions and only one major protein, PrP 27-30, exhibited the same single amino-terminal sequence as that obtained with PrP 27-30 purified by high-pressure liquid chromatography. Thus, PrP 27-30 obtained from zonal sucrose gradient fractions containing infectious prions was homogeneous with respect to N-terminal amino acid sequence, indicating a single protein or a family of related proteins in the range of M_r 27,000 to 30,000 (38).

In this report, data are presented demonstrating the charge heterogeneity of PrP 27-30. Furthermore, we show the presence of a PAS-staining carbohydrate attached to PrP 27-30 and show that digestion of the denatured protein with neuraminidase or endoglycosidase H affects the charge characteristics of PrP 27-30. Our results indicate that previous attempts to demonstrate PAS-staining carbohydrate on PrP 27-30 probably failed due to an insufficient amount of the protein available for staining. The presence of sialic acid-containing oligosaccharides on PrP 27-30 helps to explain several observations about the behavior of the protein and of the scrapie prion.

MATERIALS AND METHODS

Abbreviations. CIAP, calf intestine alkaline phosphatase; endoglycosidase H, endo-β-*N*-acetylglucosaminidase H; HPAP, human placental alkaline phosphatase; Iodobeads,

N-chloro-benzenesulfonamide-derivatized polystyrene beads; NEPHGE, non-equilibrium pH gradient electrophoresis; PAGE, polyacrylamide gel electrophoresis; PAS, periodic acid-Schiff; PrP 27-30, scrapie prion protein (molecular weight, 27,000 to 30,000); SDS, sodium dodecyl sulfate.

Materials. All chemicals were of the highest grades commercially available. Neuraminidase (*Clostridium perfringens*) and ovomucoid protein were purchased from Sigma Chemical Co. (St. Louis, Mo.). Endoglycosidase H (45) was obtained from Health Research, Inc. (Albany, N.Y.). Acrylamide and *N,N'*methylene bis-acrylamide were purchased from Bio-Rad Laboratories (Richmond, Calif.). $Na^{125}I$ was purchased from Amersham Corp. (Arlington Heights, Ill.).

Source of scrapie prions and purification. A hamster-adapted isolate of the scrapie prion was a gift from Richard Marsh (29). It was passaged and prepared as described by Prusiner et al. (37, 39). The prions were purified as described by Prusiner et al. (37), with modifications (40). Before cleavage with CNBr, PrP 27-30 and a related protein (molecular weight, 23,000 to 26,000) were further purified by molecular sieve chromatography on tandem 60- and 30-cm TSK-2000 SW columns (38). PrP 27-30 and the related protein were separated from each other by electrophoresis through a 15% polyacrylamide gel. The proteins were eluted from appropriate gel fragments identified by using the radioiodinated proteins as markers and concentrated by precipitation (Bolton et al., in press).

Radioiodination of PrP 27-30. Four procedures were used to chemically label PrP 27-30 with ^{125}I. In general, proteins were concentrated 10-fold before iodination by precipitation with SDS and quinine hemisulfate or by sedimentation from sucrose gradient fractions diluted with distilled water (R. K. Durbin, personal communication; Bolton et al., in press).

Samples in 0.1 M sodium borate–0.1% SDS (pH 8.5) were iodinated with methyl-3,5-di-[^{125}I]iodo-*p*-hydroxybenzimidate hydrochloride by combining 50-μl samples of the protein suspension with 100-μl samples of the reagent in methanol (47). The reaction mixture was incubated at room temperature for 24 h with occasional mixing. The radiolabeled proteins were removed from the reaction mixture by precipitation with SDS and quinine hemisulfate (Durbin, personal communication).

Proteins in 0.05 M sodium phosphate–0.1% SDS (pH 7.5) were iodinated directly with 1.0 mCi of $Na^{125}I$ by the chloramine T procedure of Hunter and Greenwood (22), with minor modifications.

Proteins in 0.1 M sodium borate (pH 8.5) with or without 0.1% SDS were radioiodinated with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate by the procedure of Bolton and Hunter, with minor modifications (3, 4; Bolton et al., in press).

Sucrose gradient fractions without prior concentration were radioiodinated with 1 to 2 mCi of $Na^{125}I$ with Iodobeads (Pierce Chemical Co., Rockford, Ill.). After the addition of sodium phosphate buffer (pH 6.5) to a final concentration of 30 mM, the proteins were iodinated by the procedure of Markwell (28). The labeled proteins were removed from the reaction mixture by sedimentation in a microcentrifuge (Bolton et al., in press).

NEPHGE-SDS-PAGE two-dimensional gel electrophoresis. Samples (10 or 20 μl) in 9.5 M urea, 5% β-mercaptoethanol, 2% Nonidet P-40, and 2% Ampholytes were subjected to NEPHGE in the first dimension as described by O'Farrell et al. (34). The proteins were separated at 400 V for 4 to 4.5 h. The extruded first-dimension gels were incubated in 62.5 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, and 0.002% bromphenol blue (pH 6.8) at room temperature for 30 to 60 min before quick freezing in a dry ice-ethanol bath and storage at −70°C. The frozen first-dimension gels were thawed by brief incubation at 37°C and electrophoresed immediately in the second dimension in 15% acrylamide gels as described previously (34). Analyses by SDS-PAGE in one dimension were performed as described by Laemmli (26). Gels were stained with silver by the method of Morrissey (33).

The pH gradient in the NEPHGE gels was estimated by measuring the pH of the eluted Ampholytes with a Radiometer pH meter. Briefly, extruded first-dimension gels electrophoresed with sample buffer only were cut into 1-cm segments. The segments were placed into a glass tube containing 1 ml of double-distilled water; the tubes were purged with nitrogen, sealed, and incubated at room temperature for several hours before measuring the pH.

PAS staining. Samples of sucrose gradient-purified prions containing approximately 250 μg of protein or 100 μg of ovomucoid protein were electrophoresed on a 12.5% SDS–polyacrylamide gel as described previously (26). The gel was stained with the PAS reagent by the method of Glossman and Neville, with minor modifications (9, 12). The PAS-stained gels were photographed and then counterstained with Coomassie brilliant blue R-250 and photographed again.

Alkaline phosphatase treatment of PrP 27-30. Purified prion rods were radioiodinated by using Iodobeads as described above. The pellet was suspended in digestion buffer (0.1 M NaCl, 0.015 M Tris-HCl, 1 mM dithiothreitol, pH 7.5) and heated to 100°C for 15 min to disaggregate the rods. The suspension was cooled to 30°C and distributed into working samples. The samples were incubated for 30 min at 30°C in the digestion buffer alone or in digestion buffer with 25 U of CIAP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml or 25 U of HPAP (Sigma) per ml. In addition, samples were incubated with 25 U of CIAP or HPAP per ml in the presence of 5 mM *p*-nitrophenyl phosphate (Sigma). The digestions were terminated by heating the samples to 100°C for 5 min, and the samples were analyzed by NEPHGE-SDS-PAGE after 10-fold dilution with NEPHGE sample buffer.

CNBr cleavage of PrP 27-30. SDS-PAGE-purified PrP 27-30 was precipitated with quinine hemisulfate (Durbin, personal communication) and suspended in 70% formic acid at a protein concentration of approximately 0.2 mg/ml. One-tenth volume of CNBr (50 mg/ml) was added to the protein suspension in a glass vial containing a Teflon-coated magnetic stirring bar and sealed with a Teflon septum (Pierce Chemical Co., Rockford, Ill.). The vial was purged with nitrogen, and the reaction was performed overnight in the dark with continuous stirring (5, 6, 11). The reaction was terminated by diluting the suspension 10-fold with distilled water and quick freezing in a dry ice-ethanol bath. The cleaved protein was stored at −70°C, lyophilized, and analyzed by SDS-PAGE and NEPHGE-SDS-PAGE.

RESULTS

Detection of charge isomers by radioiodination and silver staining. In our initial studies designed to investigate the charge of PrP 27-30, sucrose gradient fractions prepared from scrapie-infected hamster brains were chemically modified with methyl-3,4-di-[^{125}I]-iodo-*p*-hydroxybenzimidate hydrochloride. This reagent was used because it has been reported to iodinate proteins while preserving their native

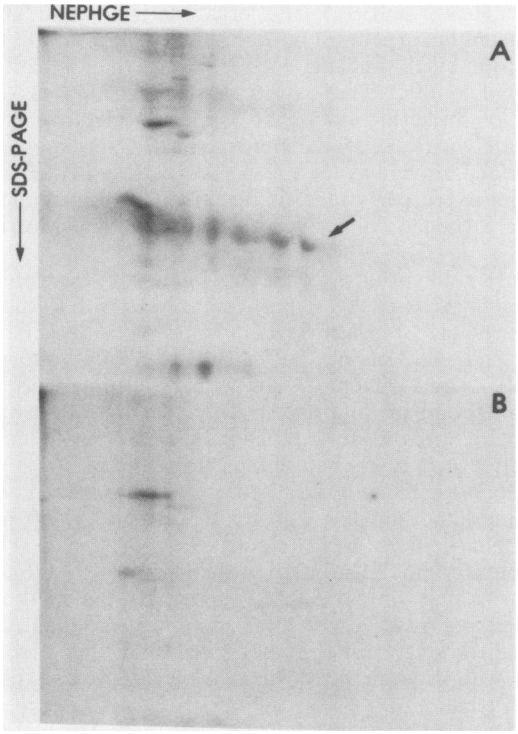

FIG. 1. NEPHGE-SDS-PAGE of scrapie-infected and normal brain sucrose gradient fractions. Samples of scrapie-infected (A) or normal brain (B) sucrose gradient fractions were radioiodinated with methyl-3,5-di-[^{125}I]-iodo-*p*-hydroxybenzimidate hydrochloride at 25°C for 24 h. The fractions were concentrated by precipitation with SDS and quinine hemisulfate (Durbin, personal communication), suspended in NEPHGE sample buffer, and electrophoresed for 3,000 Vh in the first dimension. The SDS-PAGE in the second dimension was performed in 15% polyacrylamide gels. The gels were fixed, stained, and dried as described previously (4; Bolton et al., in press). The autoradiograph was exposed at room temperature for 40 days. The arrow indicates the most alkaline of the major charge isomers.

charge (47). A series of charge isomers migrating in the size range of PrP 27-30 was found in fractions from scrapie-infected brain, but not from normal brain (Fig. 1). Other proteins present in these samples did not appear as a series of charge isomers, although some broadening of the protein spots was apparent. The low labeling efficiency of this reagent made it impractical to pursue detailed analysis of PrP 27-30 by NEPHGE-SDS-PAGE.

Improved purification methods provided fractions having a specific infectivity of between $10^{9.4}$ and $10^{10.3}$ 50% infective dose units per mg of protein and containing primarily one protein, PrP 27-30. The apparent charge isomers of PrP 27-30 were readily evident in these fractions containing the extensively purified protein (Fig. 2). Silver staining the NEPHGE-SDS-PAGE gels of these fractions conclusively demonstrated that the presence of multiple charged species was not an artifact due to radiolabeling (Fig. 2). At least eight different charge isomers within the size range of PrP 27-30 were evident in the silver-stained gel. These charge isomers have isoelectric points ranging from approximately pH 4.6 to 7.9.

The resistance of PrP 27-30 to digestion by proteases was used to determine whether these charge species were isomers of PrP 27-30. Sucrose gradient fractions were radioiodinated with Na^{125}I by the chloramine T method. The high efficiency of labeling permitted detection of proteins such as PrP 27-30, which were present in low concentration in partially purified sucrose gradient fractions (4, 37; Bolton et al., in press). Charge isomers of the same size as PrP 27-30 were detected as a faint smear of radioiodinated proteins in scrapie fractions before protease digestion (Fig. 3A). After digestion with proteinase K, the sample contained significantly less detectable protein; prolonged exposure of the autoradiograph clearly revealed the presence of a series of spots similar to those described above (Fig. 3B).

Additional samples were radiolabeled with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate or with Na^{125}I by using Iodobeads. Analyses of these samples demonstrated that the migration of the major charged species of PrP 27-30 was not influenced by these chemical labeling procedures (Fig. 3C and D). Some minor charge modification of PrP 27-30 may have occurred after these procedures as evidenced by a noticeable broadening of the individual protein spots. The increased sensitivity for detecting PrP 27-30 obtained with radioiodination prompted us to use this method despite the possibility of introducing minor artifacts.

Disaggregation procedures. During the course of our investigation into the charge heterogeneity in PrP 27-30, we became concerned that procedures used to disaggregate the protein, which exists in rod-shaped or fibrillar structures in sucrose gradient fractions, might generate multiple charge isomers. We have found that boiling proteins used as molecular weight standards in SDS-PAGE sample buffer produced minor charge and size variants detectable by the NEPHGE-SDS-PAGE two-dimensional technique. To explore this question, we sought additional methods for disaggregating PrP 27-30 from its aggregated fibrillar state. Although considerable data suggested that the scrapie prion behaves like a hydrophobic particle, several recent observations show

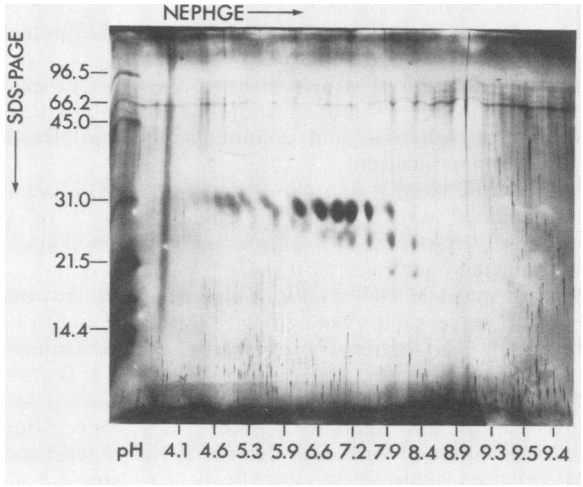

FIG. 2. Unmodified PrP 27-30 shows charge heterogeneity. A 200-μl sample of a zonal sucrose gradient fraction containing highly purified scrapie prions was diluted with 500 μl of 0.1 M sodium phosphate (pH 6.5) and sedimented for 60 min in a microcentrifuge at 4°C. The resulting pellet was suspended in 10 μl of 0.1 N HCl at 25°C by repeated pipetting with a micropipette for 2 to 3 min. The sample was then neutralized with 10 μl of 0.1 N NaOH, and 20 mg of urea was added followed by 20 μl of NEPHGE sample buffer. The proteins were separated in the NEPHGE first dimension for 1,800 Vh. The second-dimension SDS-PAGE gel contained 15% acrylamide. The gel was stained with silver by the method of Morrissey (33).

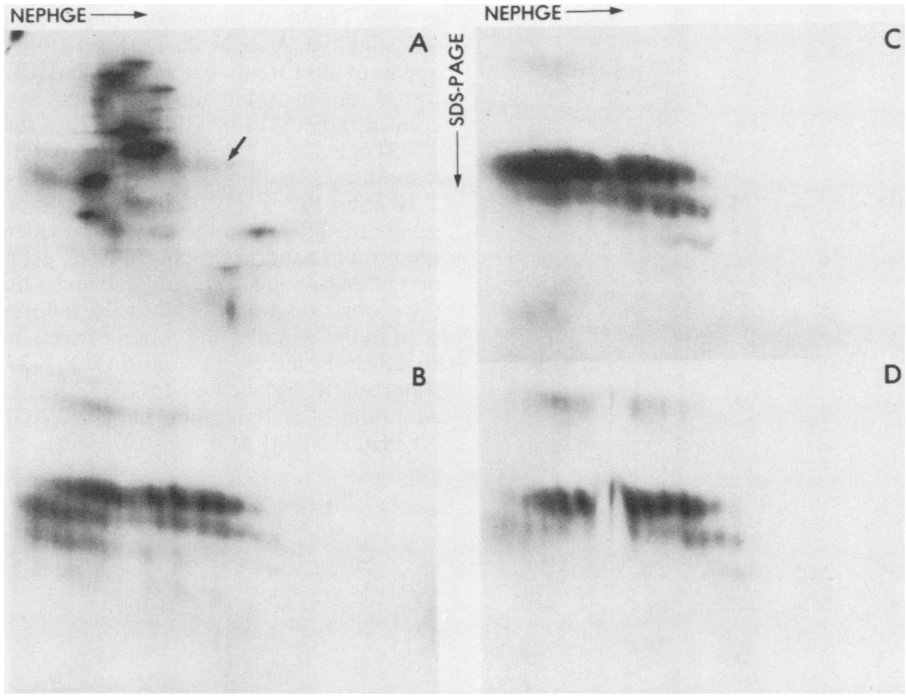

FIG. 3. Protease digestion and radioiodination do not alter charge heterogeneity. A sample of a scrapie sucrose gradient fraction was radioiodinated with $Na^{125}I$ by using chloramine T, and the proteins were removed from the remaining free iodine by precipitation with methanol (Bolton et al., in press). The resulting pellet was suspended in 10 mM Tris-hydrochloride–0.2% Sarkosyl (pH 7.4) and incubated at 25°C for 30 min in buffer alone (A) or 100 μg of proteinase K per ml (B). After this treatment, the proteins were again precipitated with methanol and separated by NEPHGE-SDS-PAGE. (A) Scrapie sucrose gradient fraction incubated in Tris-hydrochloride alone. The arrow indicates faintly visible charge isomers. The autoradiograph was exposed for 0.5 h. (B) Scrapie sucrose gradient fraction incubated in 100 μg of proteinase K per ml. The autoradiograph was exposed for 5.0 h. (C) A sample of a scrapie zonal sucrose gradient fraction containing highly purified prions was concentrated by sedimentation to a pellet in a microcentrifuge and radiolabeled with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate. The radioiodinated proteins were moved from the unreacted reagent by sedimentation to a pellet as before. The pellet was suspended in 0.1 N HCl, neutralized, and prepared for electrophoresis as described in the legend to Fig. 2. The autoradiograph was exposed for 15 h at 25°C. (D) A sample of a scrapie zonal sucrose gradient fraction containing highly purified prions was radioiodinated with $Na^{125}I$ by using Iodobeads. The radiolabeled proteins were removed from unreacted iodine by sedimentation to a pellet in a microcentrifuge for 60 min at 4°C. The pellet was suspended in 0.1 N HCl, neutralized, and prepared for electrophoresis as described in the legend to Fig. 2. The autoradiograph was exposed for 2 h at 25°C.

that the aggregation of PrP 27-30 may also be significantly altered by procedures that modify ionic interactions. Thus, we explored the disaggregation of PrP 27-30 at extreme pH values.

Disaggregation of rods containing PrP 27-30 was measured by determining the migration of the protein into 15% SDS-PAGE gels after treatment with NaOH or HCl (Fig. 4A). Suspending the rods in 0.1 N NaOH or 0.1 N NaOH containing 1% SDS was clearly effective at disaggregating PrP 27-30, as was 0.01 N NaOH containing 1% SDS. Disaggregation was less when 0.01 N NaOH, 0.1 N HCl, or 0.1 N HCl containing 1% SDS was used. Suspending the rods in 0.01 N HCl resulted in little measurable disaggregation, but inclusion of 1% SDS in the suspension aided monomerization of PrP 27-30. SDS-PAGE sample buffer was effective in disaggregating the prion rods at 100°C (Fig. 4A, lane 9) but had little effect at 25°C (data not shown). Treatment with 25 mM NaCl was equally ineffective (Fig. 4A, lane 10).

We also measured the effect of these disaggregation procedures on the conformation of PrP 27-30 by examining the protease resistance of the protein after treatment. In the native state, PrP 27-30 is resistant to digestion by a variety of proteases, but becomes sensitive to digestion upon denaturation (4, 31; Bolton et al., in press). Suspending PrP 27-30 in 0.1 N NaOH or 0.1 N NaOH containing 1% SDS by repeated pipetting at room temperature denatured the protein, resulting in digestion with proteinase K (Fig. 4B). Similar treatment with 0.01 N NaOH apparently did not denature the protein, as shown by its resistance to digestion. The addition of 1% SDS to the 0.01 N NaOH promoted denaturation and digestion.

Suspending PrP 27-30 in 0.1 N HCl containing 1% SDS, but not in 0.1 N HCl alone, denatured the protein, allowing significant digestion by proteinase K (Fig. 4B). PrP 27-30 also was not denatured after exposure to 0.01 N HCl, but exposure to 0.01 N HCl containing 1% SDS promoted denaturation and digestion by proteinase K.

Two-dimensional NEPHGE-SDS-PAGE analyses of fractions disaggregated by three of these procedures are shown in Fig. 5. Heating PrP 27-30 to 100°C for 5 min in SDS-PAGE sample buffer or suspending PrP 27-30 at room temperature in 0.01 N NaOH with 1% SDS or in 0.1 N HCl containing 1% SDS produced essentially identical results. Thus, these procedures resulted in disaggregation of prion rods and at least partial denaturation of PrP 27-30 and promoted penetration of the protein into the first-dimension NEPHGE gel (Fig. 5B and C).

Modification by acid and alkali. Our studies on the effects of acid and alkali on PrP 27-30 were extended by incubating

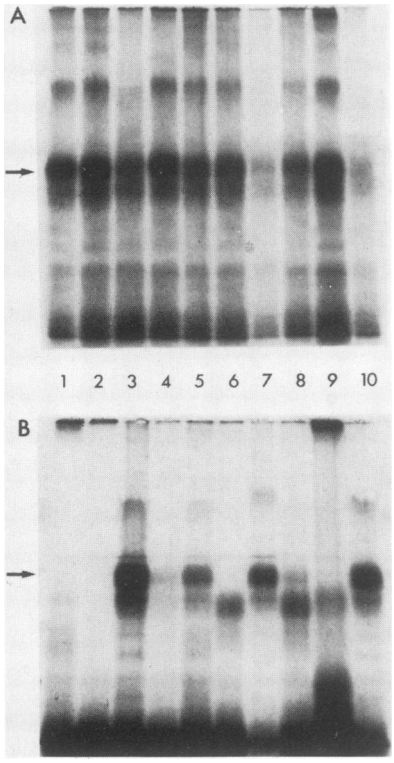

FIG. 4. Disaggregation and denaturation of PrP 27-30 in acid and alkali. A sample of a scrapie sucrose gradient fraction was radioiodinated with $Na^{125}I$ by using Iodobeads. The radiolabeled material was distributed into 10 samples, and each was sedimented to a pellet. The pellets were suspended in acid or alkali by repeated pipetting and then neutralized and analyzed for disaggregation of prion rods or denaturation of PrP 27-30. (A) Disaggregation of prion rods was measured as a function of the migration of PrP 27-30 into a 15% SDS-PAGE gel after addition of an equal volume of 2× SDS-PAGE sample buffer at 25°C. The autoradiograph was exposed for 3 h at 25°C. (B) Denaturation of PrP 27-30 was measured as a function of the susceptibility of the protein to digestion by proteinase K. Tris-hydrochloride (pH 7.4) was added to a final concentration of 10 mM, and proteinase K was added to a concentration of 100 μg/ml. The samples were incubated at 25°C for 30 min. The digestion was terminated by adding phenylmethylsulfonyl fluoride to 1 mM, an equal volume of 2× SDS-PAGE sample buffer was added, and immediately the samples were boiled for 5 min. The autoradiograph was exposed at 25°C for 16.3 h. The arrows show the position of PrP 27-30. The acid and alkali treatments were as follows. Lanes: 1, 0.1 N NaOH; 2, 0.1 N NaOH–1% SDS; 3, 0.01 N NaOH; 4, 0.01 N NaOH–1% SDS; 5, 0.1 N HCl; 6, 0.1 N HCl–1% SDS; 7, 0.01 N HCl; 8, 0.01 N HCl–1% SDS; 9, SDS-PAGE sample buffer, 100°C for 5 min; 10, 25 mM NaCl.

the protein in HCl or NaOH at higher temperatures. Disaggregation of PrP 27-30 was measured by increased penetration of the protein in polyacrylamide gels as described above. Radioiodinated PrP 27-30 was concentrated by sedimentation in a microcentrifuge. The pellets were suspended in NaOH or HCl solutions by repeated pipetting and incubated at 60°C for 30 min or 100°C for 15 min (Fig. 6A). At 60°C, either 0.1 or 0.01 N NaOH was as effective at disaggregating PrP 27-30 as boiling the sample in SDS-PAGE sample buffer. Heating with 0.001 N NaOH was ineffective. Some modification of PrP 27-30 was apparent after treatment with 0.1 N NaOH as seen by the increased smearing of PrP 27-30 in the gel during electrophoresis. Heating PrP 27-30 to 60°C in 0.1 N HCl was effective in disaggregating the protein, but lower concentrations of HCl were only marginally effective. A slight shift toward more rapid migration was apparent after treatment with 0.1 N HCl. Incubation at 60°C for 30 min in water alone followed by the addition of 2× concentrated SDS-PAGE buffer to a final concentration of 2% SDS and 5% β-mercaptoethanol did not result in significant disaggregation as measured by this technique.

Heating PrP 27-30 to 100°C in 0.1 N NaOH resulted in severe modification of the protein, evidenced by smearing of the protein band in the SDS-PAGE gel (Fig. 6A). Modification of PrP 27-30 also resulted from boiling in 0.1 N HCl, but this alteration was qualitatively different; no change was seen in the width of the protein band, but its migration was significantly increased (Fig. 6A). PrP 27-30 resuspended in water alone and heated to 100°C for 15 min exhibited no alteration of its migration in SDS-PAGE gels (Fig. 6A) or NEPHGE-SDS-PAGE gels (Fig. 6B).

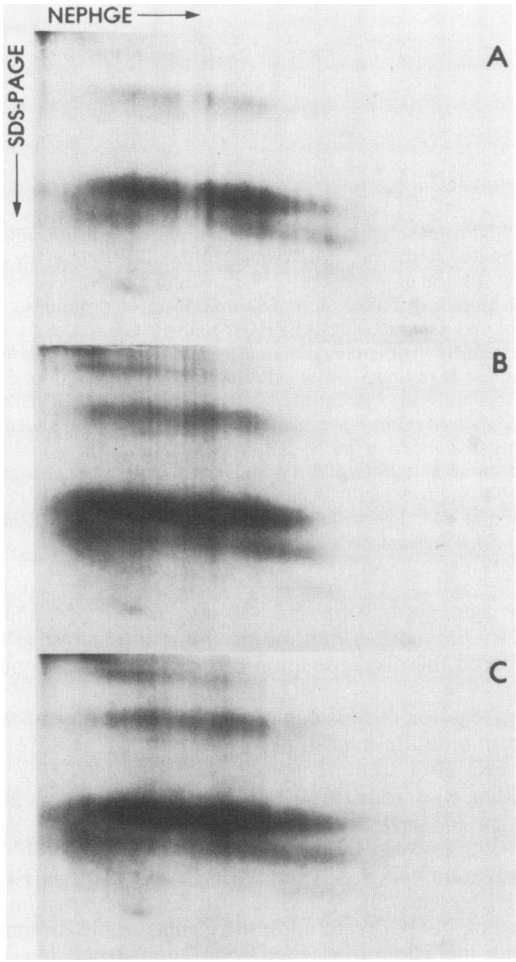

FIG. 5. Disaggregation procedures do not affect charge heterogeneity. Samples of a scrapie sucrose gradient fraction were radioiodinated with $Na^{125}I$ by using Iodobeads, and the proteins in these samples were removed from the unreacted iodine by sedimentation to a pellet. The pellets were suspended in (A) SDS-PAGE sample buffer followed by heating to 100°C for 5 min, (B) 0.01 N NaOH–1% SDS at 25°C for 1 to 2 min, or (C) 0.1 N HCl–1% SDS at 25°C for 1 to 2 min. Samples B and C were neutralized with the conjugate acid and base, and then 20-μl samples of each were prepared for NEPHGE-SDS-PAGE by adding an equal volume of NEPHGE sample buffer and 20 mg of urea. The autoradiographs were exposed for 17 h at 25°C.

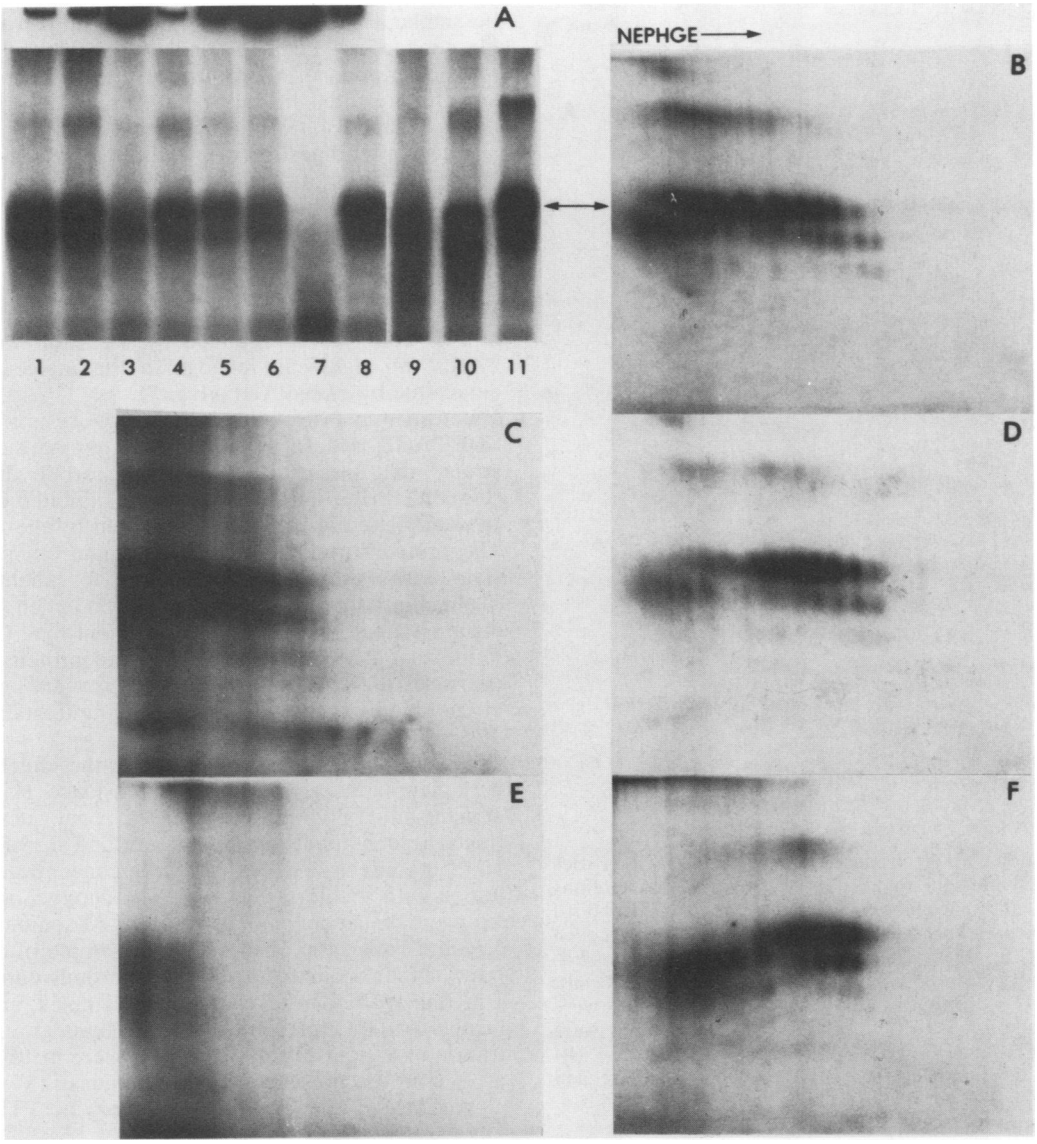

FIG. 6. Modification of PrP 27-30 by heating in acid and alkali. (A) Proteins in samples of a scrapie sucrose gradient fraction were radiolabeled with $Na^{125}I$ by using Iodobeads and concentrated by sedimentation to a pellet. The pellets were suspended in acid or alkali and heated to 60°C for 30 min (lanes 1 through 7) or to 100°C for 15 min (lanes 9 through 11). Disaggregation or modification of PrP 27-30 was assessed from its migration in a 15% SDS–PAGE gel. In lanes 1 through 8, the wells of the stacking gel are shown to indicate radiolabeled material that failed to enter the gel. The double arrow indicates the position of PrP 27-30. Lanes: 1, 0.1 N NaOH; 2, 0.01 N NaOH; 3, 0.001 N NaOH; 4, 0.1 N HCl; 5, 0.01 N HCl; 6, 0.001 N HCl; 7, water only; 8, heated to 100°C for 5 min in SDS-PAGE sample buffer; 9, 0.1 N NaOH; 10, 0.1 N HCl; 11, water only. (B through F) NEPHGE-SDS-PAGE separation of proteins treated under selected conditions as in A. (B) Proteins heated to 100°C for 15 min in water. The autoradiograph was exposed for 34 h. (C) Proteins heated to 60°C for 30 min in 0.1 N NaOH. The autoradiograph was exposed for 51 h. (D) Proteins heated to 60°C for 30 min in 0.1 N HCl. The autoradiograph was exposed for 39 h. (E) Proteins heated to 100°C for 15 min in 0.1 N NaOH. The autoradiograph was exposed for 37 h. (F) Proteins heated to 100°C for 15 min in 0.1 N HCl. The autoradiograph was exposed for 34 h.

Modification of PrP 27-30 during heating to 60°C for 30 min in 0.1 N NaOH was confirmed by NEPHGE-SDS-PAGE electrophoresis (Fig. 6C). In the first dimension, the charge isomers were shifted to a more acidic pH, and increased smearing was observed in the first and second dimensions. Boiling PrP 27-30 in 0.1 N NaOH caused severe modification of the protein. Electrophoresis in the NEPHGE-SDS-PAGE system confirmed the smeared, indistinct pattern observed with SDS-PAGE. In the two-dimensional system, PrP 27-30 appeared to be shifted to more acidic forms (Fig. 6E), but poor resolution made interpretation of this observation uncertain.

Incubation in 0.1 N HCl at 60 or 100°C had a different effect. The number of charge isomers was reduced, but distinct charged species were still observed, giving the impression that the acidic isomers of PrP 27-30 were converted to species with a more alkaline isoelectric point (Fig. 6D and F). The apparent decrease in the molecular weight of PrP 27-30 that was observed in SDS-PAGE after heating to 60°C in HCl (Fig. 6A) was accompanied by a shift toward more alkaline isoelectric points in the NEPHGE-SDS-PAGE system. These changes were seen more clearly after incubation at 100°C; four major charged species having isoelectric points between pH 6.9 and 7.9 are shown in Fig. 6F. These

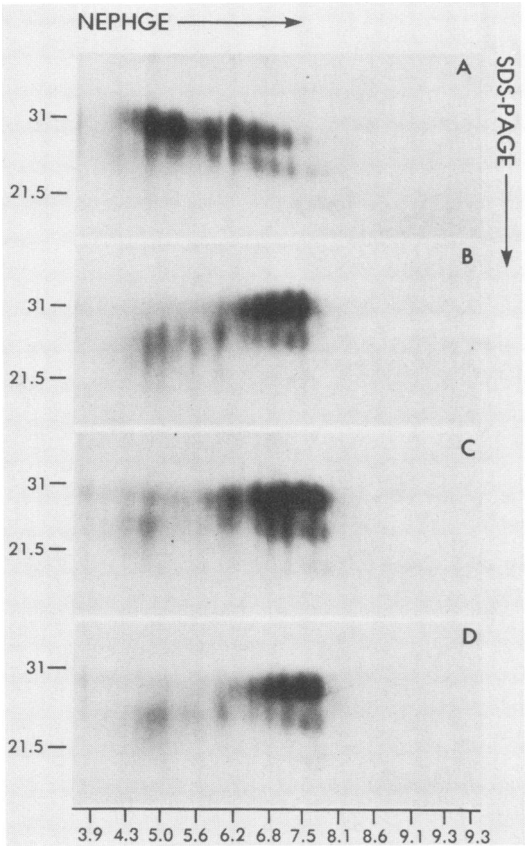

FIG. 7. Digestion with glycosidases alters charge characteristics of PrP 27-30. Proteins from zonal rotor sucrose gradient fractions were radioiodinated by the chloramine T procedure and then boiled in 0.1 M sodium acetate (pH 5.5) for 15 min. The proteins were then incubated at 37°C for 12 h at a protein concentration of approximately 0.7 mg/ml in the presence of neuraminidase (62 U/ml), endoglycosidase H (3 U/ml), or both enzymes. The digestions were terminated by heating to 100°C for 5 min, and the samples were prepared for NEPHGE-SDS-PAGE. (A) Digestion buffer alone. (B) Neuraminidase. (C) Endoglycosidase H. (D) Neuriminidase and endoglycosidase H. The autoradiographs were exposed for 16 h.

isomers coincide with the most alkaline forms of PrP 27-30 seen previously (Fig. 2 and 6B). These data are in agreement with the general observation that the alkaline species of PrP 27-30 migrated at smaller apparent molecular weights than did the acidic species (Fig. 1, 2, 3, 5, and 6B).

Digestion with glycosidases alters charge characteristics. Digestion of PrP 27-30 by neuraminidase or endoglycosidase H resulted in a significant alteration of the charge characteristics as seen by NEPHGE-SDS-PAGE. PrP 27-30 incubated in the presence of buffer alone produced the characteristic two-dimensional pattern of multiple charge isomers. At least eight discrete charge isomers were observed, ranging in isoelectric pH from approximately pH 4.6 to 7.5 (Fig. 7A).

Incubating the protein in the presence of neuraminidase caused an apparent shift in the isoelectric points of the isomers and reduced the number of isomers seen from eight to four major species having isoelectric points between pH 6.5 and 7.5 (Fig. 7B). The effect produced by digestion with endoglycosidase H was qualitatively similar to that caused by neuraminidase, except that six major isomers were observed (Fig. 7C). These species exhibited isoelectric points ranging from approximately pH 6.5 to 7.9. The isomers produced by digestion with endoglycosidase H appeared to be similar to those produced by digestion with neuraminidase, except that an additional species was found having an isoelectric point of approximately 7.9. Digestion with both enzymes resulted in the apparent conversion of the eight normal charge isomers to four major species, similar to what was seen after digestion with neuraminidase alone (Fig. 7D). However, three of these species appeared to coincide with the three most alkaline isomers produced by treatment with neuraminidase (Fig. 7B), whereas the fourth species aligned more closely with the most alkaline isomer produced by digestion with endoglycosidase H (Fig. 7C). We noted that, although significant alterations in the charge characteristics of PrP 27-30 were produced by digestion with these enzymes, no significant change in the molecular size was detectable by SDS-PAGE (Fig. 7).

Detection by PAS staining. Electrophoresis of PrP 27-30 by SDS-PAGE and subsequent PAS staining clearly demonstrated that the protein contained carbohydrate residues (Fig. 8A). PrP 27-30 and ovomucoid exhibited the pink color characteristic of glycoproteins stained by the PAS method. The proteins used as molecular weight standards did not stain with PAS (Fig. 8A), except for slight staining of ovalbumin, but could be easily seen when this gel was counterstained with Coomassie brilliant blue (Fig. 8B). We found that PrP 27-30 and ovomucoid protein retained the characteristic pink color of the PAS reagent and apparently did not bind Coomassie blue when counterstained with that dye.

An attempt was made to measure the effect of digestion with neuraminidase and endoglycosidase H on the PAS staining of PrP 27-30. Samples of zonal sucrose gradient fractions containing approximately 250 μg of protein and $10^{9.6}$ 50% infective dose units were concentrated by precipitation with trichloroacetic acid and deoxycholate. The pellets were resuspended in 25 μl of 0.1 M sodium acetate (pH 5.5) and boiled for 15 min. A 5-μl sample of an analogous fraction radioiodinated with ^{125}I at a protein concentration of 1 mg/ml was added to each of the samples, and they were heated to 100°C for 15 min. The proteins at a final concentration of approximately 6.4 mg/ml were incubated for 12 h at 37°C in the presence of neuraminidase (54 U/ml), endoglycosidase H (2.5 U/ml), or both enzymes. The digestions

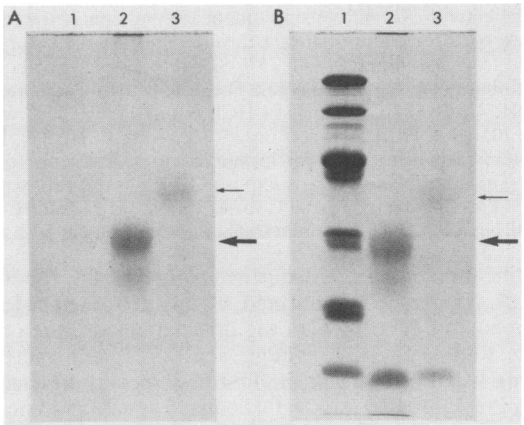

FIG. 8. PAS staining of PrP 27-30. Samples of zonal rotor sucrose gradient-purified scrapie prions were electrophoresed into an SDS-polyacrylamide gel. (A) Stained with PAS. (B) Same gel counterstained with Coomassie blue. Lanes: 1, molecular weight protein standards; 2, zonal rotor sucrose gradient scrapie fraction containing primarily PrP 27-30; 3, ovomucoid protein.

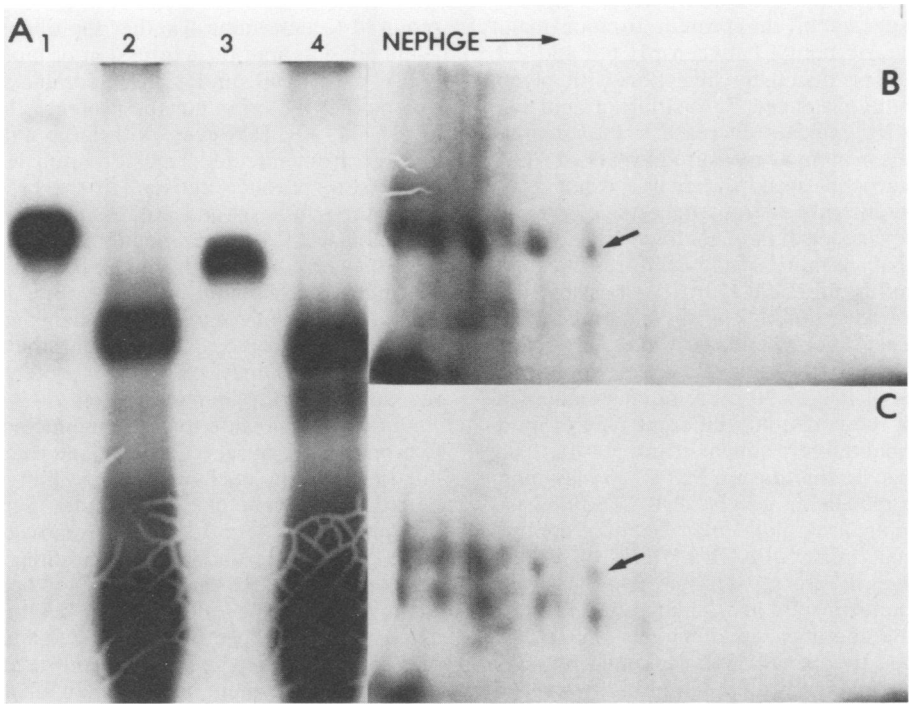

FIG. 9. CNBr cleavage of PrP 27-30 and a related protein. Purified fractions were prepared and cleaved with CNBr as described in the text. (A) Lanes: 1, gel-purified PrP 27-30; 2, CNBr cleavage fragments of PrP 27-30; 3, PrP-related protein, M_r of 24,000 to 27,000; 4, CNBr cleavage fragments of PrP-related protein. The separation was performed on a 20% SDS–PAGE gel. The autoradiograph was exposed for 45.5 h at 25°C. (B) NEPHGE-SDS-PAGE separation of PrP 27-30 CNBr cleavage fragments. (C) NEPHGE-SDS-PAGE separation of PrP-related protein CNBr cleavage fragments. The autoradiographs in B and C were exposed at 25°C for 13 days.

were stopped by heating the samples to 100°C for 30 min after adding an equal volume of SDS-PAGE sample buffer containing 2% SDS and 5% β-mercaptoethanol. Analysis of these samples by SDS-PAGE revealed no differences in the PAS staining characteristics or the electrophoretic mobility of PrP 27-30 during SDS-PAGE (data not shown).

Charge heterogeneity was not altered by digestion with alkaline phosphatase. Radioiodinated PrP 27-30 was heated in digestion buffer to 100°C for 15 min to disaggregate the prion rods and denature the protein. Digestion with CIAP or HPAP (25 U/ml) for 30 min did not alter the charge heterogeneity of PrP 27-30 (data not shown). Each phosphatase was shown to be active in the presence of PrP 27-30 by cleavage of phophate from *p*-nitrophenyl phosphate in reaction mixtures to which this substrate was added.

CNBr cleavage. Cleavage of PrP 27-30 with CNBr produced one major fragment (M_r, 17,100 to 21,900), which also was heterogeneous with respect to charge (Fig. 9). The charge isomers of this fragment migrated coincidentally with the isomers from the larger of two fragments generated by CNBr cleavage of a related protein that migrates ahead of PrP 27-30 in SDS-PAGE systems (Bolton et al., in press). Other studies have suggested that this smaller protein (PrP 23-26) represents a proteinase K digestion product of PrP 27-30 produced during purification of the prion (Bolton et al., in press). It is of interest that the differences in isoelectric pH found between each of the charge isomers of the CNBr fragments were greater than those demonstrated by the charge isomers of PrP 23-26, PrP 27-30, or acid-modified PrP 27-30.

DISCUSSION

The data presented in this report clearly demonstrate that oligosaccharides are attached to PrP 27-30 and that terminal sialic acids are responsible for at least some of the charge heterogeneity exhibited by this protein. However, it may be significant that extensive digestion of this protein by neuraminidase alone, or in conjunction with endoglycosidase H, failed to convert all of the charge isomers into a single species. Although this study does not provide direct evidence for phosphorylation as a source of charge heterogeneity, some of our observations raise the possibility that phosphorylation of tyrosine residues may contribute to the charge heterogeneity of PrP 27-30. It is possible that several different modifications may be responsible for the charge isomerization observed (14–16, 23).

Size microheterogeneity exhibited during SDS-PAGE is characteristic of many glycoproteins (10, 18, 19). This property was an important factor for the identification of PrP 27-30 because it distinguished this protein from other proteins of similar apparent M_r which were found in fractions prepared from both scrapie-infected and normal brain (4, 37). At the time of its discovery, the characteristic size heterogeneity of PrP 27-30 prompted speculation that it was a glycoprotein, but attempts to demonstrate the presence of oligosaccharides by PAS staining were unsuccessful until recently.

Sialoglycoproteins often show charge heterogeneity due to variation in the number of neuraminic acid residues added (2, 41). In this report, we have established that the presence of sialic acid on PrP 27-30 is responsible, at least in part, for the charge heterogeneity of this protein. Digestion with neuraminidase converted acidic forms of PrP 27-30 to four major species having isoelectric pH values between pH 6.5 and 7.5. Digesting PrP 27-30 with neuraminidase and endoglycosidase H also produced four major charge isomers, but these had isoelectric points between pH 6.8 and 7.9. We also found that heating PrP 27-30 in 0.1 N HCl appeared to

convert the acidic species of the protein to four major isomers having isoelectric points between pH 6.8 and 7.9. Contrary to the effect produced by digestion with glycosidases, the charge shift produced by heating in acid was also accompanied by a significant decrease in the apparent molecular weight of the protein as measured by SDS-PAGE. The remarkable similarity between the charge isomers obtained by these different procedures suggests a common chemical basis for their generation. This hypothesis is supported by reports that terminal sialic acid residues are hydrolyzed by heating in mild acid (10). It is possible that heating in acid may remove the bulk of the oligosaccharide chains in addition to neuraminic acid. However, it remains to be determined whether the acid treatment alters the charge characteristics of PrP 27-30 by removing sialic acid residues or whether a fundamentally different type of modification produces a qualitatively similar result (14–16, 23).

The presence of oligosaccharides on PrP 27-30 may influence estimates of its molecular weight. PrP 27-30 consistently migrates with an apparent molecular weight of approximately 27,000 to 30,000 in SDS-PAGE systems (4, 31, 37, 38, 40; Bolton et al., in press). The protein has been analyzed on gels of uniform total acrylamide concentration ranging from 13 to 20% and linear gradient gels of 5 to 20% total acrylamide. When the protein was separated by high-pressure liquid size exclusion chromatography, however, it eluted at a position suggesting an M_r of 19,500 to 21,000 (38). Subsequent analysis of the chromatographed PrP 27-30 showed that it continued to migrate with an M_r of 27,000 to 30,000 when analyzed by SDS-PAGE (38). Glycoproteins have been reported to migrate anomalously in SDS-PAGE systems, often resulting in artificially high estimates of their M_r (27, 35). However, in most cases the deviation of the observed M_r from the actual value was significant only in gels with acrylamide concentrations below 10%. The cause of the discrepancy between these estimates of M_r remains to be established (25, 38).

Equally intriguing is our observation that digestion with neuraminidase and endoglycosidase H, alone or in combination, resulted in charge modification of PrP 27-30, but did not alter its apparent M_r as measured by SDS-PAGE. This might indicate that an insignificant mass was removed from the protein during digestion by these enzymes or that the residues removed have little effect on either the Stokes radius or the amount of SDS bound to the protein (27). Assuming that the apparent shift observed indicates the removal of a minimum of four sialic acid residues from PrP 27-30, the decrease in mass should be approximately 1,200 daltons. In the absence of other counteracting effects, it seems likely that this decrease would be observable by SDS-PAGE. Leach et al. found that oligosaccharides retard the migration of glycoproteins during electrophoresis in SDS-PAGE systems primarily by decreasing the amount of SDS bound to the protein and thus decreasing the net charge of the SDS-protein complex (27). However, the overall effect of the oligosaccharides on the migration of glycoproteins in SDS-PAGE gels was not a direct function of the amount of carbohydrate attached. This is probably due to the unpredictable effect of the oligosaccharide portion on the Stokes radius of the SDS-protein complex (27, 35).

Inactivation of the scrapie agent by periodate (20, 21, 32, 43) has been reported. This property lent support to speculation that the agent might be a replicating polysaccharide, among other possibilities (8). The demonstration of oligosaccharides attached to PrP 27-30 provides a logical basis for interpreting those earlier results. Further studies will be required to determine whether the oligosaccharides on PrP 27-30 are essential for expression of biological activity.

It is clear from studies of the scrapie prion and PrP 27-30 that neither the prion nor the protein is highly immunogenic (7, 21, 24, 30). However, with large amounts of PrP 27-30 isolated from infected hamster brain it recently has been possible to produce antisera to the protein in rabbits (1). Since the synthesis and processing of the oligosaccharides found on PrP 27-30 are undoubtedly performed by the host cell, the structure of the oligosaccharide chains probably resembles those found on many other host cell glycoproteins. If these oligosaccharide chains cover a substantial portion of the surface of the protein, their similarity to other host structures may effectively protect the protein from recognition by the immune system.

Our results indicate that neuraminic acids are responsible for some of the observed charge heterogeneity of PrP 27-30, but they do not exclude the possibility that other charge modifications may also contribute. Serine and threonine phosphates are reported to be removed from proteins by heating in alkali under the conditions we used but are relatively stable to degradation by heating in acid (17). Removal of serine or threonine phophates by heating in alkali would be expected to produced a shift toward alkaline pH. This appears to be in direct contrast to our observations with PrP 27-30, where heating in an alkali resulted in a shift toward forms having an acidic isoelectric point. This observation was somewhat obscured by the apparent modification and degradation of the protein which occurred during the treatment. Covalent modification of proteins heated in strong alkali is well documented (46), but the relevance of the modifications to the charge heterogeneity of PrP 27-30 remains uncertain.

Heating PrP 27-30 in the presence of acid reduced the charge variation concomitant with a reduction in molecular size. It has been reported that tyrosine phosphate bonds are cleaved under these conditions (17). Whether boiling in 0.1 N HCl affects the net charge of PrP 27-30 by removing tyrosine phosphates or by cleaving terminal sialic acid residues is uncertain. Digesting PrP 27-30 with two different alkaline phosphatases had no effect on charge distribution. These enzymes have been reported to be more active in cleaving tyrosine phosphates than serine and threonine phosphates (44). However, radioiodination at tyrosine residues of PrP 27-30 may have inhibited cleavage of tyrosine phosphate bonds by CIAP and HPAP.

Amino acid analyses of homogeneous PrP 27-30 indicate the presence of 18 methionine residues within the molecule, assuming that it contains 240 amino acid residues (38). After cleavage of PrP 27-30 with CNBr, we detected one major fragment and two or three minor fragments. Failure to detect any PrP 27-30 after cleavage indicates that at least one of the methionine sites in the protein is cleaved uniformly. It is not clear whether the discrepancy between the number of methionine residues and the number of CNBr fragments is due to close spacing of methionine residues resulting in fragments of small size or to a complete lack of cleavage at some of the methionine residues. Our data indicate that at least some of the groups responsible for charge heterogeneity in PrP 27-30 are stable to incubation in formic acid during cleavage with CNBr and reside on the major peptide fragment generated by this chemical cleavage procedure. These charged groups also are found on PrP 23-26, the major CNBr fragment of this protein, and a smaller CNBr fragment that is not seen after CNBr cleavage of PrP 27-30. These similarities between the CNBr fragments of PrP 23-26 and PrP 27-30

support our hypothesis that these proteins are related (Bolton et al., in press). Other studies have shown that these proteins possess cross-reacting antigenic determinants (1) and closely related peptide maps (D. C. Bolton and S. B. Prusiner, unpublished experiments).

An understanding of the cause of the charge heterogeneity of PrP 27-30 should provide a basis for more rapid or efficient methods for purifying PrP 27-30. Determining the composition of the oligosaccharides of this unusual protein should facilitate structural studies. Charge heterogeneity in the major scrapie prion protein may have mechanistic and theoretical implications as well. Post-translational modification could be a required step in the conversion of inert PrP 27-30 to a biologically active protein or may play a role in protecting the protein from degradation during the long incubation period of the disease. Glycosylation as well as other possible covalent modifications may be important in regulating the expression of PrP 27-30 or other macromolecules that play a role in the disease process.

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