Properties of the Scrapie Prion Protein: Quantitative Analysis of Protease Resistance†

Bruno Oesch,*,‡ Margit Jensen,§ Povl Nilsson,§ and Jens Fogh§

Brain Research Institute, University of Zürich, August-Forelstrasse 1, 8029 Zürich, Switzerland, and Novo Nordisk, Niels Steensen Vej 1, 2820 Gentofte, Denmark

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ABSTRACT: The disease-specific isoform of the prion protein (PrP^{Sc}) is an essential part of the infectious particle which causes spongiform degeneration in various mammalian species. PrP^{Sc} differs from PrP of normal animals (PrP^{C}) by its relative protease resistance. The physical nature of this difference is still unknown. We analyzed the protease resistance of PrP^{Sc} quantitatively using an enzyme-linked immunofiltration assay. PrP^{Sc} was rendered completely protease-sensitive at alkaline pH or in >1.5 M guanidinium thiocyanate (GdnSCN). Denaturation in 4 M GdnSCN completely abolished the protease resistance of PrP^{Sc} within 15 min, while denaturation in 7.2 M urea showed a slower time course. In the presence of ethanol, PrP^{Sc} was protected from denaturation by GdnSCN or alkaline pH. Denaturation curves were used to calculate the free energy (ΔG_D) as a function of different denaturant concentrations. Linear regression of ΔG_D values was used to extrapolate the free energy in the absence of denaturants (ΔG_{H_2O}), yielding similar values ($\Delta G_{H_2O,GdnSCN} = -2.3$ kcal/mol; $\Delta G_{H_2O,urea} = -3.1$ kcal/mol). The linear relationship between ΔG_D and the denaturant concentration is suggestive of a two-state model involving the conformational change of a single protein domain. This is also reflected in the small number of side chains (11.6) additionally exposed to the solvent upon conversion of PrP^{Sc} to its protease-sensitive isoform. Our results suggest that only minor rearrangements of the structure of PrP are needed to abolish the protease resistance of PrP^{Sc}.

The disease-specific isoform of the prion protein (PrP^{Sc}) is part of the infectious particle causing scrapie in sheep, bovine spongiform encephalopathy in cattle, or Creutzfeldt–Jakob disease in humans. PrP^{Sc} differs from the normal cellular prion protein (PrP^{C}) by its relative protease resistance (Bolton et al., 1982; Oesch et al., 1985). The molecular changes leading to this difference in physicochemical properties are unknown. PrP^{Sc} to date has never been separated from infectivity (McKinley et al., 1983; Gabizon et al., 1988; DeArmond et al., 1987; Sklaviadis et al., 1989). This led to the proposal that the infectious particle would be composed of specifically altered PrP molecules (Prusiner, 1991).

Various hypotheses regarding these alterations have been disproved. The amino acid sequence of PrP^{Sc} is identical to that predicted from cDNA or genomic nucleic acid sequences (Oesch et al., 1985; Chesebro et al., 1985; Stahl et al., 1993), and the infectious particle does not encode an altered PrP gene (Oesch et al., 1985). In cell culture, PrP^{C} is converted into PrP^{Sc} posttranslationally (Caughey et al., 1989; Borchelt et al., 1990). Inhibition of asparagine-linked glycosylation did not prevent the synthesis of protease-resistant PrP^{Sc} (Taraboulos et al., 1990). No difference in covalent modifications of PrP^{Sc} and PrP^{C} was observed using mass spectrometry (Stahl et al., 1991, 1993). As an alternative, it was proposed that PrP^{C} might interact with a second component (most likely a nucleic acid), leading to a modification of the properties of PrP (Bruce & Dickinson, 1987; Hope et al., 1986; Weissmann, 1991b). Prion-specific nucleic acids are still elusive (Oesch et al., 1988; Kellings et al., 1992), while two peptides have been observed reproducibly in PrP^{Sc} preparations (Stahl et al., 1993). These peptides may be indicative of other proteinaceous components present in prion preparations such as nucleic acid binding proteins (Sklaviadis et al., 1993) or proteoglycans which have been colocalized with PrP amyloid deposits (Nochlin et al., 1989; Snow et al., 1990). However, the role of these compounds in scrapie remains to be clarified.

The lack of a molecular explanation for the observed differences between PrP^{Sc} and PrP^{C} led to the proposal that they differ in conformation (Basler et al., 1986; Stahl & Prusiner, 1991; Stahl et al., 1993). By infrared spectroscopy, a high content of β-sheet structure was detected for PrP 27–30, the protease-resistant core of PrP^{Sc} (Caughey et al., 1991; Gasset et al., 1993). A reduced content of β-sheet structure is found in PrP^{C}, suggesting differences in secondary structure between the two isoforms of PrP (Pan et al., 1993).

The relative protease resistance of PrP^{Sc} is currently the only way to distinguish the two forms of PrP (Oesch et al., 1985). Serban et al. (1990) developed an assay to detect PrP^{Sc} that involved immobilization of proteins on nitrocellulose followed by protease digestion, denaturation, and immunodetection with monoclonal antibodies. To quantitate the amount of PrP^{Sc}, we have refined an enzyme-linked immunofiltration assay (ELIFA) for PrP^{Sc} (Prusiner et al., 1990). Protease resistance of PrP^{Sc} was reduced at basic pH or by incubation in chaotropic reagents while the presence of ethanol protected PrP^{Sc} from denaturation. We have also analyzed the thermodynamic parameters for the conversion of PrP^{Sc} to a protease-sensitive form, suggesting only a minor difference in free energy between the two isoforms.

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* To whom correspondence should be addressed. e-mail: oesch@hifo.unizh.ch.

‡ University of Zürich.

§ Novo Nordisk.

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MATERIALS AND METHODS

Materials. Scrapie brain homogenates, purified PrP 27–30, and 13A5 monoclonal antibodies were a kind gift of Dr. S. B. Prusiner, University of California, San Francisco. Avidin and biotin were purchased from Boehringer Mannheim. Proteinase K and human serum albumin (HSA) were from Sigma. Streptavidin coupled to peroxidase and the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for peroxidase were from Kirkegaard and Perry Laboratories, Gaitherburg, MD; other chemicals were purchased from Fluka, Buchs, Switzerland. Nitrocellulose (0.2 μm) and the ELIFA blotting apparatus were from Pierce.

Standard ELIFA Procedure. Samples were applied to nitrocellulose filters (0.2 μm) in the ELIFA apparatus in a 100-μL final volume containing 1 M guanidinium thiocyanate (GdnSCN) and 5 mM DTT. Appropriate dilutions (1:200 to 1:2000) were applied. Wells were rinsed with 100 μL of sample dilution buffer (1 M GdnSCN, 0.01% HSA, 150 mM NaCl, and 10 mM $NaPO_4$, pH 7.4). Filters were taken out of the block, washed in water, dried at room temperature (RT) for 30 min, and then wetted in 150 mM NaCl and 10 mM $NaPO_4$, pH 7.4 (PBS), followed by incubation in 1% KOH for 5 min. Filters were then washed in PBS followed by 0.5% HSA in 0.05% Tween 20 and PBS (PBST) for 30 min. All incubations were done at room temperature unless otherwise indicated, and filters were washed 3 times for 5 min with PBST between steps. Biotin in the applied samples was blocked by incubation in 25 μg/mL avidin/PBST followed by 2 μg/mL *d*-biotin/PBST for 30 min each. Biotinylated monoclonal antibody 13A5 (Prusiner et al., 1990) was incubated in PBST for 14–18 h at 4 °C on a rocking platform. After being washed in PBST, filters were incubated in 0.1 μg/mL streptavidin coupled to peroxidase for 1 h. For detection, filters were placed back in the ELIFA block and the substrate (0.2 mg/mL TMB, 0.01% H_2O_2) was pulled through into an ELISA plate placed underneath. Color was stabilized by addition of 100 μL of 1 M H_3PO_4. OD_{450} versus OD_{600} was determined in an SLT 400 ATX ELISA reader (SLT Labinstruments, Gröding, Austria).

PrP Standards. Purified PrP 27–30 was denatured in 4 M GdnSCN for 1 h at 37 °C and then diluted to 1 M GdnSCN and stored at a concentration of 2 μg/mL at −70 °C. The amount of PrP was determined by amino acid analysis as described (Stahl et al., 1990). A standard curve for various amounts of PrP (0.1–2 ng/well) was generated using GdnSCN-denatured PrP 27–30. The lower limit of detection was 0.1 ng of purified PrP 27–30. To control for variability, each filter contained duplicates of PrP standards. The parameters for the standard curve were obtained by linear regression analysis and then used for the calculation of the PrP quantity in test samples. The correlation coefficient of the standard curves was usually greater than 0.98.

Detection of PrP^{Sc}. Ten percent brain homogenates from terminally ill, scrapie-infected hamsters were centrifuged at 1000*g* for 20 min. Pelleted nuclei were discarded. The supernatant (cleared scrapie brain homogenate, cSBH) was stored at −70 °C in aliquots to be used in further experiments. The amount of PrP^{Sc} in a given sample was determined as follows: samples were digested with 50 μg/mL proteinase K for 30 min at 37 °C. Proteinase K was inactivated by the addition of 7.5 mM PMSF. Samples then were denatured in 4 M GdnSCN for 1 h at 37 °C, diluted to 1 M GdnSCN, and applied to nitrocellulose as described above. As a control, half of the native sample was immediately denatured in 4 M GdnSCN, diluted to 1 M GdnSCN, and then either digested with proteinase K, yielding the background of the particular assay, or applied untreated, giving the total amount of PrP.

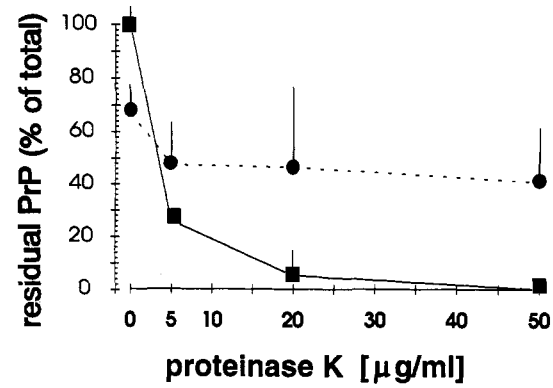

FIGURE 1: Sensitivity of PrP^{C}, PrP^{Sc}, and denatured PrP to digestion with proteinase K. Native (●) or 4 M GdnSCN denatured (■) brain homogenates were treated with the indicated concentrations of proteinase K. PrP is expressed relative to the amount of total PrP in denatured, untreated homogenates.

Treatment of cSBH with GdnSCN, Urea, Different pH, and Ethanol. cSBH (1/10th volume) was mixed with 6 M GdnSCN or 8 M urea to give a final 10-fold dilution with the appropriate denaturant concentration. Samples were incubated at 37 °C for 1 h and diluted 4-fold, and residual PrP^{Sc} was determined. The buffers for the different pH's were as follows: pH 2–6, 100 mM citric acid adjusted with NaOH; pH 7, 7.4, 8, and 8.5, 100 mM Tris base adjusted with HCl; pH 9–11, 100 mM Na_2CO_3 adjusted with HCl. Incubation at the various pH's was done at 37 °C for 1 h. For treatment with ethanol, incubation buffers were mixed to 9/10 of the final volume followed by the addition of 0.1 volume cSBH. All samples were incubated at 37 °C for 1 h followed by determination of PrP^{Sc}.

RESULTS

Enzyme-Linked Immunofiltration Assay (ELIFA) for the Scrapie-Specific Isoform of Prion Protein. PrP^{C} is defined as the protease-sensitive form of PrP, whereas PrP^{Sc} is partially protease-resistant. PrP^{C} is readily detected, but native, protease-resistant PrP^{Sc} is not recognized by antibodies. Therefore, PrP^{Sc} needs to be denatured prior to immunodetection (Kitamoto et al., 1987; Serban et al., 1990; Prusiner et al., 1990). We applied an ELIFA system to quantitate PrP. Briefly, protein samples were applied to nitrocellulose using a slot-blot apparatus. Membranes then were incubated with biotinylated anti-PrP monoclonal antibodies (13A5; Prusiner et al., 1990) followed by streptavidin coupled to peroxidase. For quantitation, the membrane was placed back on the slot-blot apparatus and soluble reaction product was pulled into ELISA plates placed underneath. Subsequently, color development was quantitated in a conventional ELISA reader.

The distinction between PrP^{C} and PrP^{Sc} was made by the digestion of samples with various amounts of proteinase K (Figure 1). Subsequently, proteinase K was blocked with PMSF, and the sample was denatured with 4 M GdnSCN and then used in the ELIFA procedure described above. PrP^{Sc} was measured relative to the total amount of PrP present in the same sample without proteinase K digestion. About 50% of total PrP could not be digested with proteinase K concentrations up to 50 μg/mL (Figure 1, circles). As a control, extracts were first denatured in 4 M GdnSCN and then treated with proteinase K, resulting in complete digestion of all PrP (Figure 1, squares). Surprisingly, the undenatured sample contained less PrP at 0 μg/mL proteinase K than the

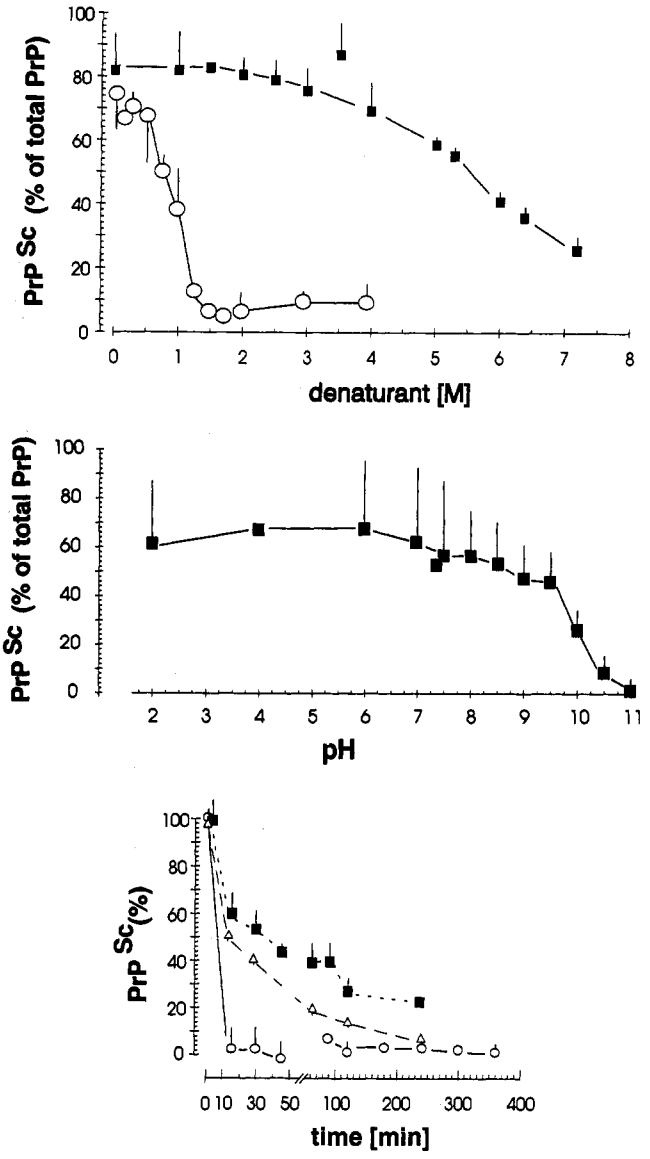

FIGURE 2: Reduction of PrP^{Sc} by incubation in GdnSCN or urea or at different pH's. (A, top) Cleared brain homogenates were incubated in various concentrations of GdnSCN (○) or urea (■) for 1 h at 37 °C followed by determination of residual protease-resistant PrP. (B, middle) Residual PrP^{Sc} after incubation at the indicated pH. (C, bottom) Time course of denaturation of PrP^{Sc} by 4 M GdnSCN (○), by 7.2 M urea (■), or at pH 11 (△). PrP^{Sc} at 0 min was set at 100%.

GdnSCN-denatured sample. We suspect that endogenous proteases degrade PrP^{C} in the native sample during the incubation at 37 °C, and that these proteases may be inactivated in the samples first denatured in GdnSCN.

Treatments Rendering PrP^{Sc} Protease-Sensitive. Brain extracts containing PrP^{Sc} were incubated in various concentrations of GdnSCN or urea (Figure 2A) or at different pH's (Figure 2B). Incubation in 1.5 M GdnSCN rendered PrP^{Sc} completely protease-sensitive. Urea at the highest concentration (7.2 M) reduced PrP^{Sc} to approx. 25% (Figure 2A). The same result was observed when the samples were incubated at 37 or 4 °C (results not shown). As a control for proteinase K activity, denatured PrP^{Sc} was treated in parallel under the same conditions, resulting in complete digestion of all PrP (results not shown). The transition midpoints for conversion of PrP^{Sc} to protease-sensitive PrP were at 0.87 M GdnSCN and 5.8 M urea, respectively.

Changes in the protease resistance of PrP^{Sc} were also measured after incubation at various pH's (Figure 2B). It has been suggested that PrP^{Sc} undergoes pH-dependent conformational changes (Gasset et al., 1993). The amount of PrP^{Sc} was unchanged following incubation at various pH's between 2 and 9.5, while it decreased at pH 10. PrP^{Sc} was no longer detectable after incubation at pH 11.

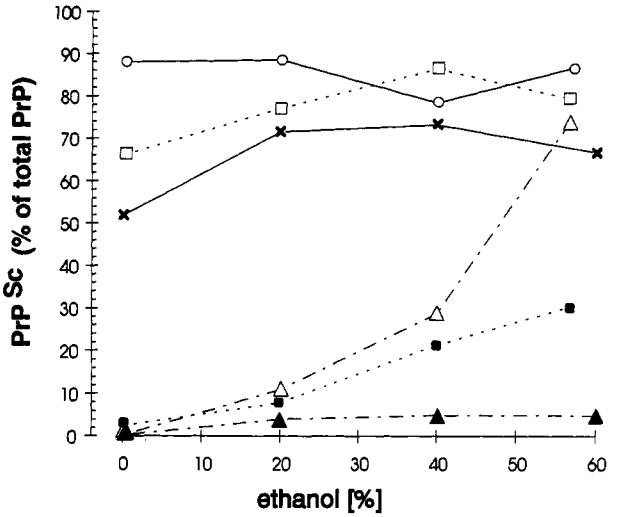

FIGURE 3: Effect of ethanol on denaturation of PrP^{Sc}. Brain extracts were incubated at pH 7 (○), pH 2 (□), pH 11 (■), 6.9 M urea (×), or 4 M GdnSCN (△, ▲) in the presence of the indicated concentrations of ethanol. For the incubations in GdnSCN, ethanol was added either simultaneously (△) or 15 min after the GdnSCN (▲).

The time course of denaturation was analyzed for treatment with GdnSCN (4 M) or urea (7.2 M) and at pH 11 (Figure 2C). Denaturation of PrP^{Sc} was complete after 15 min with GdnSCN, while denaturation by urea was slower with a half-maximal inactivation at 30 min. The time course of denaturation at pH 11 was intermediate between those with GdnSCN and urea denaturation; i.e., half-maximal denaturation was observed at 15 min (Figure 2C). Incubation at 37, 20, or 4 °C gave the same results (not shown). Gasset et al. (1993) found that an irreversible change in conformation occurs at alkaline pH. This conformational change can therefore be correlated with increased solubility of PrP^{Sc}, a loss in infectivity, and a loss of protease resistance (Gasset et al., 1993; Figure 2B).

Protection of PrP^{Sc} by the Presence of Ethanol. Ethanol is used commonly to dehydrate proteinaceous samples, often leading to denaturation (Touati et al., 1992; Pan & Briggs, 1992; Ramalingam & Bello, 1993; Fan et al., 1993; Wang et al., 1993). We were interested to see whether dehydration of PrP^{Sc} would facilitate denaturation. PrP^{Sc} was incubated in increasing concentrations of ethanol. Under standard conditions (pH 7) we did not find any change in PrP^{Sc} levels with concentrations up to 60% ethanol (Figure 3, open squares). When we used increasing concentrations of ethanol in combination with 6.9 M urea, or pH 2 we observed a slight increase in PrP^{Sc} indicating that PrP^{Sc} might be protected from denaturation in the presence of ethanol. This effect is much more pronounced upon treatment with 4 M GdnSCN: in the absence of ethanol all PrP^{Sc} is converted into the protease-sensitive form, while in the presence of 60% ethanol the denaturing effect of GdnSCN is completely neutralized (open triangles). When GdnSCN was added first followed by the addition of various concentrations of ethanol, we did not find any recovery of PrP^{Sc}, indicating that ethanol is unable to restore protease resistance (closed triangles). Similarly, treatment at pH 11 reduced PrP^{Sc} to background levels in the absence of ethanol but was less effective at higher concentrations of ethanol.

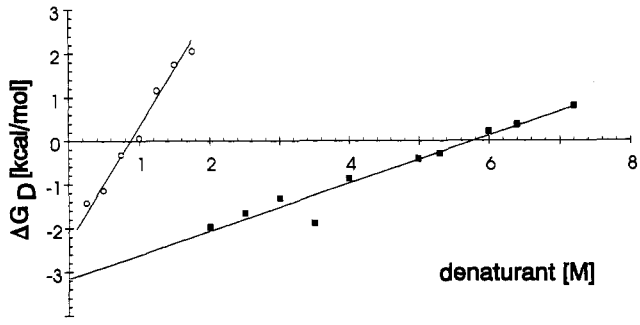

FIGURE 4: Dependence of the free energy, ΔG_D, on denaturant concentration. The denaturation curves from Figure 2 were used to calculate ΔG_D as a function of the GdnSCN (○) or urea concentration (■) as described in Results. The lines were determined by linear regression.

Transition of PrP^{Sc} to the Protease-Sensitive Isoform Requires Only Small Changes in Secondary Structure. The unfolding of proteins in denaturants has been studied extensively using spectroscopic methods (Dill & Shortle, 1991; Zerovnik et al., 1992; Dyer et al., 1992; Staniforth et al., 1993; Pappa & Cass, 1993; Ahmad et al., 1992). The resulting denaturation curves are analyzed for the number of transitions which indicate unfolding of independent domains. In addition, the free energy and the number of additionally exposed amino acid side chains can be derived. We assumed that the secondary or tertiary structure of PrP would be the cause of protease resistance. Therefore, denaturation curves were measured using the protease sensitivity of PrP as a parameter to follow the changes in conformation (Figure 2). Ideally, we would be able to derive information about the nature of the scrapie-specific part in PrP^{Sc}. We were first interested in the change of free energy (ΔG_D) upon denaturation of PrP^{Sc} as a function of the concentration of GdnSCN or urea. ΔG_D is calculated according to the relationship

$$\Delta G = -RT \ln K \quad (1)$$

where K is the ratio of protease-resistant to protease-sensitive PrP, which has been determined experimentally (Figure 2). The ΔG_D values were plotted versus the denaturant concentration itself (Figure 4). Linear regression analysis revealed correlation coeffients of $r = 0.988$ (GdnSCN) and $r = 0.971$ (urea) indicative of a single transition midpoint for both denaturants. Thus, the conversion of PrP^{Sc} to the protease-sensitive form of PrP appears to depend on the conformational change of a single domain. Extrapolation of ΔG_D values to a value in the absence of denaturant (ΔG_{H_2O}) yielded similar ΔG_{H_2O} values for both denaturants ($\Delta G_{H_2O,GdnSCN} = -2.3$ kcal/mol; $\Delta G_{H_2O,urea} = -3.1$ kcal/mol; Figure 4).

Assuming that most of the energy requirement would be due to exposure of previously buried amino acid side chains (Staniforth et al., 1993), the number of newly exposed side chains (n_e) was calculated according to the equation

$$\Delta G_D = \Delta G_{H_2O} + n_e \Delta G_{s,d} \quad (2)$$

where $\Delta G_{s,d}$ is the difference in solvation energy required to expose an average side chain as a function of the denaturant concentration. $\Delta G_{s,d}$ can be calculated according to the formula

$$\Delta G_{s,d} = (\sum \Delta G_{si} n_i)/n \quad (3)$$

where ΔG_{si} is the free energy required to expose a particular side chain, n_i is the number of side chains of type i in the solvent-excluded core, and n is the total number of side chains in the core. ΔG_{si} values for different concentrations of urea were taken from Staniforth et al. (1993). The exposure of side chains in PrP^{Sc} to the solvent is unknown; hence we have applied average values determined from 55 proteins in the Brookhaven Protein Data Bank. It has been shown that these values can be applied to proteins with unknown secondary structure (Staniforth et al., 1993). These calculations were applied to the protease-resistant core of PrP^{Sc} (amino acids 89–231). Solving eqs 2 and 3 for concentrations of 4–7.2 M urea, we calculated the number of additionally exposed amino acid side chains (n_e) to be 11.6 ± 0.49. Thus, the conversion of PrP^{Sc} to its protease-sensitive form seemed to require only a minor change in conformation.

DISCUSSION

The protease resistance of PrP^{Sc} is the major property which is specific for the infectious particle. The nature of the presumed alterations is defined by exclusion: (1) There are no substitutions in the amino acid sequence (Oesch et al., 1985; Hope et al., 1986; Basler et al., 1986; Stahl et al., 1993). (2) No PrP^{Sc}-specific posttranslational modifications have been found; both forms of PrP have N-linked carbohydrates and a glycolipid anchor (Haraguchi et al., 1989; Stahl et al., 1987). It is not known whether modifications of two arginines at the N-terminus of PrP^{Sc} are also found in PrP^{C} (Hope et al., 1986; Stahl et al., 1993). (3) No molecules other than PrP^{Sc} are linked to infectivity (Bolton et al., 1982; Hope et al., 1986; Gabizon et al., 1987). Therefore, it was proposed that PrP^{Sc} and PrP^{C} differ in their conformation (Basler et al., 1986; Stahl et al., 1993).

Taking these previous findings as a basis for our analysis of denaturation curves of PrP^{Sc}, we assumed that the incubation in denaturants would lead to an alteration of PrP^{Sc} conformation. Our calculations revealed a difference in free energy of 2.3–3.1 kcal/mol and the exposure of 11.6 additional amino acid side chains for the transition from PrP^{Sc} to the protease-sensitive form of PrP. The relatively small change in free energy and additionally solvent-exposed amino acid side chains makes it unlikely that there is a complete unfolding of the polypeptide chain but rather the unfolding of a specific domain. Unfolding of other proteins typically exposes 40–80 amino acid side chains per domain; i.e., phosphoglycerate kinase (PGK) has three transitions with ΔG_{H_2O} values of 3.8, 10.8, and 8.2 kcal/mol and exposure of 55.5, 84.8, and 51 additional side chains, respectively (Staniforth et al., 1993). This represents a total of 191.3 additionally exposed side chains, which is equivalent to the calculated number of solvent-excluded amino acid side chains of the native molecule. Therefore, all the side chains are exposed to the solvent in the fully denatured protein. Comparing these values to the denaturation parameters of PrP^{Sc}, we conclude that only minor perturbations of the structure of PrP lead to the conversion of PrP^{Sc} to protease-sensitive PrP. The secondary structure of PrP^{Sc} and PrP^{C} has also been investigated by infrared spectroscopy (Caughey et al., 1991; Gasset et al., 1993; Pan et al., 1993). A high proportion of β-sheet structure was present in purified PrP^{Sc}, while the secondary structure of PrP^{C} appears to be predominantly α-helical (Pan et al., 1993). It will therefore be important to analyze the folding of specific domains of PrP by independent methods.

What fraction of the energy difference between PrP^{Sc} and protease-sensitive PrP can be attributed to the scrapie-specific part of PrP^{Sc}? Our calculations above suggest that a specific domain of PrP is folded differently in PrP^{Sc} and PrP^{C} and that

this difference in folding may be responsible for the protease resistance of PrP^{Sc}. ΔG values for the denaturation of this domain in PrP^{C} are not available; however, the region between amino acids 106 and 126 spontaneously forms amyloid which is partially protease resistant (Gasset et al., 1992; Selvaggini et al., 1993). The protease-resistant conformation of this region therefore seems to be energetically favored. The same domain in PrP^{C} may be unable to adopt this conformation. PrP^{Sc} may therefore be the energetically more stable form; however, the formation of PrP^{Sc} would normally be prevented due to a higher activation energy (Weissmann, 1991a).

Alternative explanations for the protease resistance of PrP^{Sc} suggest an association of PrP with other cellular (or viral) components such as nucleic acids or proteoglycans (Snow & Wight, 1989; Snow et al., 1989; Czub et al., 1988; Diringer et al., 1991; Sklaviadis et al., 1993). It also has been suggested that nucleation-dependent polymerization of PrP is directly involved in the pathogenic mechanism of scrapie (Jarrett & Lansbury, 1993). PrP^{Sc} would be in an equilibrium between an aggregated form (amyloid) and a soluble form. If the aggregation was the reason for the protease resistance of PrP^{Sc}, we could interpret the difference in free energy, ΔG_{H_2O}, as a function of the equilibrium between free and amyloid-bound PrP^{Sc}. Under normal conditions (37 °C) this equilibrium would be on the side of the amyloid by a factor of 40 or higher ($\ln([\text{amyloid}]/[\text{free}]) = -\Delta G/RT$). The number of additionally exposed amino acids could then be interpreted as the size of the contact area between PrP molecules. These conclusions are particularly interesting in view of mutations in the prion protein linked to the occurrence of prion diseases in humans. Mice transgenic with mutant PrP (Pro → Leu at codon 102; Hsiao et al., 1990) did not produce significant amounts of protease-resistant PrP even though these animals had spontaneous prion disease. Mutant prion proteins may not be driven into a different conformation but rather might change more easily between conformations or, in the latter model, between the free and amyloid states.

We have reported here that ethanol stabilized PrP^{Sc} against denaturation by GdnSCN (Figure 3). In other proteins (β-globin, apocytochrome *c*, monellin, ubiquitin) alcohols restrict the conformational flexibility (Acharya et al., 1992; Fan et al., 1993; Pan & Briggs, 1992). This leads to a decreased susceptibility of β-globin, apocytochrome *c*, and the streptococcal PepM49 protein to proteolysis (Acharya et al., 1992). The gradual effect of increasing concentrations of ethanol suggests that the observed protease resistance is not due to precipitation of proteins. However, the aggregated state of PrP^{Sc} may be stabilized by the presence of ethanol. Alternatively, alcohols like ethanol, trifluoroethanol, or *n*-propanol induce α-helical structures. The presence of 50% ethanol induces the change of monellin from all β-sheet to α-helix (Fan et al., 1993). We would therefore have expected a destabilization of PrP^{Sc}, which is known to have a large content of β-sheet structure (Caughey et al., 1991; Gasset et al., 1993), while PrP^{C} is predominantly α-helical (Pan et al., 1993). Since we observe a stabilization of PrP^{Sc}, we cannot easily reconcile these findings with structural predictions.

The comparison of conditions to inactivate prions and to reduce protease resistance of PrP^{Sc} shows an excellent correlation. Infectivity and the protease resistance of PrP^{Sc} are reduced at alkaline pH and by urea (Gasset et al., 1993; Prusiner et al., 1981, 1993; Mould et al., 1965). Denaturation of prions by 1 M GdnSCN for 1 h did not reduce infectivity, while in 2 M GdnSCN infectivity was reduced 1000-fold (Prusiner et al., 1993). This correlates quite well with the reduction in protease resistance of PrP^{Sc} observed in our experiments even though we would have predicted a loss of ~50% of infectivity at 1 M GdnSCN after 1 h. This may be due to the relative imprecision of the infectivity assay, where a reduction by a factor of 2 is very difficult to determine.

CONCLUSIONS

We have characterized the nature of the protease resistance of PrP^{Sc} through the analysis of unfolding induced by GdnSCN or urea. The most striking result is the apparently low free energy required to convert PrP^{Sc} into a protease-sensitive form. Only a small number of amino acids are exposed additionally upon destruction of protease resistance. These seemingly minor requirements to destroy the protease resistance of PrP^{Sc} contrast with the high stability of prions against procedures which readily inactivate other infectious particles and may reflect the proteinaceous rather than viral nature of the infectious particles causing scrapie, bovine spongiform encephalopathy, or Creutzfeldt–Jakob disease.

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