INFLUENCE OF GUANIDINE ON PROTEINASE K RESISTANCE *IN VITRO* AND INFECTIVITY OF SCRAPIE PRION PROTEIN PRP^{Sc}

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Received March 30, 2005; accepted January 16, 2006

Summary. – As the scrapie prion protein PrP^{Sc} is rich in ß-sheets it aggregates into prion rods, which show infectivity and proteinase K (PK) resistance. Consequently, dissociation of prion rods and breakdown of β-sheets in PrP^{Sc} by denaturation results in loss of both infectivity and PK-sensitivity. In this study, the effects of guanidine (Gdn), which solubilizes and denatures proteins by breaking down their higher structure, on the solubility, the PK-resistance *in vitro* and the infectivity of PrP^{Sc} of scrapie strain 263K was examined. The infectivity was assayed by intracerebral inoculation into hamsters. Brain tissues of scrapie-infected hamsters were used for preparation of homogenates and crude extracts of PrP^{Sc}. A treatment of PrP^{Sc} with Gdn enhanced its PK-sensitivity in a dose-dependent manner. The PK-resistance *in vitro* of PrP^{Sc} denatured with lower concentrations of Gdn (<2.5 mol/l) could partially resume by renaturation. Gdn markedly reduced or, at higher concentrations, even destroyed the infectivity of PrP^{Sc}. On the other hand, the infectivity of PrP^{Sc} inactivated by denaturation could be partially restored by renaturation. These results confirmed our assumption that all the alternations in the PK-resistance and the infectivity of PrP^{Sc} caused by Gdn resulted from changes in its higher structure. However, it should be emphasized that a complete loss of PK-resistance of PrP^{Sc} may not necessarily mean its full non-infectivity.

Key words: scrapie; prions; PrP^{Sc}; guanidine; proteinase K; infectivity

Introduction

Several lines of evidences have indicated that the infectious agent of transmissible spongioform encephalopathies (TSE) or prion diseases consists largely, if not completely, of an abnormal, detergent-insoluble, partially PK-resistant protein PrP^{Sc}, which is derived from a normal, host-coded, detergent-soluble, PK-sensitive protein PrP^{C} (Weissmann, 1999; Prusiner, 1998). Both PrP^{C} and PrP^{Sc} are encoded by the same *prnp* gene, do not differ in post-translational modification and have the same primary structure. They differ only in conformation, higher structure (Prusiner, 1998; Riek *et al.,* 1996; James, 1997).

Some denaturation studies indicate that the infectivity of scrapie agents correlate partially with their specific features, e.g. the protease resistances, the state of aggregation and the ability to convert PrP^{C} to PRP^{Sc} (Caughey *et al.*, 1995, 1997; Kocisko *et al.*, 1994). Moreover, PrP^{Sc} aggregates with characteristic partial PK-resistance of amino acids 90–232 have been always associated with infectivity and converting activity (Caughey *et al.*, 1995; Diringer *et al.*, 1983; McKinley *et al.*, 1983; Kocisko *et al.*, 1996). Some studies have shown that natural or recombinant PrP^{C} could become PK-resistant under certain conditions *in vitro* (e.g. following a treatment with bivalent metal ions Cu and Mn, detergents SDS and sodium N-lauroyl sarkosinate at low concentrations or reducing agents at low pH) in the absence of PrP^{Sc}, but could not become infectious in bioassays (Jackson *et al.,*

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Abbreviations: Gdn = guanidine; PK = proteinase K; PrP^{C} = cellular prion protein; PrP^{Sc} = scrapie prion protein; TSE = transmissible spongiform encephalopathies

1999; Nandi and Leclerc, 1999; Brown *et al.*, 2000; Morillas *et al.*, 2001; Quaglio *et al.*, 2001; Xiong *et al.*, 2001). Cell-free conversion studies *in vitro* have yielded PrP^{Sc}-like molecules, PrP^{Res} with PK-resistance, but these molecules have not been proven infective (Kocisko *et al.*, 1994; Kaneko *et al.*, 1995; Hill *et al.*, 1999). These findings suggest that the conversion of PrP^{C} into a PK-resistant and detergent-insoluble protein is not the only crucial step in prion replication and TSE pathogenesis. Moreover, in some cases, the prion infectivity could be demonstrated in the absence of detectable PK-resistant PrP^{Sc} (Hsiao *et al.*, 1994; Lasmezas *et al.*, 1997). This suggests that each prion strain could be associated with a characteristic PK-resistant core formed by a defined PrP^{Sc} conformation (Telling *et al.*, 1996; Collinge *et al.*, 1996).

Gdn, frequently used for solubilization of proteins, denatures them by breaking down their higher structure. Gdn in high concentrations induces a loss of scrapie infectivity, accompanied by an irreversible loss of the PK-resistance *in vitro* (Caughey *et al.*, 1997; Kocisko *et al.*, 1994). Literature data quote diverse Gdn concentrations causing reversible changes in infectivity or PK-resistance *in vitro* (Kocisko *et al*., 1994, 1996; McKenzie *et al.*, 1998; Callahan *et al.*, 2001). However, not only the Gdn concentration, but also other factors influencing Gdn experiments could affect their results (McKenzie *et al.*, 1998; Zou and Cashman, 2002). E.g., the PrP^{Sc} used in some studies (Caughey *et al.*, 1991; Bolton *et al.*, 1987) was obtained by a high-speed gradient centrifugation, a method hardly available in each laboratory; furthermore, there immediately arisis a question whether such a laboratory-prepared PrP^{Sc} is identical with the PrP^{Sc} present in the brain infected with scrapie. Recently, a study has put forward that the prion infectivity might depend upon or at least be largely facilitated by the presence of other components of prion rods (Shaked *et al.*, 2001). Whether certain factors present normally in the brain may influence the reversibility of PrP aggregation, and furthermore, whether different purification procedures may affect prion rods, consequently alter their structure or biochemical properties, e.g. the infectivity, the resistance to proteinases or the refolding process, are questions awaiting solution.

In order to analyze the effect of Gdn on the scrapie PrP^{Sc}, brain homogenates, probably at most ensuring native status of prion rods, and crude extracts of PrP^{Sc} were prepared from hamsters infected with the scrapie strain 263K. After treatment with different concentrations of Gdn, the PK-resistance *in vitro* and the infectivity of PrP^{Sc} in these preparations were evaluated. The results showed that Gdn in dose-dependent manner reduced or even destroyed both the PK resistance and the infectivity of the agent. Removal of Gdn from the preparations, leading to renaturation of the agent, restored both activities.

Materials and Methods

Scrapie. A hamster-adapted strain of scrapie, 263K (Kimberlin and Walker, 1977, 1978) has been passaged several times in this laboratory (Gao *et al.*, 2004; Zhang *et al.*, 2004).

Antibodies. A PrP-specific monoclonal antibody 3F4 was obtained from Dako Company (Denmark). A horseradish peroxidase-conjugated anti-mouse IgG was obtained from Santa Cruz Biotechnology (Denmark).

Brain homogenates. To prepare 10% brain homogenates, scrapie-infectd female Syrian hamsters showing typical scrapie manifestations were sacrificed. Their brain tissues were collected and homogenized in a lysis buffer (100 mmol/l NaCl, 10 mmol/l EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mmol/l Tris-HCl pH 7.5) at 4°C. Aliquots of brain homogenates were stored at -70°C for further use.

Crude extracts of PrP^{Sc} were prepared as described earlier (Chen *et al.*, 1997; Zhang *et al.*, 2002). Briefly, 800 µl of 10% brain homogenate was clarified at 3,000 x g for 10 mins and pelleted at 20,000 x g for 90 mins. The pellets were suspended in 80 µl of distilled water and stored at -70°C until used.

Gdn treatment. A stock solution of 7.5 mol/l Gdn (guanidine hydrochloride, Genview, USA) was employed. Gdn was added to crude extract (80 µl) to final concentration of 1.0–6.0 mol/l and adjusted with with TN buffer (10 mmol/l Tris-HCl and 130 mmol/l NaCl, pH 7.0) to final volume of 400 µl (Kocisko *et al*., 1994).

If not otherwise quoted, samples were incubated at 37°C for 12 hrs. One aliquot was immediately mixed with five volumes of cooled methanol and kept at -20°C for 2 hrs. The precipitated proteins were pelleted by centrifugation at 12,000 x g for 30 mins and resuspended in TN buffer (the denatured sample). In another aliquot, Gdn was diluted dropwise with TN to a final concentration of 0.75 mol/l. After 24 or 48 hrs total proteins were collected by the methanol precipitation and resuspended as described above (the renatured sample). After treatments with or without 50 µg/ml PK at 37°C for 1 hr, the samples were mixed with a 2x loading buffer, boiled and subjected to Western blot analysis .

Quantitative Western blot analysis. The proteins separated by SDS-PAGE (15%) were blotted onto nitrocellulose membranes using a Trans-Blot apparatus. After blocking with 5% non-fat milk in TBST buffer (a Tris-buffered saline pH 7.6 containing 0.05% Tween 20) overnight at 4°C, the blots were incubated with a PrP-specific monoclonal antibody (3F4) for 2 hrs at room temperature. PrP bands were visualized with a horseradish peroxidase-conjugated anti-mouse IgG. Quantitative analysis of the immunoblots was carried out using a computer-assisted software Image Total-Tech (Pharmacia). Briefly, the immunoblots were scanned (Typhoon, Pharmacia) and saved in a TIF format. A recombinant hamster PrP (Zhang *et al.*, 2002) was used as a standard.

Analysis of solubility of PrP in Gdn. The brain extracts treated with various concentrations of Gdn were pelleted at 20,000 x g at 4°C for 90 mins, the obtained pellets (after solubilization with TN buffer) and supernatants were precipitated by five volumes of ice-cold methanol, and each preparation was subjected to SDS-PAGE and quantitative Western blot analysis.

Bioassay of infectivity of PrP^{Sc}. Two-week-old female Syrian hamsters were intracerebrally inioculated with PrP^{Sc} samples treated or

untretaed with Gdn. Each dose corresponding to 2.1 x 10^{-4} g of infected brain tissue was supplemented with ampicillin (100 μg/ml) and injected into parietal lobe to a depth of 4–5 mm. In order to maintain PrP^{Sc} in the denatured status, all samples were diluted with PBS 1 min before inoculation. All hamsters were feed at liberty in cages. Ataxia was considered a clinical manifestation of experimental scrapie.

Necropsy was performed within 8 hrs after natural death or immediately after sacrificing almost dead or live animals under the condition of ether anesthesia. The brains were removed surgically and used for preparation of the homogenates.

Results

Gdn solubilizes PrP^{Sc}

To see the influence of Gdn on the solubility of PrP^{Sc}, crude extracts of PrP^{Sc} from brains of scrapie-infected hamsters were treated with a various concentration of Gdn for 12 hrs, soluble as well as insoluble proteins were purified and assayed for PrP^{Sc} content by quantitative Western blot analysis. The results showed (Fig. 1) that the solublilty of PrP increased with the Gdn concentration. In 4 mol/l and 5 mol/l Gdn PrP^{Sc} was completely soluble.

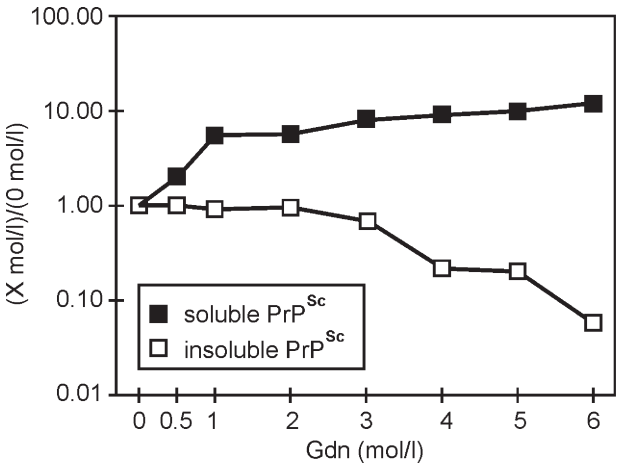

Fig. 1

Solubility of PrP^{Sc} in Gdn

The ordinate: the ratios (X mol/l)/(0 mol/l) of the amounts of PrP corresponding to individual concentrations of Gdn (X mol/l) to those corresponding to water (0 mol/l).

Gdn enhances the PK-sensitivity of PrP^{Sc}

To address the PK-sensitivity of PrP^{Sc} brain homogenates (Fig. 2A) or crude extracts of PrP^{Sc} (Fig. 2B) these were first treated with various concentrations of Gdn (1.0–6.0 mol/l) for 12 hrs and assayed for PrP^{Sc} by quantitative Western blot analysis. As expected, three PrP^{Sc}-specific bands in the range of 30–35 K representing diglycosyl, monoglycosyl and aglycosyl isoforms were observed in all samples. Their intensities did not differ significantly. Besides these three

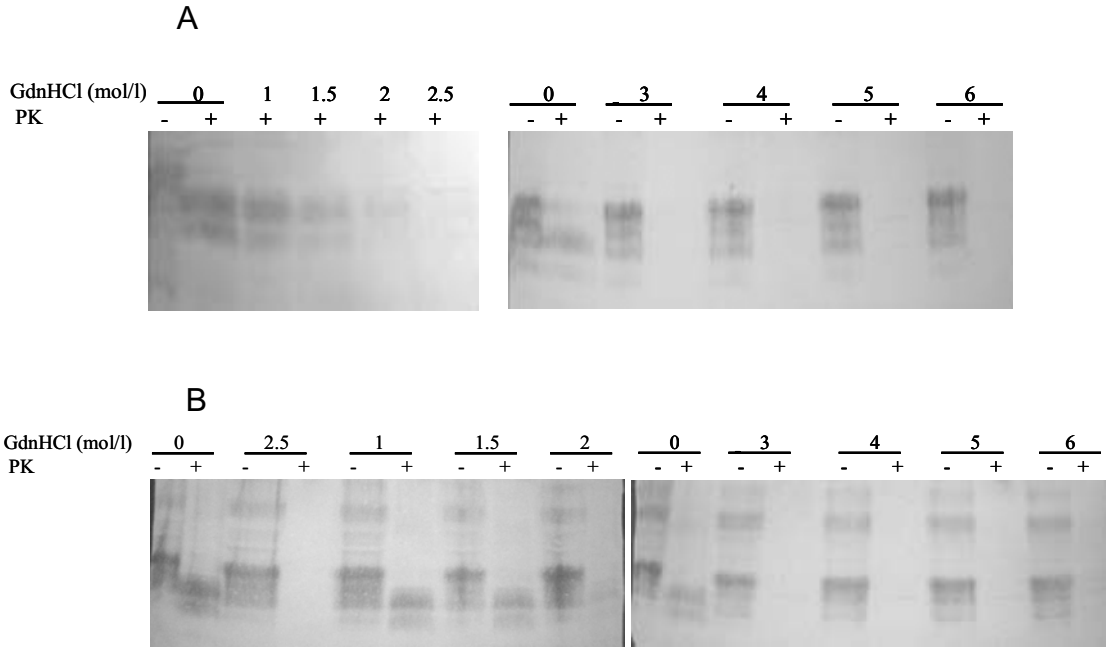

Fig. 2

Effect of Gdn on the PK-resistance *in vitro* of PrP^{Sc}

Western blot analysis. Brain homogenates (A) and crude extracts of PrP^{Sc} (B).

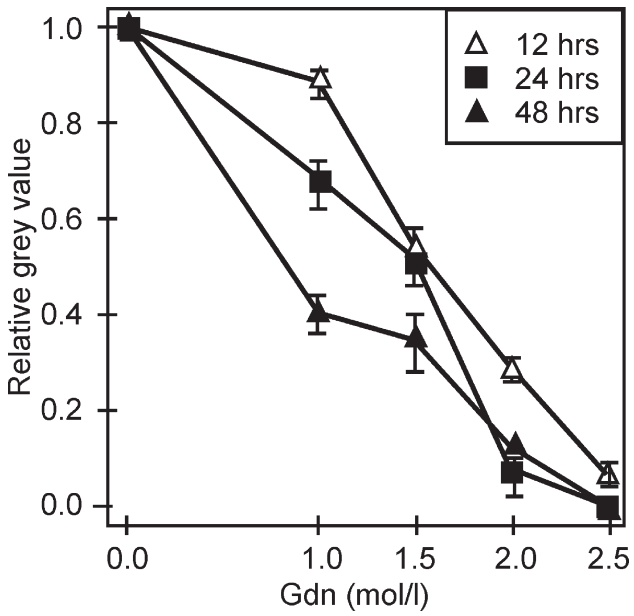

Fig. 3

PK-resistance i*n vitro* of PrP^{Sc} denatured with Gdn

The ordinate: PrP^{Sc} amounts (averages from 2–3 independent experiments).

bands also high molecular material of 45–90 K was clearly seen in all samples. These results suggested that the treatment of PrP^{Sc} with various concentrations of Gdn did not change its characteristic electrophoretic pattern, glycosylation and immunoreactivity.

Next, the PK sensitivity of Gdn-treated and thus denatured PrP^{Sc} was examined. The results showed that while 1.0–2.0 mol/l Gdn led to PK-resistant bands of 20–27 K (identical with controls), Gdn in concentrations ≥ 2.5 mol/l resulted in disappearance of these bands (Fig. 3).

The intensity of PK-resistant bands depended on Gdn concentration. Namely, 1, 1.5 and 2 mol/l Gdn caused a reduction to 89%, 52% and 29% of control, respectively. These results showed that the treatment of PrP^{Sc} with Gdn facilitated its proteolysis with PK.

To see whether the duration of denaturation of PrP^{Sc} with Gdn influences its PK-sensitivity the incubation time was prolonged to 24 and 48 hrs. The results showed that the intensity of PK-resistant PrP signals for 1, 1.5 and 2 mol/l Gdn dropped to 68%, 52% and 8% after 24 hrs, and to 41%, 35% and 12% after 48 hrs, respectively (Fig. 4). The PK-resistant signal dropped to undetectable level with 2.5 mol/l Gdn after 24 and 48 hrs. All these results confirmed the enhancing effect of Gdn on the PK-sensitivity of PrP^{Sc}.

Renaturation partially resumes the PK-resistance of PrP^{Sc} denatured with Gdn

To see whether renaturation may influence the PK-sensitivity of PrP^{Sc} denatured with Gdn, the PrP^{Sc} preparations containing Gdn were gradually (in 24–48 hrs) diluted with TN to final concentration of 0.75 mol/l Gdn, digested with PK and assayed for PrP^{Sc} by the quantitative Western blot analysis. The results revealed that with Gdn in concentrations ≥3 mol/l no PK-resistant PrP signals were observed. However, with 1.0–2.5 mol/l Gdn, PK-resistant signals increased (Fig. 4). A prolongation of the renaturation time from 24 to 48 hrs resulted in a slight increase of the PK-resistance (Fig. 5). It seems that a part of PrP^{Sc} that loosed PK-resistance in lower concentration of Gdn was able to resume its activity during renaturation.

The infectivity of PrP^{Sc} denatured with Gdn can be only partially restored by renaturation

The effects of denaturation with various concentrations of Gdn and subsequent renaturation on the infectivity of PrP^{Sc} was examined using brain homogenates. All the animals inoculated with untreated PrP^{Sc} (positive control)

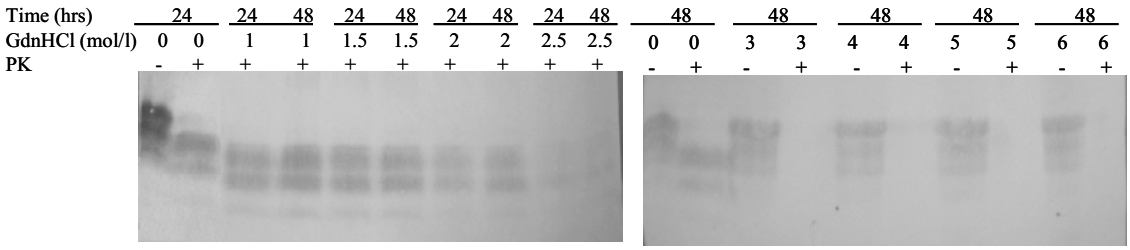

Fig. 4

Effect of renaturation on the PK-resistance *in vitro* of PrP^{Sc} denatured with Gdn

Western blot analysis.

became ill with the mean incubation time of 79.1 ± 8.6 days and their brain tissues exhibited the presence of PK-resistant PrP^{Sc} in Western blots. On the other hand, the brain tissues from healthy animals did not show any PK-resistant PrP^{Sc} bands (Table 1).

In the infectivity experiments with PrP^{Sc} denatured with Gdn, 1.5 mol/l Gdn resulted in a prolonged incubation time (120 ± 14.1 days), while 3 mol/l and 6 mol/l Gdn did not produce any clinical signs of the disease within 200 days post inoculation (Table 1). It suggests that the infectivity of PrP^{Sc} denatured with Gdn is markedly reduced or even fully destroyed in dependence on concentration.

In the infectivity experiments with Gdn-denatured and subsequently renatured PrP^{Sc}, the Gdn concentration turned out to be crucial. While the only effect with 1.5 mol/l Gdn was a slightly shorter incubation time (107.7 ± 4.8 days) for renatured vs. denatured preparation, 3 mol/l and 6 mol/l Gdn had marked effects on all the three markers examined: clinical manifestations and PrP^{Sc} appeared in significant number of animals and the incubation time markedly decreased (Table 1).

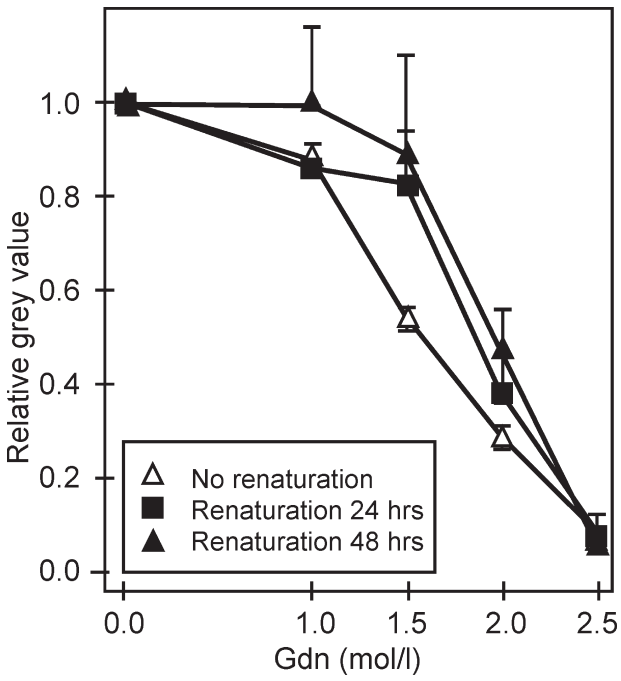

Fig. 5

Effect of prolonged renaturation on the PK-resistance *in vitro* of PrP^{Sc} denatured with lower concentrations of Gdn

The ordinate: PrP^{Sc} amounts (averages from 2–3 independent experiments).

Discussion

Conformational conversion of PrP^{C} to PrP^{Sc} is commonly considered the major cause of PK-resistance *in vitro*, solubility, infectivity as well as self-propagating ability of PrP^{Sc} (Prusiner, 1998; Caughey *et al.*, 1995; Diringer *et al.*, 1983; McKinley *et al.*, 1983). Gdn, an unspecific solvent, markedly affects the conformation (higher structure) of proteins generally and those associated with neuro-degeneration disorders like scrapie, Alzheimer disease etc (Safar *et al*., 1993; Roher *et al*., 1988) particularly, but does not influence their primary structure. Unlike the effects of NaOH and NaClO on PrP^{Sc} isolated from brain tissues of animals infected with scrapie, characteristic by disappearance of the signals of PrP^{Sc} from Western blots in the case of higher concentrations of these agents regardless of the PK-treatment (Yao *et al.,* 2004), the signal intensities and the electrophoresis patterns of the PrP^{Sc} prior to PK treatment remained unchanged in all preparations of Gdn.

Table 1. Clinical manifestation, presence of PrP^{Sc} and incubation time in hamsters inoculated with brain homogenates subjected to denaturation with Gdn and renaturation

Inoculum		Clinical	Incubation time	PrP^{Sc}
Denaturation (Gdn mol/l)	Renaturation	manifestation[a,b]	(days)	presence[b]
0[d]	–	8/8	79.1 ± 8.6	8/8
1.5	–	5/6	121.8 ± 14.1[c]	5/6
1.5	+	4/5	107.5 ± 4.8[c]	4/5
3.0	–	0/4	> 180	0/4
3.0	+	5/6	130.6 ± 28.6[c]	5/6
6.0	–	0/5	>180	0/5
6.0	+	2/5	118 ± 0.0	2/5

[a]Ataxia.
[b]Positive/total.
[c]$P \leq 0.01$ vs. positive control.
[d]Positive control.

It may imply that all the alternations both in PK resistance and infectivity of PrP^{Sc} following Gdn treatment result from the changes in its higher, secondary and/or tertiary structure (Gasset *et al.*, 1993; Safar *et al.*, 1993).

One of special characteristics of PrP^{Sc} is its insolubility in common protein solvents, so that PrP^{Sc} can be separated from PrP^{C} by a low-speed centrifugation (Meyer *et al.*, 1986). It is believed that an increased β-sheet content partially contributes to this feature of PrP^{Sc} (Caughey *et al.*, 1991). However, the solubility of PrP^{Sc} like other proteins with aggregating tendency can be improved through increasing the concentration of solvents. The variable distribution of PrP^{Sc} in soluble and insoluble forms in solutions of different Gdn concentrations implies that the ß-sheet content of PrP^{Sc} was probably reduced by Gdn through breaking inter-strand hydrogen bonds and, thereby, the solubility of PrP^{Sc} increased.

In this study, we observed that a renaturation of PrP^{Sc} denatured with 2 mol/l Gdn was possible but that with ≥2.5 mol/l Gdn was not. Thus the threshold Gdn concentration enabling a reversible denaturation observed in this study was slightly different from those reported by Kocisko *et al.* (1996) (≥3.5 mol/l) and Kaneko *et al.* (1995) (≥3 mol/l).

These discrepancies can arise from different PrP^{Sc} preparation methods leading to differences in the PK-sensitivity of PrP^{Sc}. It has been shown that different PrP^{Sc} preparation methods may lead to different changes in the higher structure of PrP^{Sc} and this influence the homogeneity and size of prion rods (Caughey *et al.*, 1991; Gasset *et al.*, 1993). In this study, we found also the optimum Gdn concentration for reversible denaturation of PrP^{Sc} (1.5 mol/l). However, taking into account the results of Caughey *et al.* (1999), the optimum concentration apparently varies in dependence on the method of preparation of PrP^{Sc}.

It is believed that the PK-resistance and the infectivity of PrP^{Sc} raises with the increase in the ß-sheet content in its secondary structure (McKinley *et al.*, 1983; Gasset *et al.*, 1993). A breakdown of the ß-sheet and/or the tertiary structure of PrP^{Sc} through denaturing agents leads to partial or full loss of those activities; however, such a change may be reversed by renaturation (Caughey *et al*., 1997). Gdn (i) has the ability to destroy the higher (secondary and/or tertiary) structure of proteins generally and to unfold ß-sheets particularly, and (ii) has a strong hydratation effect; all this leads to an increased solubility of proteins generally and hydrophobic proteins particularly.

Remarkable relationships between structure and biological functions of PrP^{Sc} have been repeatedly observed in this as well as other studies (Caughey *et al.*, 1997; Safar *et al.*, 1993).

The rescue of the infectivity of denatured PrP^{Sc} by renaturation strongly suggests that the restoring of higher structure of PrP^{Sc} reestablishes its biological activity. Whether some substances like chaperons (Stockel and Hartl, 2001) or others of non-protein nature (e.g. polysaccharides) (Shaked *et al.*, 2001) contribute to the recovery of the infectivity remains unsettled.

The resistance to proteolysis is a widely used property in distinguishing PrP^{Sc} from PrP^{C}. The PK-resistance of PrP^{Sc} is somehow related to its infectivity. However, the absence of PK-resistance does not necessarily exclude the possibility of infectivity of a PrP^{Sc} sample. As a matter of fact, the detection of PK-resistance *in vitro* of PrP^{Sc} depends on the sensitivity of the used method. In one report, a weakening or disappearance of PK-resistant PrP signal in Western blot following a treatment with 2.5–3.0 mol/l Gdn was interpreted as caused by a too high concentration of Gdn that made the antibody-binding-epitope sensitive to PK; in fact, the above phenomenon was caused by the existence of a critically PK-resistant C-terminal domain of PrP^{Sc} (Kocisko *et al.*, 1996). The rescue of the infectivity of Prp^{Sc} even in higher concentrations of Gdn strongly suggests that, to eliminate the infectivity of a prion, it is better to use the chemicals that completely destroy the prion protein, such as NaOH or NaClO in high concentration (Weber and Rutala, 2002). Furthermore, the results of the infectivity assays of PrP^{Sc} described in this study demonstrated that the determination of infectivity was more reliable than the detection of PK-resistance.

Acknowledgements. The authors thank Ms Hui-Ying Jiang and Wei Zhou for excellent technical assistance and Mr Tong-Xing Zhao for animal bioassays. This work was supported by the grant No. 30130070 from the Chinese National Natural Science Foundation, the National High-tech Development Project No. 2001AA215391, the National Science and Technology Task Force Project No. 2001BA712A04-2 and the EU Project QLRT 2000 01441.

References

Bolton DC, Bendheim PE, Marmorstein AD, Potempska A (1987): Isolation and structural studies of the intact scrapie agent protein. *Arch. Biochem. Biophys.* **258,** 579–590.

Brown DR, Hafiz F, Glasssmith LL, Wong BS, Jones IM, Clive C, Haswell SJ (2000): Consequences of manganese replacement of copper for prion protein function and proteinase resistance. *EMBO J.* **19,** 1180–1186.

Callahan MA, Xiong L, Caughey B (2001): Reversibility of scrapie-associated prion protein aggregation. *J. Biol. Chem.* **276,** 28022–28028.

Caughey B, Kocisko DA, Raymond GJ, Lansbury PT Jr (1995): Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. *Chem. Biol.* **2,** 807–817.

Caughey B, Raymond GJ, Kocisko DA, Lansbury PT Jr (1997): Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated

prion protein in guanidine denaturation studies. *J. Virol.* **71,** 4107–4110.

Caughey B, Raymond GJ, Priola SA, Kocisko DA, Race RE, Bessen RA, Lansbury PT Jr, Chesebro B (1999): Methods for studying prion protein (PrP) metabolism and the formation of protease-resistant PrP in cell culture and cell-free systems. *Mol. Biotechnol.* **13,** 45–55.

Caughey BW, Dong A, Bhat KS, Ernst D, Hayes SF, Caughey WS (1991): Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy. *Biochemistry* **30,** 7672–7680.

Chen SG, Parchi P, Brown P, Capellari S, Zou W, Cochran EJ, Vnencak-Jones CL, Julien J, Vital C, Mikol J, Lugaresi E, Autilio-Gambetti L, Gambetti P (1997): Allelic origin of the abnormal prion protein isoform in familial prion diseases. *Nat. Med.* **3,** 1009–1015.

Collinge J, Beck J, Campbell T, Estibeiro K, Will RG (1996): Prion protein gene analysis in new variant cases of Creutzfeldt-Jakob disease. *Lancet* **348,** 56.

Diringer H, Gelderblom H, Hilmert H, Ozel M, Edelbluth C, Kimberlin RH (1983): Scrapie infectivity, fibrils and low molecular weight protein. *Nature* **306,** 476–478.

Gao JM, Gao C, Han J, Zhou XB, Xiao XL, Zhang J, Chen L, Zhang BY, Hung T, Dong XP (2004): Dynamic analyses of PrP and PrP^{Sc} in brain tissues of golden hamsters infected with scrapie strain 263K revealed various PrP forms. *Biomed. Environ. Sci.* **17,** 8–20.

Gasset M, Baldwin MA, Fletterick RJ, Prusiner SB (1993): Perturbation of the secondary structure of the scrapie prion protein under conditions that alter infectivity. *Proc. Natl. Acad. Sci. USA* **90**, 1–5.

Hill AF, Antoniou M, Collinge J (1999): Protease-resistant prion protein produced in vitro lacks detectable infectivity. *J. Gen. Virol.* **80,** 11–14.

Hsiao KK, Groth D, Scott M, Yang SL, Serban H, Rapp D, Foster D, Torchia M, Dearmond SJ, Prusiner SB (1994): Serial transmission in rodents of neurodegeneration from transgenic mice expressing mutant prion protein. *Proc. Natl. Acad. Sci. USA* **91,** 9126–9130.

Jackson GS, Hosszu LL, Power A, Hill AF, Kenney J, Saibil H, Craven CJ, Waltho JP, Clarke AR, Collinge J (1999): Reversible conversion of monomeric human prion protein between native and fibrilogenic conformations. *Science* **283,** 1935–1937.

James TL, Liu H, Ulyanov NB, Farr-Jones S, Zhang H, Donne DG, Kaneko K, Groth D, Mehlhorn I, Prusiner SB, Cohen FE (1997): Solution structure of a 142-residue recombinant prion protein corresponding to the infectious fragment of the scrapie isoform. *Proc. Natl. Acad. Sci. USA* **94,** 10086–10091.

Kaneko K, Peretz D, Pan KM, Blochberger TC, Wille H, Gabizon R, Griffith OH, Cohen FE, Baldwin MA, Prusiner SB (1995): Prion protein (PrP) synthetic peptides induce cellular PrP to acquire properties of the scrapie isoform. *Proc. Natl. Acad. Sci. USA* **92,** 11160–11164.

KIimberlin RH, Walker CA (1978): Evidence that the transmission of one source of scrapie agent hamsters involves separation of agent strains from a mixture. *J. Gen. Virol.* **39,** 487–496.

KIimberlin RH, Walker CA (1977): Characteristics of a short incubation model of scrapie in the golden hamster. *J. Gen. Virol.* **34,** 295–304.

Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, Caughey B (1994): Cell-free formation of protease-resistant prion protein. *Nature* **370,** 471–474.

Kocisko DA, Lansbury PT Jr, Caughey B (1996): Partial unfolding and refolding of scrapie-associated prion protein: evidence for a critical 16-kDa C-terminal domain. *Biochemistry* **35,** 13434–13442.

Lasmezas CI, Deslys JP, Robain O, Jaegly A, Beringue V, Peyrin JM, Fournier JG, Hauw JJ, Rossier J, Dormont D (1997): Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science* **275,** 402–405.

McKenzie D, Bartz J, Mirwald J, Olander D, Marsh R, Aiken J (1998): Reversibility of scrapie inactivation is enhanced by copper. *J. Biol. Chem.* **273,** 25545–25547.

McKinley MP, Bolton DC, Prusiner SB (1983): A protease-resistant protein is a structural component of the scrapie prion. *Cell* **35,** 57–62.

Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA, Prusiner SB (1986): Separation and properties of cellular and scrapie prion proteins. *Proc. Natl. Acad. Sci USA* **83,** 2310–2314.

Morillas M, Vanik DL, Surewicz WK (2001): On the mechanism of alpha-helix to beta-sheet transition in the recombinant prion protein. *Biochemistry* **40,** 6982–6987.

Nandi PK, Leclerc E (1999): Polymerization of murine recombinant prion protein in nucleic acid solution. *Arch. Virol.* **144,** 1751–1763.

Prusiner SB (1998): Prions. *Proc. Natl. Acad. Sci. USA* **95,** 13363–13383.

Quaglio E, Chiesa R, Harris DA (2001): Copper converts the cellular prion protein into a protease resistant species that is distinct from the scrapie isoform. *J. Biol. Chem.* **276,** 11432–11438.

Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K (1996): NMR structure of the mouse prion protein domain PrP (121-321). *Nature* **382,** 180–182.

Roher AE, Palmer KC, Chau V, Ball MJ (1988): Isolation and chemical characterization of Alzheimer's disease paired helical filament cytoskeletons: differentiation from amyloid plaque core protein. *J. Cell Biol.* **107,** 2703–2716.

Safar J, Roller PP, Gajdusek DC, Gibbs CJ Jr (1993): Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein. *J. Biol. Chem.* **268,** 20276–20284.

Safar J, Roller PP, Gajdusek DC, Gibbs CJ Jr (1993): Thermal stability and conformational transitions of scrapie amyloid (prion) protein correlate with infectivity. *Protein Sci.* **2,** 2206–2216.

Shaked GM, Meiner Z, Avraham I, Taraboulos A, Gabizon R (2001): Reconstitution of prion infectivity from solubilized protease-resistant PrP and nonprotein components of prion rods. *J. Biol. Chem.* **276,** 14324–14328.

Stockel J, Hartl FU (2001): Chaperonin-mediated de novo generation of prion protein aggregates. *J. Mol. Biol.* **313,** 861–872.

Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R, Mastrianni J, Lugaresi E, Gambetti P, Prusiner SB (1996): Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science* **274,** 2079–2082.

Weber DJ, Rutala WA (2002): Managing the risk of nosocomial transmission of prion diseases. *Curr. Opin. Infect. Dis.* **15,** 421–425.

Weissmann C (1999): Molecular genetics of transmissible spongiform encephalopathies. *J. Biol. Chem.* **274,** 3–6.

Xiong LW, Raymond LD, Hayes SF, Raymond GJ, Caughey B (2001): Conformational change, aggregation and fibril formation induced by detergent treatments of cellular prion protein. *J. Neurochem.* **79,** 669–678.

Yao HL, Han J, Zhang BY, Gao JM, Guo YJ, Zhang J, Xiao XL, Nie K, Cai CX, Dong XP (2004): Effects of several chemical and physical treatments on the protease resistance of scrapie strain 263K. *Chin. J. Virol.* **20,** 40–45.

Zhang FP, Zhang J, Zhou W, Zhang BY, Hung T, Dong XP (2002): Expression of PrP^{C} as HIS-fusion form in a baculovirus system and conversion of expressed PrP-sen to PrP-res in a cell-free system. *Virus Res.* **87,** 145–153.

Zhang J, Chen L, Zhang BY, Han J, Xiao XL, Tian HY, LI BL, Gao C, Gao JM, Zhou XB, Ma GP, Liu Y, Xu CM, Dong XP (2004): Comparison study on clinical and neuropathological characteristics of hamsters inoculated with scrapie strain 263K in different challenging pathways. *Biomed. Environ. Sci.* **17,** 65–78.

Zou WQ, Cashman NR (2002): Acidic pH and detergents enhance in vitro conversion of human brain PrP^{C} to a PrP^{Sc}-like form. *J. Biol. Chem.* **277,** 43942–43947.