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Studies on prions: from biochemistry of the recombinant prion protein to properties of the infectious form

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Table of contents

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ZUSAMMENFASSUNG

Unter Prionenerkrankungen versteht man infektiöse Krankheiten wie Scrapie bei Schafen, bovine spongiforme Enzephalopathie (BSE) bei Rindern und Creutzfeld-Jakob Krankheit (CJD) beim Menschen. Entsprechend der „Nur Eiweiss"-Hypothese wird angenommen, dass das infektiöse Agens, das Prion, nur oder hauptsächlich aus einer abnormalen Form, PrP^{Sc}, des gutartigen zellulären Prionproteins, PrP^{C}, besteht. PrP^{C} und PrP^{Sc} zeigen keine Unterschiede in ihrer kovalenten Struktur. Jedoch ist PrP^{C} ein Monomer und hauptsächlich α-helikal, wohingegen PrP^{Sc} ein unlösliches Oligomer darstellt und im Vergleich zu PrP^{C} erhöhten β-Faltblatt-Anteil zeigt. Die Struktur von rekombinanten Prionproteinen, die PrP^{C} entsprechen, wurde gelöst. Sie bestehen aus einer ungefalteten N-terminalen Hälfte und einer gefalteten, überwiegend α-helikalen C-terminalen Domäne. Die hohe Aggregationstendenz von PrP^{Sc} verhinderte bisher detaillierte Strukturaufklärungen dieser Form.

Prioninfektiosität ist resistent gegenüber dem klassischen Desinfektionsmittel Formaldehyd. Ausgehend von dieser Tatsache wurde im ersten Teil dieser Arbeit versucht, die Konformation von PrP^{Sc} durch kovalente intramolekulare Vernetzung mit Formaldehyd zu stabilisieren. Der zuvor gezeigte Effekt, dass Formaldehydbehandlung Prioninfektiosität gegen Autoklavieren bei hoher Temperatur resistent macht, konnte jedoch nicht reproduziert werden. Die Behandlung von rekombinantem Prionprotein mit geringen Formaldehydkonzentrationen führte zu intramolekularen Crosslinks, und mit hohen Formaldehydkonzentrationen zu intermolekularen Vernetzungen, wobei hochmolekulare Aggregate gebildet wurden. Die optimale Konzentration für intramolekulare Crosslinks wurde auf PrP(27-30) DLPCs, eine durch Lipide und Detergens solubilisierte Form des infektiösen, proteaseresistenten Kerns von PrP^{Sc}, angewendet. Nach Denaturierung mit Guanidiniumchlorid oder SDS wurden lösliche und unlösliche Fraktionen getrennt. Anschliessende Infektiositätstests mit den löslichen Fraktionen zeigten die höchste Infektiosität nach Behandlung mit 1.3 mM Formaldehyd und Denaturierung mit 2% SDS. Dies könnte ein erster Hinweis für die Existenz einer niedermolekularen, evtl. sogar monomeren Form der infektiösen Einheit sein.

Es wird vermutet, dass PrP^{C} *in vivo* Kupfer bindet. Eine vorausgehende Studie zeigte, dass durch Zugabe von Kupfer die Renaturierung von denaturiertem und daher nicht-infektiösem Scrapie-Material zu infektiösen Prionen verbessert werden kann. Die

analoge Reaktion wurde im zweiten Teil dieser Arbeit mit dem rekombinanten Mausprionprotein PrP(23-231) durchgeführt, das niemals zuvor infektiös gewesen war. Das Protein aggregierte und zeigte eine gewisse Proteinase K-Resistenz, was Eigenschaften von PrP^{Sc} widerspiegelt. Jedoch wurde keine Infektiosität gefunden.

Im dritten Teil der Arbeit wurde gezeigt, dass die Reduktion der Disulfidbrücke von rekombinantem Mausprionprotein, mPrP(23-231), zu einer signifikanten Strukturänderung führt. Während das Protein mit intakter Disulfidbrücke hauptsächlich α-helikale Struktur zeigt, liefert das reduzierte Protein bei saurem pH CD-Spektren, die typisch sind für β-Faltblatt Strukturen. Bei erhöhter Ionenstärke kommt es zur Ausformung von fibrillären Aggregaten des reduzierten Proteins. Die reduzierte Form könnte daher prinzipiell ein Intermediat bei der Bildung von PrP^{Sc} sein. Nimmt man an, dass dieses Intermediat bei der Bildung von PrP^{Sc} beteiligt ist, so müssen mindestens drei Schritte auftreten: Reduktion, Aggregation und Reoxidation des Prionproteins. Die Geschwindigkeit der Reduktion erwies sich als extrem langsam, was damit übereinstimmt, dass eine vollständige Entfaltung des Proteins nötig ist, um die Disulfidbrücke für Reduktionsmittel zugänglich zu machen. Saurer pH, wie er in Endosomen, dem möglichen Bildungsort von PrP^{Sc}, auftritt, begünstigt jedoch die Reduktion. Bei erhöhter Ionenstärke ($\geq$ 0.2 M) tritt anschliessend die Aggregation spontan ein. Reoxidation wurde sowohl für lösliches wie auch für aggregiertes, reduziertes mPrP(23-231) gezeigt. Bei Proteinase K-Verdau der reoxidierten Fibrillen zeigte sich jedoch nicht die für PrP^{Sc} typische Proteaseresistenz. Daher erscheint eine transiente Reduktion der Disulfidbrücke während der Bildung von PrP^{Sc} unwahrscheinlich. Falls reduziertes PrP dennoch bei der Entstehung von PrP^{Sc} eine Rolle spielt, ist eine Reduktion bei saurem pH im reduzierenden Milieu von Endosomen am wahrscheinlichsten. Dort wäre auch eine Katalyse der Reduktion durch Disulfidoxidoreduktasen denkbar. Dies wurde am Beispiel der Katalyse der Reduktion von PrP durch die bakteriellen Disulfidoxidoreduktasen DsbA, DsbC und Thioredoxin verifiziert.

Bei neutralem pH aggregiert reduziertes mPrP(23-231) unspezifisch. Die gefaltete Domäne des Prionproteins, mPrP(121-231), ist nach Reduktion bei allen pH-Werten unlöslich. Nur durch Zugabe von denaturierenden Reagenzien wie Harnstoff tritt Solubilisierung auf. Bei pH 4.0 und 2 M Harnstoff wurden durch CD-Analysen β-Faltblatt-artige Spektren für reduziertes mPrP(121-231) identifiziert, wie sie auch für

das reduzierte Vollängenprionprotein, mPrP(23-231), gefunden werden. Daher kann angenommen werden, dass sowohl bei reduziertem mPrP(121-231) als auch bei reduziertem mPrP(23-231) zumindest Teile der α-helikalen Region der entsprechenden oxidierten Proteine in β-Faltblatt-Struktur übergehen.

In einem weiteren Projekt konnte schliesslich gezeigt werden, dass Stahldraht nach Inkubation in Prion-infiziertem Hirnhomogenat von Mäusen und anschliessendem extensiven Waschen noch sehr hohe Mengen gebundener Infektiosität aufweist. Diese Experimente zeigen neben der Tatsache, dass Prionen enorm fest an rostfreien Stahl binden, dass in der Humanmedizin wesentlich effektivere Sterilisierungsmethoden für chirurgische Instrumente nötig sind, um in der Chirurgie ein Infektionsrisiko mit Prionen auszuschliessen.

SUMMARY

Prion diseases are infectious disorders like scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeld-Jakob disease (CJD) in humans. According to the "protein only" hypothesis the infectious agent, the prion, is believed to consist only or mainly of PrP^{Sc}, an abnormal form of the benign cellular prion protein, PrP^{C}. The covalent structures of PrP^{C} and PrP^{Sc} are identical but the two forms differ in conformation. PrP^{C} is a predominantly α-helical monomer whereas PrP^{Sc} represents an insoluble oligomer possessing increased β-sheet content compared to PrP^{C}. The high-resolution structure of recombinant PrP corresponding to PrP^{C} has been solved. The protein consists of an unfolded N-terminal tail and a folded, predominantly α-helical C-terminal domain. The high aggregation tendency of PrP^{Sc} hindered so far detailed structural analysis of this isoform.

Prion infectivity is resistant to formaldehyde treatment, a classical disinfection method. Therefore, in the first part of this thesis, it was tried to fix the PrP^{Sc} conformation by covalent intramolecular crosslinking with formaldehyde. The previously shown effect of strong resistance of formaldehyde treated prion infectivity against strong heat autoclaving, however, could not be reproduced. Treatment of recombinant prion protein with low concentrations of formaldehyde resulted in intramolecular crosslinks and with high concentrations of formaldehyde in intermolecularly crosslinked aggregates. The optimal concentration for intramolecular crosslinks was applied to PrP(27-30) DLPCs, a detergent-solubilized form of the infectious, protease resistant core of PrP^{Sc}. After denaturation with guanidinium chloride or SDS soluble and insoluble fractions were separated. Subsequent bioassays of the soluble fractions showed the highest infectivity after crosslinking with 1.3 mM formaldehyde and treatment with 2% SDS. This might be a first indication for the existence of a small molecular, eventually even monomeric form of the infectious unit.

It is assumed that PrP^{C} binds Cu^{2+} *in vivo*. A previous study has shown that the addition of Cu^{2+} enhances the renaturation of denatured, non-infectious scrapie material into infectious prions. In the second part of this thesis, a similar reaction was performed with recombinant mouse prion protein, mPrP(23-231), that was never infectious. Although the protein showed aggregation and some Proteinase K-resistance, characteristics reminiscent of PrP^{Sc}, no infectivity was gained.

In the third part of this work, it was shown that upon reduction of the single disulphide bond recombinant full-length mouse prion protein, mPrP(23-231), exhibits a significant structural change. While its structure is mainly α-helical with intact disulphide bridge, the reduced form displays β-sheet-like CD-spectra at acidic pH. At high ionic strength the reduced protein forms amyloid fibrils, perhaps suggesting that the reduced state is an intermediate during PrP^{Sc} formation.Three steps would thus seem necessary: reduction, aggregation and reoxidation of the prion protein. Reduction of mPrP(23-231) with dithiothreitol proved to be extremely slow, consistent with the requirement of complete unfolding of the protein to make the disulphide bond accessible for the reducing agent. Acidic pH, as found in endosomes, the potential sites of PrP^{Sc} formation, favors the reaction. At increased ionic strength (≥ 0.2 M) fibrillar aggregation did spontaneously occur, and reoxidation was shown for both soluble and aggregated reduced mPrP(23-231). However, upon Proteinase K digestion the reoxidized fibrils did not exhibit the PrP^{Sc}-typical protease resistance. For this reason, the proposed transient reduction of the disulphide bridge during the formation of PrP^{Sc} appears unlikely to take place. In case that reduced PrP still plays a role in the formation of PrP^{Sc}, reduction would presumably occur at acidic pH in the reducing environment of endosomes, where catalysis of the reduction by disulphide oxidoreductases is conceivable. This was exemplary verified by catalysation of the reduction of mPrP(23-231) by the bacterial disulphide oxidoreductases DsbA, DsbC and thioredoxin.

At neutral pH reduced mPrP(23-231) aggregates non-specifically. The folded C-terminal domain of the prion protein, mPrP(121-231), is insoluble after reduction at all pH values. Solubilization occurs only upon addition of denaturing agents like urea. At pH 4.0 and 2 M urea β-sheet-like far-UV CD spectra are displayed for reduced mPrP(121-231) as for reduced full-length mPrP(23-231). It therefore seems likely that in both reduced proteins at least parts of the α-helical region of the oxidized proteins form β-sheet structure.

In a further project, stainless steel wire was incubated in prion-infected mouse brain homogenates, and then washed extensively, before bioassay for residual infectivity. The wire was found to retain very high amounts of infectivity. Besides demonstrating the fact that prions can adhere enormously firmly to stainless steel, these experiments underscore the need for more effective sterilization procedures of surgical instruments that may have been exposed to prion infectivity.

LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
bp	base pair
BSE	bovine spongiform encephalopathy
CD	circular dichroism
CJD	Creutzfeld Jakob disease
CNS	central nervous system
CWD	chronic wasting disease
DLPCs	detergent lipid protein complexes
Dpl	doppel, homolog of PrP
ER	endoplasmatic reticulum
FDCs	follicular dendritic cells
FFI	fatal familial insomnia
FSE	feline spongiform encephalopathy
FSI	fatal sporadic insomnia
FTIR	Fourier-transformed infrared spectroscopy
GdnHCl	guanidinium chloride
GPI	glycosyl phosphatidyl inositol
GSS	Gerstmann Sträussler Scheinker syndrome
HPLC	high performance liquid chromatography
i.c.	intracerebral
i.p.	intraperitoneal
i.v.	intravenous
ID_{50}	infectious dose where 50% of the infected animals die
LRS	lymphoreticular system
NMR	nuclear magnetic resonance
nvCJD	new variant Creutzfeld Jakob disease
PAGE	polyacrylamide gel electrophoresis
PK	Proteinase K
Prnd	prion gene complex, downstream, coding for Dpl
Prnp	gene coding for PrP
PrP	prion protein
PrP^{C}	cellular form of PrP
PrP^{Sc}	scrapie form of PrP
PrP(27-30)	PK-resistant core of PrP^{Sc} (27-30 kDa)
$PrP^{0/0}$	PrP knockout
$PrP^{0/+}$	PrP heterozygous
$PrP^{+/+}$	PrP homozygous
SCID	severe combined immunodeficient
SDS	sodium dodecylsulfate
TME	transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathy
UV	ultraviolet

1. INTRODUCTION

1.1. The infectious agent

Early studies

Since the occurrence of the bovine spongiform encephalopathy (BSE) crisis in Great Britain, starting in 1986, the term "prion" has become widely known by the public. Still, researchers do not know the exact nature of the infectious agent for this disease, belonging to the so-called transmissible spongiform encephalopathies (TSEs). Besides the infectious form, these diseases can occur sporadically or may be genetically inherited. A growing body of evidence now supports the "protein-only" hypothesis. It was first proposed in 1967 by Alper and coworkers, by Griffith, as well as by Pattison and Jones that the scrapie (= TSE of sheep) agent might exclusively consist of protein.

The reasons for these speculations were the abnormal properties of this transmissible agent in sheep that made it very different from all known viruses and bacteria, the common causes of transmissible diseases. For example, the scrapie agent survives in necrotic tissue, shows extraordinary resistance to heat (it "survives" 120°C steam autoclaving (Brown *et al.*, 1982), and infectivity is not destroyed by harsh chemicals such as formaldehyde (Pattison, 1965a), ethanol (Hartley, 1967), and chloroform (Lavelle, 1972). It is also resistant to non-denaturing detergents, nucleases, proteases and glycosidases (Hunter *et al.*, 1969; Hunter and Millson, 1967; Millson *et al.*, 1976). UV-irradiation revealed that the target size of the infectious material to inactivation at 254 nm is relatively small, too small for a virus (Alper *et al.*, 1967; Alper *et al.*, 1966). And infectivity proved to be more sensitive to irradiation at 237 nm (Latarjet *et al.*, 1970), suggesting that no nucleic acid was involved.

Purification of the infectious agent

Early attempts to purify the infectious agent failed, as the virtually non-ideal and unusual physical behaviour strongly hampered enrichment trials.

However, in the early 1980s, Prusiner and coworkers published an enrichment procedure (Prusiner *et al.*, 1980; Prusiner *et al.*, 1982a). A 100-fold enrichment of infectivity was achieved by a series of detergent extractions, limited digestion with proteases and nucleases, and differential centrifugation steps, including a sucrose gradient. This purified transmissible agent of spongiform encephalopathies was named

prion, shorthand for "proteinaceous infectious particle" (Prusiner, 1982), because a proteinase-resistant protein was the main and only identifiable component of the purified preparation and accordingly termed prion protein (PrP).

Two forms of this protein exist: In normal brain the prion protein is completely digested by Proteinase K (PK), whereas in infected brain a fraction of the protein is only partly cleaved. The two species were hence designated PrP^{C} (cellular form) and PrP^{Sc} (scrapie-associated form). PrP^{Sc} can only be isolated in aggregated form and upon PK digestion the N-terminus is removed up to amino acid 88. The remaining protease resistant core is designated PrP(27-30) for its apparent size on SDS-gels (Prusiner *et al.*, 1984). As all subunits of the PrP^{Sc} oligomer are digested with Proteinase K in exactly the same manner, PrP^{Sc} must be an ordered aggregate.

Still some researchers assume that a virus is the causative agent of TSEs, but despite enormous efforts, no nucleic acid with a size above 100 bp could be identified at a particle to infectivity ratio ≥ 1 (Diringer *et al.*, 1997; Kellings *et al.*, 1992; Kellings *et al.*, 1993; Kellings *et al.*, 1994; Oesch *et al.*, 1988).

Recent investigations have shown that highly-purified prion preparations contain about 1.5% lipids. These lipids seemed to be non-essential for infectivity but rather just representative of caveolae, the site where the GPI-anchored cellular prion protein is located on the cell membrane (Klein *et al.*, 1998). However, there exist several speculations about an involvement of lipids in infectivity based on the following findings: 1) Radiolysis of the scrapie agent by UV became more efficient in oxygen-saturated water, conditions that target especially lipids (Alper, 1997). 2) Dispersion of prions in detergent-lipid-protein-complexes (DLPCs) or liposomes increased infectivity by about two orders of magnitude (Gabizon *et al.*, 1987). 3) Preparations of "Hyper" and "Drowsy", two distinct mink prion strains, differ in their density, arguing for differences in their lipid content (Bessen and Marsh, 1992).

In addition, a previously unknown, inert homopolysaccharide consisting of mainly 1,4-linked glucose units has been found in purified infectious prion preparations and has been proposed to be an essential scaffold for prions (Appel *et al.*, 1999).

"Protein only" hypothesis

These results led to the establishment of the "protein only" hypothesis that states that the prion is devoid of informational nucleic acid and consists of protein as the

propagating infectious agent (Prusiner, 1989). This is additionally supported by genetic evidence (see below). However, as even in the purest samples, the ratio of PrP molecules to infectious units is about 10^5 (Bolton *et al.*, 1991), it is impossible to exclude the presence of some other component or covalent modification that might be required for infectivity (Chesebro, 1998; Farquhar *et al.*, 1998; Weissmann, 1991).
A proof of the "protein only" hypothesis would be in the view of most researchers, the production of infectious PrP^{Sc} *in vitro* from previously non-infectious protein (Aguzzi and Weissmann, 1997). So far, this could not be achieved (see also "*In-vitro*-conversion" and chapter 3:"Cu^{2+}-conversion").

Bioassays

Until today infectivity can only be assayed and detected in living animals. Initially sheep and goats were used for the tests on transmissibility, where the animals had to be observed for over 18 months post-inoculation before the onset of the disease and the presence of infectivity was only determined qualitatively (Hunter, 1972). The transmission of scrapie to the laboratory animals hamster and mouse showing relatively short scrapie incubation times of 70-200 days was an important advance for studies on infectivity as it helped to quantify the amount of infectivity by end point titration experiments (Chandler, 1961; Kimberlin, 1976; Kimberlin and Walker, 1977; Marsh and Kimberlin, 1975). Four to six animals were inoculated for each of about eight logarithmic dilutions, and the concentration of infectious units was calculated from the infectivity rate after the longest possible incubation time (200-400 days). Although these endpoint titration experiments in mice revealed some properties of the scrapie agent, development of an effective purification protocol was difficult, because the interval between execution of the experiment and availability of the results was still about one year (Prusiner, 1988; Siakotos *et al.*, 1976). It was only after the establishment of the incubation time assay for hamster-adapted scrapie, which took advantage of the fact that incubation time and infectivity concentration are reversibly related (Prusiner *et al.*, 1982b; Prusiner *et al.*, 1980), that the number of animals and the time required for assaying fractions during the enrichment of the agent decreased and that the studies on the nature of the infectious agent were accelerated.
For prion diseases, the infectivity parameter is equal to mortality as TSE diseases are as yet invariably lethal. A single infectious unit is defined as the mean lethal dose, the so-

called LD_{50}, the inoculum amount that kills 50% of subjects in the test system. This unit of activity, however, does not say anything about the physical properties of the agent. Thus, for non-established TSE-agents, where no incubation time curve has been determined, end-point titration is still the only method to determine infectivity amounts.

1.2. The prion protein (PrP)

Identification of the Prnp gene

Sequencing of purified tryptic peptides of the prion protein isolated from scrapie infected brain (Prusiner *et al.*, 1984) enabled the identification of the PrP cDNA (Chesebro *et al.*, 1985; Oesch *et al.*, 1985) and subsequently the gene for the prion protein, Prnp (Basler *et al.*, 1986). The gene contains 3 exons, with the entire coding sequence included in the third exon. Surprisingly, the gene encoding PrP proved to be a host gene. Meanwhile homologous Prnp genes have been identified in all mammals examined (Schatzl *et al.*, 1995; Schatzl *et al.*, 1997; Wopfner *et al.*, 1999) and the sequences show in general > 90% pairwise identity.

The prion protein from chicken as well as that of several other birds have also been cloned (Gabriel *et al.*, 1992; Wopfner *et al.*, 1999). These sequences show only around 30% homology to mammalian PrP. Sea turtle prion protein, the only identified reptile PrP has 40, respectively 58% identity to mammalian and avian prion proteins (Simonic *et al.*, 2000).

Biosynthesis

The full PrP amino acid sequence, depicted in Figure 1, reveals a protein that has a N-terminal signal sequence of 22 amino acids, cleaved from the primary translation product after directing it to the endoplasmatic reticulum (ER), and N-glycosylation sites occuring at Asn181 and Asn197. Cys179 and Cys214 form a disulphide bond. At Ser231 a glycosyl-phoshatidyl-inositol (GPI)-anchor is attached that fixes the protein to the outer surface of the plasma membrane and the C-terminal prosequence from amino acids 232-253 is cleaved off. A further feature of the prion protein are the characteristic Gly-Pro-rich octapeptides, five repeats of an 8 amino acid motif. These octarepeats were postulated to bind copper ions (Viles *et al.*, 1999).

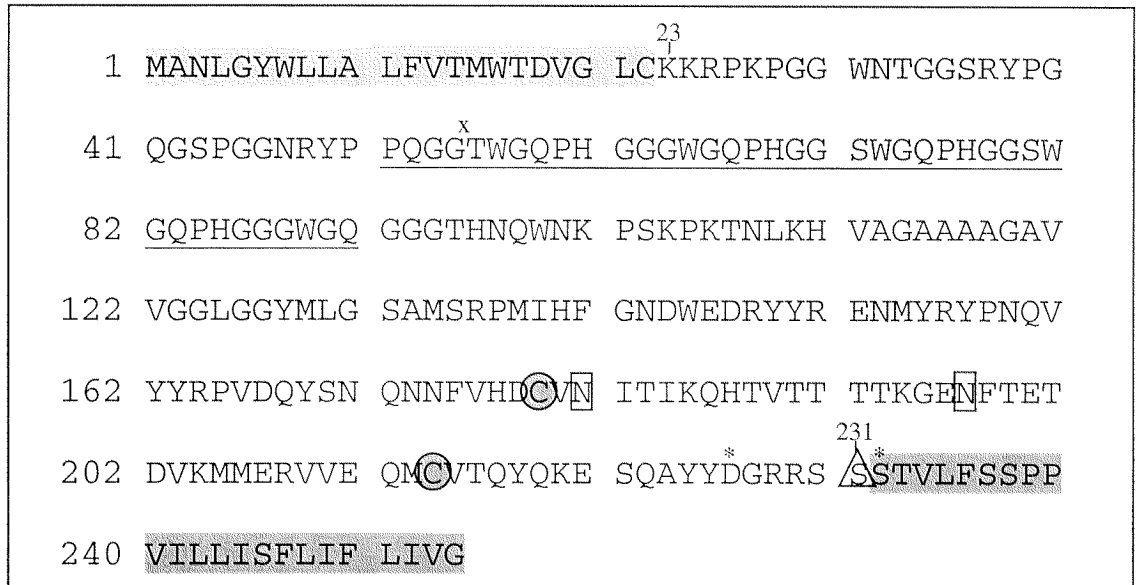

Figure 1: Amino acid sequence of the mouse prion protein
N-terminal signal sequence; octapeptide repeats; C Cystein residues C179 and C214 form a disulfide bond; N Asparagine residues N181 and N197 may be N-glycosylated; S to serine residue S231 a GPI-anchor is attached; C-terminal pro-sequence; x: residue 55 is deleted in mouse PrP; *: D and S are additional residues in mouse PrP compared to the human sequence and are not numerated.

Whereas the majority of PrP biosynthesis leads to a GPI-anchored form, the channel for PrP export into the ER may disassemble during biogenesis, resulting in different transmembrane forms of PrP (De Fea *et al.*, 1994; Hay *et al.*, 1987a; Hegde *et al.*, 1998; Yost *et al.*, 1990). Furthermore there is evidence for a secretory form of the prion protein (Hay *et al.*, 1987b; Lopez *et al.*, 1990).

The GPI-anchor targets PrP to caveolae-like domains on the cell membrane (Taraboulos *et al.*, 1995). The prion protein at the cell surface is subject to endocytosis (Borchelt *et al.*, 1992; Caughey *et al.*, 1991) and recycling (Figure 2).

The protein is expressed constitutively in the brain and to a lesser extent in most other organs such as heart, skeletal muscle, liver and the lymphoreticular system, independent of the disease (Bendheim *et al.*, 1992).

The development of tissue culture systems persistently infected by scrapie has enabled studies of the subcellular pathways followed by PrP in its normal biosynthesis and conversion to PrP^{Sc} (Clarke and Haig, 1970a; Clarke and Haig, 1970b; Race *et al.*, 1987; Rubenstein *et al.*, 1984). In these *in vitro* systems propagation of the scrapie agent

is continuously maintained. Most of the biosynthetic pathways of PrP^{C} (see Figure 2) and PrP^{Sc}-formation were determined by help of these systems.

PrP^{Sc} is derived posttranslationally from PrP^{C} (Taraboulos *et al.*, 1992). It is formed in diseased brain after PrP^{C} has reached the cell surface and is endocytosed, and can be localised to acidic lysosomes, where it can be N-terminally truncated by lysosomal proteases (Caughey *et al.*, 1991; McKinley *et al.*, 1991b; Taraboulos *et al.*, 1992).

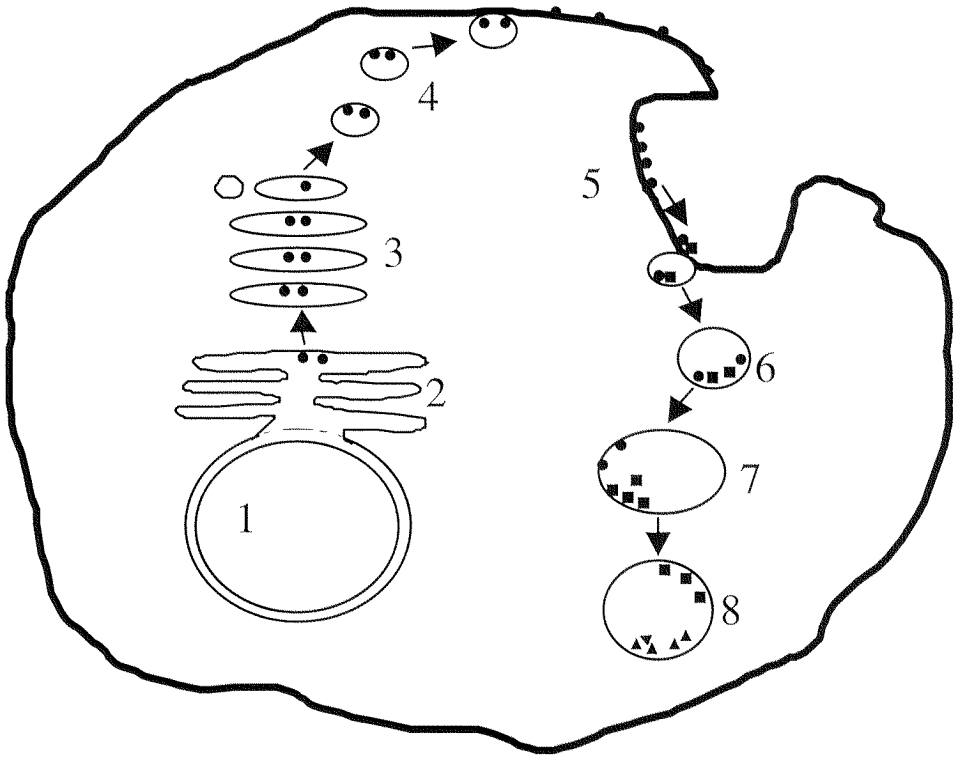

Figure 2: Biosynthesis of PrP^{C} and its conversion to PrP^{Sc}.
Like other membrane proteins PrP^{C} is synthesized in the rough ER, and after passage through the Golgi network reaches through secretory vesicles the cell surface. PrP^{C} on the plasma membrane is internalized into early endosomes. PrP^{Sc}-formation seems to occur in a compartment accessible from the plasma membrane: rafts (regions of the plasma membrane that are enriched for cholesterol and glycosphingolipids and to which GPI-anchored proteins are targeted) or endosomes. PrP^{Sc} synthesis probably occurs through the interaction of PrP^{C} with existing PrP^{Sc}. Subsequently PrP^{Sc} might be N-terminally trimmed by an acidic protease to form PrP(27-30), which accumulates primarily in lysosomes.
1) nucleus, 2) endoplasmatic reticulum (ER), 3) transgolgi network, 4) secretory vesicles, 5) caveolae-like domain/raft, 6) uncoated vesicle, 7) acidic endosome, 8) lysosome, ● PrP^{C}, ■ PrP^{Sc}, ▲ PrP(27-30); adapted from Caughey 1991 and Prusiner 1999.

Differences between PrP^C and PrP^{Sc}

As described above, PrP^C and PrP^{Sc} are two different forms of one protein, encoded from one chromosomal locus (Basler *et al.*, 1986). Despite extensive searches no covalent differences between the two isoforms could be found (Stahl *et al.*, 1990). Both types exist in un-, mono- or diglycosylated forms, containing various high mannose glycosyl residues and both, PrP^C and PrP^{Sc} contain a GPI-anchor (Stahl *et al.*, 1992). However there are biophysical and biochemical properties that significantly distinguish the two species: 1) PrP^C is soluble in non-ionic detergents whereas PrP^{Sc} is not. 2) PrP^C is readily digested by proteases, whereas PrP^{Sc} shows high partial resistance, and is only cleaved up to about amino acid 90 to form the protease-resistant core PrP(27-30). 3) Optical spectroscopy shows differences in secondary structure content between the two isoforms, Fourier-transformed infrared spectroscopy (FTIR) and circular dichroism (CD) measurements revealed that PrP^C contains about 40% α-helix and little β-sheet, whereas PrP^{Sc} is composed of about 30% α-helix and 45% β-sheet (Pan *et al.*, 1993; Pergami *et al.*, 1996). 4) PrP^C is monomeric, while PrP^{Sc} can only be isolated in an aggregated oligomeric form. 5) Regarding the localisation in the cell, PrP^C is prevalently found on the surface, attached to the cell membrane by the GPI-anchor, whereas PrP^{Sc} accumulates in endosomes of scrapie-infected cells (Taraboulos *et al.*, 1990b). 6) After Proteinase K treatment PrP^{Sc} forms amyloid-like fibrils, not produced by PrP^C in uninfected brains (McKinley *et al.*, 1991a; Merz *et al.*, 1981).

For more than 25 years, it has been widely accepted that the amino acid sequence specifies one biologically active conformation of a protein. Yet, in prion diseases we are faced with the possibility that one primary structure for PrP might adopt at least two different conformations, i.e., PrP^C and PrP^{Sc}, with entirely different biological funcions.

Structure

Purification of large amounts of PrP^C from eukaryotic cells for structure determination proved to be extremely problematic, and PrP^{Sc} aggregates instantly upon isolation. Additionally, both PrP^C and PrP^{Sc} show heterogeneous glycosylation, which complicates structural analysis of these naturally occurring isoforms. Therefore, most of the recent structural work on PrP has been performed with recombinant prion proteins produced in *Escherichia coli* (Hornemann and Glockshuber, 1996; Hornemann *et al.*, 1997; Mehlhorn *et al.*, 1996; Zahn *et al.*, 1997; Zhang *et al.*, 1997). Solution structures

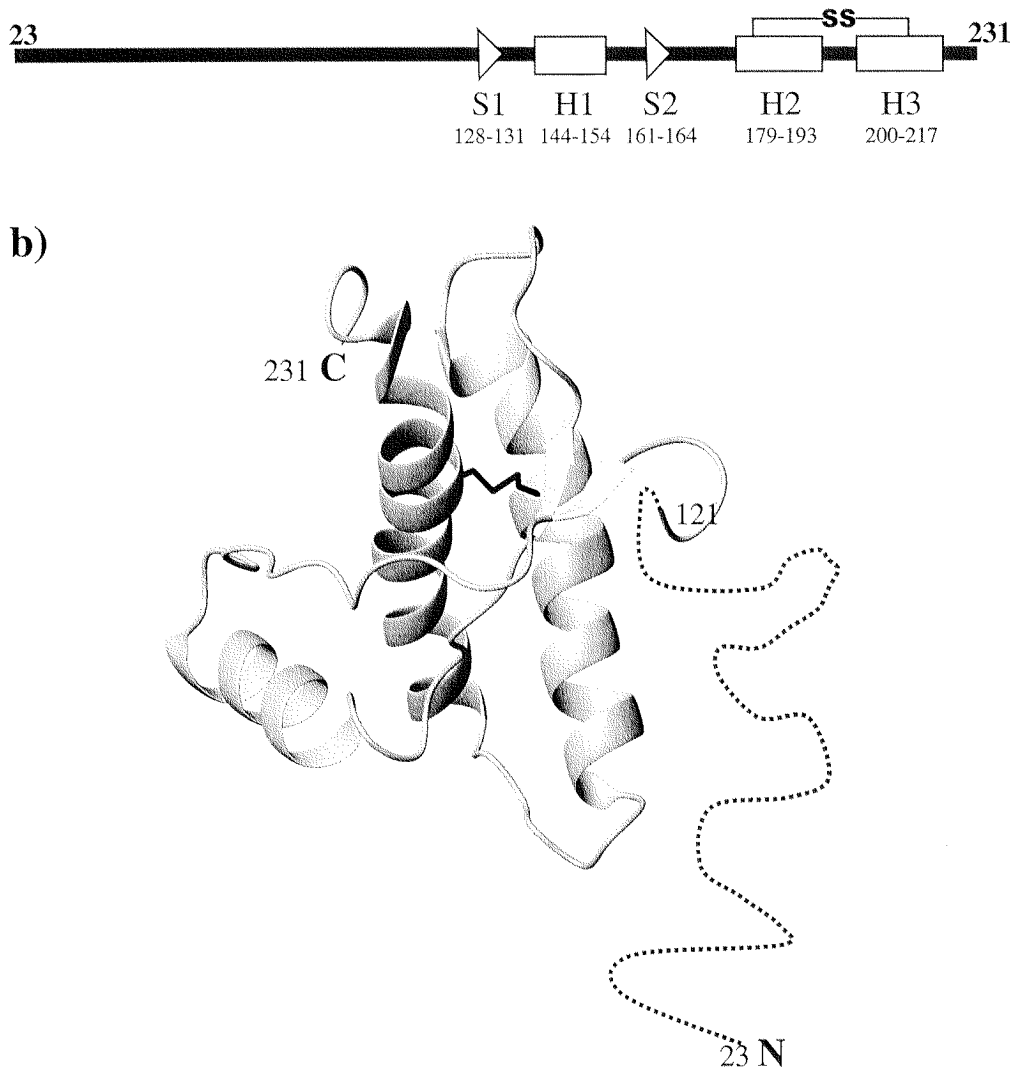

Figure 3: Three dimensional stucture of the recombinant mouse prion protein mPrP(23-231); a) Schematic presentation of the primary structure and identification of the secondary structure elements. mPrP(23-231) contains three α-helices (H1, H2 and H3) and a two-stranded antiparallel β-sheet (S1 and S2). The single disulphide bridge between Cys179 and Cys214 is indicated. b) NMR-structure (Riek, 1997), showing the folded C-terminal domain (residues 126-231) and the flexibly-disordered N-terminal tail (residues 23-125).

of recombinant PrP from mouse, hamster, human and cow species were determined by NMR (Donne *et al.*, 1997; James *et al.*, 1997; Lopez Garcia *et al.*, 2000; Riek *et al.*, 1996; Riek *et al.*, 1997; Zahn *et al.*, 2000). The resultant structural data revealed that all proteins are very similar as expected from the high degree of sequence identity (generally above 90%) among all known mammalian prion protein sequences (Schatzl

et al., 1997). The full-length proteins (mPrP(23-231), haPrP(29-231), huPrP(23-230), and boPrP(23-230)) contain two dissimilar segments, the N-terminally unfolded tail from amino acid 23-124 and the folded C-terminal domain spanning amino acids 125-231. The latter consists of three α-helices and a short, two-stranded β-sheet. α-helices 2 and 3 are covalently linked via the single disulphide bond of PrP (see Figure 3). In the hamster PrP structure, helices 2 and 3 are slightly elongated compared to mPrP, and the loop comprising residues 165-171 is more ordered (James *et al.*, 1997). These slight differences may have functional significance for the species barrier. Comparison of human with mouse and hamster PrP shows that helix 3 coincides closely with the hamster protein whereas the loop 167-171 is shared with murine PrP (Zahn *et al.*, 2000). The bovine PrP structure is essentially identical to that of the human prion protein, but there are differences in surface distribution of electrostatic charges, that might influence the transmission of BSE to humans (Lopez Garcia *et al.*, 2000).

The structural results described here and the spectroscopic properties of recombinant PrP (Hornemann and Glockshuber, 1996) are in full agreement with the data of PrP^C purified from eukaryotic cells (Pan *et al.*, 1993). This indicates that the three-dimensional structure of recombinant PrP(23-231) is identical with that of natural PrP^C, and hence the glycosylation (at Asn181 and Asn197) and fusion with the GPI-anchor (at Ser231) have at most a minor influence on the structure and folding of PrP^C.

Numerous recent studies indicate that PrP^C binds Cu^{2+} ions *in vivo* and *in vitro*, (Hornshaw *et al.*, 1995a; Hornshaw *et al.*, 1995b; Miura *et al.*, 1999; Miura *et al.*, 1996; Stockel *et al.*, 1998; Viles *et al.*, 1999) and that the highly flexible octapeptide repeat region, containing a histidine residue in 4 of the 5 repeats, is mainly responsible for Cu^{2+} binding (Cohen, 1999b).

Folding

It follows from the "protein only" hypothesis that understanding of how PrP^C unfolds and refolds into PrP^{Sc} is of key importance for elucidating the mechanism of prion propagation and possibly of other amyloid-associated degenerative illnesses.

Recombinant PrP indeed proved to exhibit a high degree of structural plasticity depending on pH, ionic strength and denaturant concentrations (Jackson *et al.*, 1999a; Jackson *et al.*, 1999b; Mehlhorn *et al.*, 1996; Post *et al.*, 1998; Swietnicki *et al.*, 1997; Zhang *et al.*, 1997). Under appropriate conditions the recombinant proteins can adopt

conformations with scrapie-like characteristics, but so far no infectivity as postulated by the "protein only" hypothesis could be produced *in vitro*.

Oxidized (disulphide-intact) mPrP(121-231) and mPrP(23-231) as well as human PrP(90-231) fold cooperatively and reversibly in denaturant-dependent un- and refolding transitions at neutral pH according to the two-state model of folding (Hornemann and Glockshuber, 1998; Hosszu *et al.*, 1999; Liemann and Glockshuber, 1999; Swietnicki *et al.*, 1997). This suggests that PrP^{C} also folds reversibly. This would explain the fact that unfolding of PrP^{Sc} with high concentrations of denaturant leads to irreversible loss of infectivity as unfolded PrP^{C} and unfolded PrP^{Sc} are identical and yield PrP^{C} after removal of the denaturant.

In general the presence of the disulphide bond favours the retention of the α-helical structure but folding pathways and stability of oxidized mPrP(121-231) are also pH dependent, shifting to lower urea concentration with decreasing pH. At acidic pH, the transitions are no longer one step processes, involving at least three molecular species at equilibrium – requiring at least one unfolding intermediate. Analysis showed that this intermediate is populated to 95% in 3,5 M urea and reveals spectroscopically β-sheet content (Hornemann and Glockshuber, 1998). Also oxidized human PrP(90-231) forms a stable monomeric folding intermediate with an alternative, β-sheet like conformation. Further studies showed that this intermediate is oligomeric, possibly a dimer (Jackson *et al.*, 1999a; Swietnicki *et al.*, 2000). In the presence of high ionic strength it polymerizes *in vitro* into large molecular weight aggregates of fibrillar morphology (Swietnicki *et al.*, 2000).

In contrast to the disulphide-intact protein reduced PrP can interconvert between α-helical and β-sheet-like structures, depending on pH (Jackson *et al.*, 1999b; Mehlhorn *et al.*, 1996; Zhang *et al.*, 1997). At acidic pH, CD-spectra typical for β-sheet proteins are found. Upon increase of ionic strength at pH 4, reduced human PrP(90-231) forms fibrillar aggregates with slightly increased PK resistance, which is however below that of PrP^{Sc} (Jackson *et al.*, 1999b).

These findings are of biological significance as in the cell during the cycle of synthesis, transport, internalisation and degradation, the protein will be exposed to oxidizing conditions (ER) and reducing conditions (endosomes and lysosomes) and both neutral (ER, cell surface) and acidic pH (pH 4-6 in endosomes and lysosomes).

Stability of PrP^C and its genetic variants

A possible mechanism for inherited human prion diseases proposed by Prusiner and colleagues was that the corresponding point mutations decrease the thermodynamic stability of PrP^C, and facilitate its conversion to the PrP^{Sc} conformation and/or increase the stability of PrP^{Sc} (Cohen, 1999a; Cohen *et al.*, 1994; Huang *et al.*, 1995). However thermodynamic measurements demonstrated a destabilisation only in some of these mutations (Liemann and Glockshuber, 1999). Consequently, it is presently not possible to deduce a clear-cut mechanism of spontaneous prion generation in inherited TSEs from the stabilities of mutant PrP variants and data on their expression in cultured cells (Lehmann and Harris, 1995; Lehmann and Harris, 1996; Liemann and Glockshuber, 1999; Singh *et al.*, 1997; Swietnicki *et al.*, 1998). In principle, however, three mechanisms may apply: destabilization of PrP^C, stabilization of PrP^{Sc} and improved kinetics of PrP^{Sc} oligomerization.

1.3. Models for PrP^{Sc} formation

Regardless of whether or not PrP^{Sc} alone is the infectious agent, the conversion of PrP^C to PrP^{Sc} does take place, and appears to be the crucial mechanism for disease development, that could also be of importance to other neurodegenerative diseases in which accumulation of abnormally-folded proteins is observed.

Many theoretical models describing the conformational conversion of PrP^C to PrP^{Sc} have been proposed (Cohen, 1998; Come *et al.*, 1993; Gajdusek, 1988; Jarrett and Lansbury, 1993; Prusiner, 1991b; Weissmann, 1996) referring to two generally distinguished mechanisms.

Refolding model

In the kinetically controlled "refolding model" a PrP^{Sc} monomer (or dimer) promotes conformational conversion of a partially destabilised PrP intermediate to a PrP^{Sc} form (see Figure 4a) (Weissmann, 1996). The resulting PrP^{Sc} is more stable than PrP^C, but kinetically inaccessible (Cohen and Prusiner, 1998; Harrison *et al.*, 1997). Spontaneous conversion is very rare, due to a high activation energy barrier. Amyloid formation is not required for replication. Transmissibility is provided by PrP^{Sc} acting as template to direct refolding.

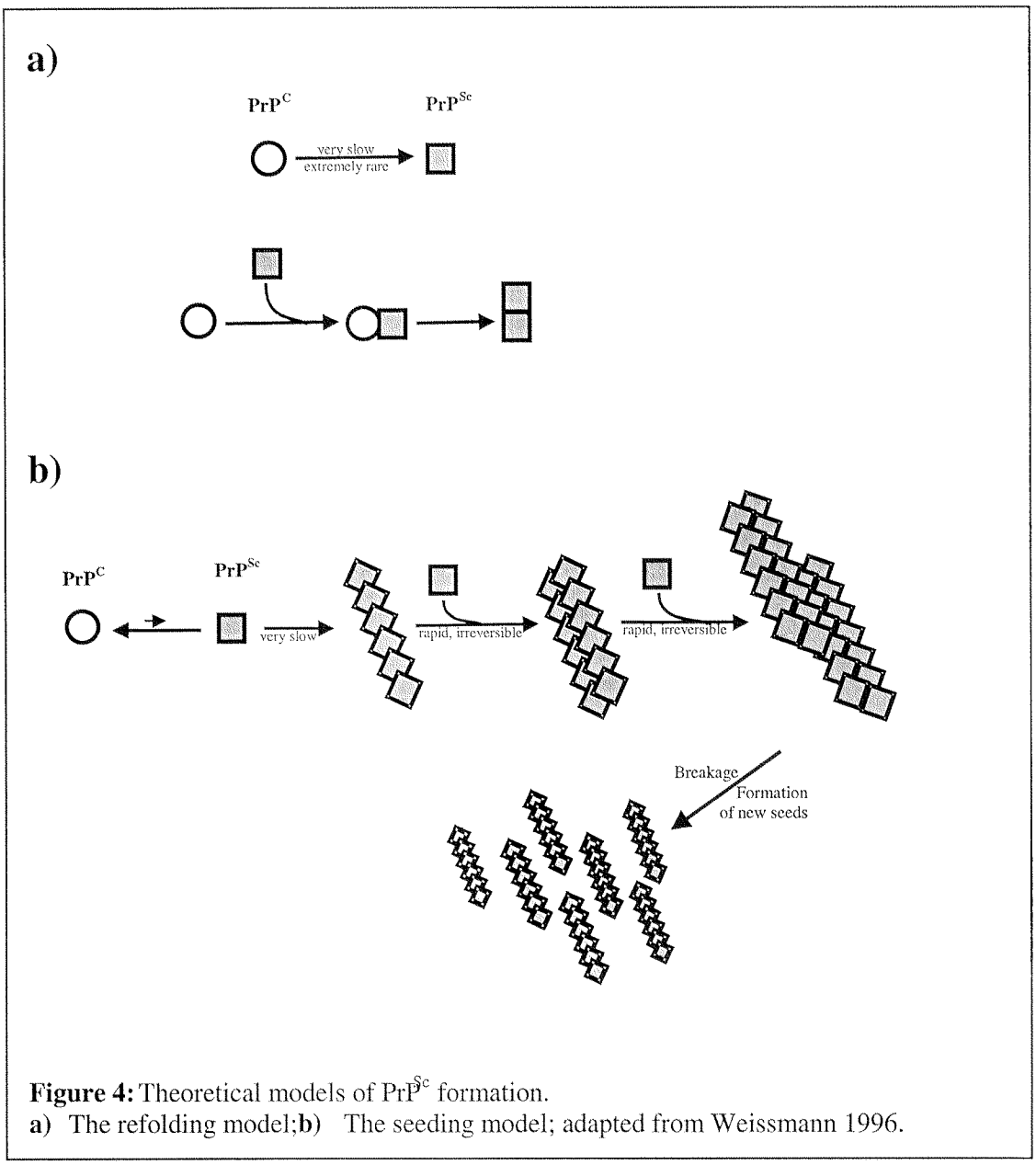

Figure 4: Theoretical models of PrP^{Sc} formation.
a) The refolding model;**b)** The seeding model; adapted from Weissmann 1996.

Seeding model

In the alternative "seeding model", the formation of PrP^{Sc} is initiated by an aggregate of PrP^{Sc}, acting as a seed for nucleation dependent polymerisation (Figure 4b) (Weissmann 1996). PrP^{C} is permanently in fast equilibrium with a monomeric precursor of PrP^{Sc}. In the absence of a stable nucleus, PrP^{C} is thermodynamically strongly favoured. Only by very slow oligomerization of several of the monomeric precursors, a PrP^{Sc} nucleus is formed which then pulls further PrP^{Sc} monomers from the equilibrium into the oligomer. The oligomerization process is irreversible inasmuch as no monomers can be

reformed. But for propagation fragmentation of a large aggregate into smaller oligomers is required for the generation of new seeds in this model. In comparison to the "refolding model" that necessarily requires a physical contact between PrP^{C} and PrP^{Sc} the "seeding model" does not.

Eigen (1996) undertook a simulation of the kinetics of the different models. He emphasised that aggregation is necessarily involved in both mechanisms, but also that both mechanisms are practically kinetically indistinguishable. The most convincing experimental argument for an aggregate or at least small oligomer as the infectious unit is that enrichment of infectivity always yields aggregated PrP^{Sc} (so called prion-rods or scrapie associated fibrils) (Prusiner, 1980), and that disruption of these rods causes loss of infectivity (Riesner *et al.*, 1996).

On the other hand, there are arguments for small oligomers of PrP^{Sc} as infectious unit. Treatment of the scrapie agent with ionizing radiation and subsequent analysis of infectivity suggested that the target size of the infectious scrapie agent is only around 100 kDa (Alper *et al.*, 1966; Gabizon and Prusiner, 1990). And for liposomes bearing prions it was calculated that they contain, on the average, 2-4 PrP^{Sc}-molecules per liposome (Gabizon *et al.*, 1987).

In summary, it is still unknown at the moment, what the infectious unit really is, or in other words, what the smallest infective entity is. However, it seems likely that one infectious prion unit is not equal to any oligomer of defined stoichiometry, but can vary in molecular size. Probably the agent can be more or weaker aggregated or in closer or less association with lipids and possibly other cocomponents.

In-vitro-conversion

Although the preparation of infectious material *in vitro* has not been reported so far, an interesting reaction, the so-called *in-vitro*-conversion has been described. By mixing radioactively labelled recombinant PrP^{C} from cultured eukaryotic cells with brain isolates from scrapie infected animals, PrP^{C} is recruited into a protease-resistant PrP isoform (Kocisko *et al.*, 1994). Consistent with the "seeding model", these cell-free conversion studies indicate that PrP^{Sc} aggregates are necessary for the reaction (Caughey *et al.*, 1997). Strikingly, both strain and species specificity, as observed *in vivo*, are reproduced *in vitro*. (Bessen *et al.*, 1995; Kocisko *et al.*, 1995; Raymond *et al.*,

1997). Nevertheless, an excess of PrP^{Sc} is necessary to form protease resistant PrP and as infectivity quantification requires concentration differences of 2-3 orders of magnitude, generation of new infectivity could not be detected so far.

To overcome this problem, experiments were done trying to make use of the species barrier: Conventional mice are not susceptible to hamster scrapie but transgenic mice expressing a chimeric hamster-mouse prion protein are. They produce the corresponding chimeric PrP^{Sc} molecules and infectivity pathogenic for conventional mice. Chimeric hamster-mouse PrP^{C} can be converted by hamster PrP^{Sc} into protease resistant chimeric PrP. However, no infectivity was detected upon bioassay in mice (Hill *et al.*, 1999).

1.4. Prion diseases

Scrapie, chronic wasting disease and transmissible mink encephalopathy

Transmissible spongiform encephalopathies (TSEs) comprise a group of disorders that affect humans and other mammals. Although most TSEs are rare, many different forms of the disease exist (see Table 1). Scrapie in sheep is the most common prion disease, known for centuries. Symptoms are ataxia, incoordination, unusual restlessness, rubbing, etc. It is an enzootic fatal neurodegenerative disorder of unknown aetiology. Epidemiological studies have been conducted on the potential risk of transmission of the scrapie agent to humans, but have never supported any causal relationship (Chatelain *et al.*, 1981; Laplanche, 1999).

Free-ranging and captive mule deer and Rocky Mountain elk can get chronic wasting disease (CWD). The prevalent clinical syndrome of this prion disease is wasting and eventual death. CWD is endemic only in Colorado and Wyoming. Its mode of transmission is unknown as well as a potential risk for humans (Laplanche, 1999).

Transmissible mink encephalopathy (TME) is a rare sporadic disease of ranched minks. The origin of TME was hypothesised to be the ingestion of scrapie agent. Clinical signs of TME are characterised by behavioural alterations that include confusion, loss of cleanliness and aimless circling. Somnolence and progressive debilitation occur until death. TME has also been successfully transmitted to other species like hamster, sheep, goat and cattle (Marsh and Hadlow, 1992; Marsh and Hanson, 1978).

Disease	Host	Etiology	Typical symptoms
Creutzfeld-Jakob disease (CJD) Sporadic	human	spontaneous conversion or somatic mutation of the Prnp gene (?)	dementia, coordination loss, loss of memory, motor disturbances, myoclonic, involuntary movements
Creutzfeld-Jakob disease (CJD) Familial	human	germ line mutation, many variants	
Creutzfeld-Jakob disease (CJD) Iatrogenic	human	infection by contaminated growth hormone, surgical instruments, dura mater and cornea transplants	
New variant Creutzfeld-Jakob-disease (nvCJD)	human	consumption of BSE-infected beef (?)	behavioural changes, ataxia, dysaesthesia (age of onset: 19-39)
Gerstmann-Sträussler-Scheinker-syndrome (GSS), familial	human	germ line mutations in the Prnp gene	ataxia, progressive dementia
Fatal familial insomnia (FFI)	human	germ line mutations in the Prnp gene	trouble sleeping followed by insomnia and dementia
Fatal sporadic insomnia (FSI)	human	spontaneous conversion or somatic mutation of the Prnp gene (?)	lethal insomnia, autonomic dysfunctions,
Kuru	human	infection via cannibalism	ataxia, tremor, incoordination, dementia
Scrapie	sheep, goats	infection of genetically susceptible animals, sporadic (?)	ataxia, metabolic wasting, rubbing
Transmissible mink encephalopathy (TME)	mink	infection through contaminated meat and bone meal from cow or sheep (?)	ataxia, metabolic wasting, itching
Chronic wasting disease (CWD)	Wapiti elk, mule deer	unknown	metabolic wasting, itching, incoordination
Bovine spongiform encephalopathy (BSE)	cow	infection through contaminated (scrapie or BSE) meat and bone meal, maternal transmission (?)	ataxia, metabolic wasting, rubbing
Feline spongiform encephalopathy (FSE)	cat	infection through contaminated beef	ataxia, metabolic wasting, rubbing
Exotic ungulate encephalopathy	Nyala-, Oryx-,Kudu-antilope	infection through contaminated meat and bone meal	ataxia, metabolic wasting, rubbing

Table 1: Mammalian prion diseases

Bovine spongiform encephalopathy and new variant Creutzfeld-Jakob disease

Bovine spongiform encephalopathy (BSE) was first described in 1986 and shortly after a great epidemic started in Great Britain. Animals show insidious onset of altered

behaviour, either fear or sometimes aggressive responses. Ataxia manifests through uncoordinated gait with falling and loss of balance. A common exposure found among all cows deceased during the epidemic, was the use of a dietary protein supplement, meat and bone meal (MBM), that was regularly fed to dairy cattle beginning at weaning. The use of MBM was stopped in 1988 by the ruminant feed ban. The epidemic continued to a peak in 1993, when more than 3000 cases per month were confirmed. By 1998 more than 177000 cases had been reported since the epidemic began. It has subsided markedly thereafter.

The origin of the epidemic itself is controversial. It is either attributed to a sporadic case of BSE or to scrapie transmitted to cattle via MBM. In the early 1980's most rendering plants abandoned the use of organic solvents and lowered the temperature in the preparation of MBM. The epidemic was most probably accelerated by the recycling of infected bovine tissues, probably prior to the recognition of BSE (Nathanson, 1999).

Throughout the BSE epidemic there were several cases of captive wild exotic animal species and a number of domestic cats that succumbed to spongiform encephalopathies. All of them are likely to have been fed with products of bovine origin (Bradley, 1997).

Most alarming was the occurrence of a new form of human TSE in Great Britain, nvCJD (new variant Creutzfeld-Jakob disease), that relates temporally and locally to the BSE crisis (Nathanson, 1999). There are now several lines of evidence that there is a causal relation between nvCJD and BSE. The agents seem to be identical as judged by strain typing (see below) (Bruce *et al.*, 1997; Collinge *et al.*, 1996; Lasmezas *et al.*, 1996b; Nathanson, 1999). Until now the number of infected persons who died of nvCJD is rather low (around 90), but steadily increasing and with the long, unpredictable incubation times of TSEs, nvCJD could become a major health problem in the future.

Transmissible spongiform encephalopathies in humans

The largest TSE-epidemic in humans so far was identified in highlanders of New Guinea (Gajdusek, 1967). The disease called kuru, which stands for "trembling", was found to be transmitted by ritual cannibalism (Gajdusek, 1977), and has declined after abandonment of this rite.

The classical neurodegenerative prion disease in man is Creutzfeld-Jakob disease (CJD). It affects about one out of a million persons per year. It can occur in three variants: sporadic, genetic or iatrogenic. Clinical features are progressive multifocal

neurologic dysfunction, myoclonic involuntary movements and severe cognitive impairment (Will, 1999). The sporadic disease starts at the earliest after age 40, whereas nvCJD, which is very atypical, has the average age of onset of 29 years. Further, nvCJD manifests different symptoms in comparison to classical CJD like personality changes, behavioural abnormalities, depression and the starting of ataxia and myoclonus before intellectual deterioration and dementia. The typical patterns of sporadic CJD in electroencephalograms, reflecting the impairment of electrical function of the brain, are absent in nvCJD (Will, 1999).

More than 20 mutations of the PrP gene have been identified in families suffering from inherited human prion disease. Besides inherited CJD, many mutations account for Gerstmann-Sträussler-Scheinker-Syndrome (GSS), a disease leading to ataxia and progressive dementia, occurring after age 40, and it is associated with PrP^{Sc} plaques in the brain of affected individuals (Gerstmann et al. 1936). Multiple affected family members were tracked, in a pattern indicating autosomal-dominant inheritance. A similar transmission has recently been described for a rare condition called FFI (fatal familial insomnia) (Gambetti *et al.*, 1993; Manetto *et al.*, 1992) that is associated with lethal insomnia and disturbances of the autonomic nervous system. Possibly this prion disease might also occur sporadically and is then called fatal sporadic insomnia (FSI) (Gambetti and Parchi, 1999).

All human prion diseases could so far be succesfully transmitted to experimental animals.

Neuropathological characteristics of prion diseases

The pathological features of TSEs are confined to the nervous system, and no inflammation or immune response is found.

Generally four neuropathological features, singly or in combination, are evident in prion diseases: 1) Death of nerve cells, also described as neuronal loss, 2) vacuolisation, leading to spongiform change (status spongiosis), 3) activation of astrocytes and microglial cells (gliosis), and 4) amyloid deposits of PrP^{Sc}. All the pathological changes in the brain vary in location and intensity, e.g. spongiform change can be absent in GSS and amyloid plaques occur only in 10-15% of all sporadic CJD cases (Will, 1999). The mechanism of pathogenesis, i.e. the events leading to vacuolisation and neuronal death, are not yet well understood (Weissmann, 1996).

In an interesting experiment Brandner and coworkers (1996b) transplanted embryonic brain tissue into a PrP knockout mouse (see below). After intracerebral infection with prions only the transplant developed disease pathology while the $PrP^{0/0}$-tissue remained unaffected, although PrP^{Sc}, produced in the transplant, diffused into the surrounding tissue. It seems therefore that PrP^{Sc} is not neurotoxic by itself (Brandner *et al.*, 1996a). Altogether the findings indicate that it is neither the absence of the normal PrP^{C}, nor the accumulation of the prion form, PrP^{Sc}, that causes neuropathology. Rather, it appears to be the intracellular accumulation of PrP^{Sc} in a neuron synthesizing PrP.

Spread of prions

Injection of prions directly into the brain is the most effective method for experimental transmission, much more effective than other delivery routes. However, the common natural routes of transmission in animals and humans are feeding, as well as intravenous (i.v.), intraperitoneal (i.p.) or intramuscular injection. Prions must find their way from these sites to the central nervous system (CNS), the only site where they elicit pathological reactions.

After TSE transmission, infectivity can initially accumulate in all components of the lymphoreticular system (LRS). Since long it is known that replication of the infectious agent in the spleen typically precedes intracerebral replication, even if it is administered intracerebrally (i.c.) (Weissmann *et al.*, 1999). Severe combined immunodeficient (SCID) mice lacking differentiated lymphocytes are not susceptible to i.p. infection, but transfer of wild-type spleen cells can restore susceptibility (Lasmezas *et al.*, 1996a). Reconstituting PrP-expression exclusively in the lymphoreticular system of $PrP^{0/0}$-mice by bone marrow transfer restored prion replication in the spleen but was not sufficient for infection of PrP-expressing brain grafts after peripheral inoculation (Blattler *et al.*, 1997). Transfer of infectivity from the spleen to the CNS is therefore crucially dependent on the expression of PrP in a tissue compartment that cannot be restored by bone marrow transfer. It was thus speculated that peripheral nerves are responsible for this transfer (Aguzzi and Weissmann, 1997).

In the nervous system, one possibility is that spread of prions occurs axonally (Fraser, 1982; Jeffrey *et al.*, 1995; Jeffrey *et al.*, 1996). Further, PrP expressing brain tissue was transplanted into the brains of $PrP^{0/0}$-mice. When the mouse was inoculated peripherally into the eye neither host nor transplant got infected (Brandner *et al.*, 1996a). It seems

therefore that spread of infectivity is dependent on the presence of PrP^C and that along the travelling route PrP^C is converted into PrP^{Sc}.

Therapy

As the understanding of prion propagation increases, it should be possible to design effective therapeutics. A first step would be an early diagnosis of infection, best before clinical symptoms are apparent. The mutations of inherited prion disease can be detected early in life or even by prenatal testing. NvCJD can early be detected by screening tonsils, as PrP^{Sc} can be found in lymphatic tissue prior to disease in the brain (Arya, 1997; Evans, 1997; Hill *et al.*, 1997b; Schreuder *et al.*, 1996). When such an early diagnosis can be made, the long incubation times are an advantage for therapeutical approaches.

Interfering with the conversion of PrP^C into PrP^{Sc} seems to be the most attractive therapeutic approach. One could either try to stabilise the structure of PrP^C or destabilise the structure of PrP^{Sc}. This has been tried with polyamines (Supattapone *et al.*, 1999b), sulfated glycans (Caughey *et al.*, 1994) or β-sheet-breaker peptides (Soto *et al.*, 2000). However, a principal difficulty is that the drugs would have to pass efficiently the blood brain barrier to enter the CNS.

A totally different therapeutic approach arose from a study on the cell types involved in prion replication in the spleen (Montrasio *et al.*, 2000). Treatment of mice with soluble lymphotoxin-β-receptor leads to the disappearance of mature follicular dendritic cells (FDCs) from the spleen and at the same time prion accumulation in the spleen was eliminated and neuroinvasion retarded. This opens the possibility to interrupt or prolong the way of prions from the periphery to the brain.

For farm animals the possibility to produce PrP knock-out strains was proposed, as these should, in reference to the $PrP^{0/0}$-mice (see below), be healthy and resistant to prion disease (Prusiner, 1998; Weissmann, 1996). However, the normal function of PrP is so far not elucidated in detail.

1.5. Characteristics of prion diseases

Incubation time

In general, TSEs have very long incubation times, measured in years or decades, but once the disease becomes clinically evident, progression to death may take as little as a few weeks or months.

The incubation time is influenced by many factors such as gene dosage, the strain of agent, the origin of the agent compared to the species infected, the amount of inoculum, the route of infection, the PrP genotype as well as additional genes (Prusiner, 1998; Weissmann, 1996; Stephenson 2000). Therefore it is presently also very difficult to make any predictions about the extent of transmission of BSE to humans.

Species barrier

The species barrier is the phenomenon in which one species tends to be more resistant to infection by prion particles generated in another species. The first passage of the infectious agent in the new host is thus almost always characterised by prolonged incubation times (Pattison, 1965b). Prions synthesised de novo are composed of the host's PrP protein and not that of the PrP^{Sc} molecules in the inoculum derived from the donor (Bockman *et al.*, 1987). On subsequent passage in a host from the same species, the incubation time shortens to that recorded for all subsequent passages. Three main factors were identified that influence the species barrier: 1) differences in PrP sequence (Collinge, 1999; Prusiner *et al.*, 1990), 2) the strain of prion (see below) (Bruce *et al.*, 1994) and 3) possibly the species specificity of protein X, a host factor proposed to be necessary for the PrP^{Sc} formation from molecular genetic studies (Telling *et al.*, 1994; Telling *et al.*, 1995).

For example, conventional mice are typically not susceptible to hamster prions (Scott *et al.*, 1989). However, mice inoculated with hamster prions were shown to exhibit no clinical disease but, upon post-mortem analysis, a significant number of individuals had histological features of TSE in the brain, as well as PrP^{Sc} (Hill *et al.*, 2000). In a second passage, transmission of disease was then shown (Hill *et al.*, 2000). Consequently, the definition of the species barrier, so far based on clinical disease, needs to be reassessed.

Prion strains

Different prion strains are varieties of prions isolated from the same species, that are distinguished by four general characteristics: 1) incubation time in inbred mouse lines, 2) lesion pattern (classification of neuropathology in different brain regions (Bruce *et al.*, 1989; Dickinson *et al.*, 1968; Fraser and Dickinson, 1973)), 3) size of PK-resistant bands, and 4) relative abundance of differently glycosylated forms.

Within the framework of the "protein only" hypothesis it is assumed that the strains differ just in the tertiary and/or quarternary structure of PrP^{Sc} (Prusiner, 1991a; Weissmann, 1996). This is supported by the finding that "Drowsy" and "Hyper", two strains of TME passaged in hamster show different resistance and cleavage sites upon digestion with Proteinase K (Bessen and Marsh, 1994).

Nevertheless, one still cannot exclude that certain PrP^{Sc} molecules from different strains carry each a strain-specific modification, e.g. specific metal ions that are associated with prions may be involved in strain specificity and strain interconversion (Wadsworth *et al.*, 1999). Alternatively, a covalent modification within the carbohydrate residues could target the prion strains to a specific subset of cells (Weissmann, 1996).

Studies altering the glycosylation of PrP give rise to speculations about its importance for the distinction of the strains. In scrapie infected cells it was shown that, after chemical blocking of glycosylation, PrP^{Sc} can still be formed from unglycosylated PrP (Taraboulos *et al.*, 1990a). However, it is not clear whether infectivity is still produced.

Abolishing the asparagine linked glycosylation sites by the mutations Thr182Ala and Thr198Ala in cultured cells resulted in synthesis of PK-resistant PrP molecules (Taraboulos *et al.*, 1990a). But it was shown that the correct trafficking of this protein was disturbed (Korth *et al.*, 2000; DeArmond *et al.*, 1997). By mutating asparagines to glutamines at the consensus site the unglycosylated protein is correctly expressed on the cell surface of cultured cells and forms protease resistant PrP after exposure to different prion strains (Korth *et al.*, 2000).

Studies with transgenic mice expressing mutated PrP at one or both glycosylation sites (Thr182Ala and/or Thr198Ala) show aberrant neuroanatomic topologies of PrP^{C} within the CNS (DeArmond *et al.*, 1997). They retain altered susceptibility to infection with different scrapie strains and modified PrP^{Sc} deposition patterns compared to wild-type mice (DeArmond *et al.*, 1997). The findings raise the possibility that glycosylation could modify the conformation of PrP and thereby alter the strain.

New variant CJD prions, propagated in humans expressing wild-type human PrP have transmission properties completely distinct from classical human CJD prions (as assessed either in transgenic or wild-type mice), but indistinguishable from those of cattle BSE (Bruce *et al.*, 1997; Hill *et al.*, 1997a).

1.6. Possible functions of PrP^C

The physiological function of the normal prion protein is presently still unknown, although various proposals have been made. As the protein possesses no significant homology to any known protein, the first hints to its function were sought from PrP knockout mice. However, mice devoid of PrP^C develop normally, and suffer from surprisingly few defects (Bueler *et al.*, 1992; Manson *et al.*, 1994). Synaptic plasticity that seems important for short-term memory and learning is impaired (Collinge *et al.*, 1994), but no behavioural abnormalities were detected (Bueler *et al.*, 1992). Certain strains of knockout mice show altered circadian rhythm and sleep behaviour (Tobler *et al.*, 1996). Another line of PrP knockout mice develop late onset ataxia with a cerebellar Purkinje cell loss (Sakaguchi *et al.*, 1996). However, this seems to be a result of additional ablation of doppel (dpl), the only very recently discovered PrP homolog (Weissmann and Aguzzi, 1999). This protein was identified from an additional coding region adjacent to the Prnp-locus (Prnd: prion gene complex, downstream). The doppel protein shows 25 % sequence identity to PrP (Moore *et al.*, 1999).

Regarding the weak phenotype of PrP knockout mice the normal function of PrP might not be obvious, as the knockout animals could adapt during early development to the lack of PrP by expression or overexpression of other proteins that complement normal PrP function (Aguzzi and Weissmann, 1997).

The attachment of PrP^C to the plasma membrane via a GPI-anchor predisposes the protein for a role in cell surface signalling, cell adhesion or transport. Many studies looked for a binding partner of PrP (Edenhofer *et al.*, 1996; Kurschner and Morgan, 1995; Oesch *et al.*, 1990; Rieger *et al.*, 1997). However, the physiological significance of these interactions of PrP, in particular with cytoplasmic proteins such as Bcl-2 or the human 37 kD laminin receptor precursor remains to be established. None of these interactions have been confirmed *in vivo*. In addition, an interaction with protein X, a possible species specific component required for prion propagation has been proposed (Telling *et al.*, 1996).

Recent investigations have focused on the Cu^{2+}-binding ability of PrP^C (Brockes, 1999). It has been shown that Cu^{2+}-ions bind to the octapeptide repeats (Viles *et al.*, 1999) and an involvement of PrP in copper transport and/or metabolism has been suggested (Pauly and Harris, 1998). Further, involvement of PrP^C in the regulation of presynaptic copper concentration and in synaptic transmission has also been proposed (Herms *et al.*, 1999), as well as a role in oxidative stress by altering the activity of Cu/Zn superoxide dismutase (Brown and Besinger, 1998). The biological relevance of these data appears to be supported by the findings of alterations in copper levels in Cu/Zn superoxide dismutase activity in PrP-deficient mice (Brown *et al.*, 1997).

Further studies show that the prion protein can prevent neuronal cell line death (Kuwahara *et al.*, 1999) and that it thus could have a role in signal transduction (Mouillet-Richard *et al.*, 2000).

In view of so many possible functions for the prion protein, one would like to know which one is correct, although it must be kept in mind that a protein can accomplish several functions simultaneously within the network of the cell.

1.7. Transgenic and knockout mice

A substantial part of the present knowledge about prion diseases arose from the possibility to generate mice that have the prion protein gene ablated, overexpress the protein, harbour mutated forms of PrP, or express the prion protein of a foreign species.

PrP knockout mice and mice expressing different levels of PrP

As described above, PrP knockout mice are overall normal mice. Nevertheless, when challenged with prions, these mice are completely protected against scrapie disease, and prions are not propagated in the brains of these $Prnp^{0/0}$ mice (Bueler *et al.*, 1993; Bueler *et al.*, 1992).

Interestingly, heterozygous $Prnp^{0/+}$ mice are partially protected, inasmuch as they show prolonged incubation times and the disease progresses much slower in $Prnp^{0/+}$ mice (Bueler *et al.*, 1994). Transgenic mice overexpressing mouse PrP succumb to scrapie even more rapidly than wild-type controls.

In summary, these findings indicate that susceptibility to scrapie increases with the PrP^C expression levels in the host (Weissmann C., 1999).

Mice expressing truncated prion proteins

By introduction of PrP transgenes harbouring defined N-terminal PrP deletions into PrP knockout mice, the PrP segments necessary for normal PrP function and for prion replication were defined (Weissmann, 1999). N-terminal deletions up to amino acid 93 (Fischer *et al.*, 1996; Flechsig *et al.*, 2000; Shmerling *et al.*, 1998) yield normal mice, which develop disease after inoculation with scrapie (Flechsig *et al.*, 2000; Weissmann, 1999), however incubation times can be longer and prion titres lower than in wild-type mice. Therefore, around 60 residues from the amino proximal region of mature PrP, including the entire octapeptide repeat region, possibly involved in Cu^{2+}-binding, are not essential for prion propagation.

In contrast, transgenic mice overexpressing PrP with deletions extending beyond amino acid 106 to position 121 and 134 spontaneously develop ataxia (Shmerling *et al.*, 1998). Introduction of one copy of the wild-type gene reversed the phenotype completely.

Deletion of regions corresponding to helix 2 and helix 3 led to a spontaneous fatal CNS disease, similar to neuronal storage disease (Muramoto *et al.*, 1997). This mutation was expressed on wild-type background.

A chimeric mouse-hamster prion protein (PrPΔ23-88Δ141-176), the so-called miniprion containing only 106 amino acids, is the smallest protein that has been shown to support prion propagation (Supattapone *et al.*, 1999a).

Mouse models for inherited prion diseases

Although many genomic mutations are known in mice, no naturally occurring mutation that causes prion disease has been described. Therefore, mutations found in inherited human prion diseases were introduced into mouse PrP.

The human PrP mutation Pro102Leu has been shown to be linked to GSS (Hsiao *et al.*, 1989). Transgenic mice expressing a chimeric hamster-mouse gene with the equivalent mutation indeed spontaneously develop neuronal degeneration, spongiosis and late astrogliosis (Hsiao *et al.*, 1990). Brain extracts from diseased mice transmitted disease to transgenic mice expressing lower levels of the same mutant gene, but not to wild-type mice (Telling *et al.*, 1996). When this Pro102Leu mutation was introduced into mice by gene targeting, no symptoms of spontaneous neurodegeneration were detected (Manson *et al.*, 1999; Moore *et al.*, 1995), indicating that the spontaneous development of prions in these mice may be an effect of PrP overproduction.

Moreover, no further mouse models for inherited human prion diseases displaying any signs of scrapie-like disease were reported. Perhaps mice do not live long enough that the occasional conversion of (mutant) PrP^{C} to the pathologic isoform would develop and cause disease.

Mice expressing PrP proteins from other species

Introduction of PrP transgenes from other species into wild-type mice resulted in reduction or abolishment of the species barrier (Bueler *et al.*, 1993; Prusiner *et al.*, 1990; Scott *et al.*, 1989). This effect is even more pronounced if the endogenous Prnp gene of the recipient mouse is ablated (Bueler *et al.*, 1993; Telling, 2000). PrP knockout but not wild-type mice harbouring human Prnp transgenes are susceptible to human prions (Telling *et al.*, 1995). Expression of bovine PrP transgenes on a $PrP^{0/0}$-background shortened the BSE incubation time. These mice are therefore presently used as indicator animals for BSE infectivity tests (Scott *et al.*, 1997).

The successful breaking of the species barrier between humans and mice has its origin in a set of studies with transgenic mice expressing chimeric PrP genes derived from hamster and mouse PrP genes (Scott *et al.*, 1992). Most efficient transmission of human CJD prions to transgenic HuPrP/$Prnp^{0/0}$-mice was achieved when the endogenous mouse PrP gene was inactivated, suggesting that mouse PrP^{C} competes with human PrP^{C} for binding to a cellular component (Telling *et al.*, 1995). This supposed species-specific factor was named protein X. It is likely to be a protein and supposedly, it binds to PrP^{C} and facilitates PrP^{Sc} formation.

Ectopic expression of PrP

The use of tissue specific promoters preceding the Prnp transgene enabled studies on the susceptibility of different cell types to prions and investigations on prion transport and pathogenicity. Mice expressing PrP under the neuron-specific NSE-promoter succumb to scrapie disease upon inoculation (Race *et al.*, 1995). The same was found for transgenic mice harboring the prion protein only in astrocytes (Raeber *et al.*, 1997). Overexpression of PrP on B- and T-cells supported infectivity in the spleen but not in the brain. Single expression of the prion protein on either T- or B-cells is not sufficient to support infectivity (Montrasio *et al.*, 2000; Weissmann, 1999).

In summary, one can conclude from these experiments that the presence of PrP on the cell surface does not suffice to support prion replication and that another factor or a special localization, e.g. within a specialized plasmamembrane region is required (Weissmann, 1999).

1.8. Other selfpropagating proteins

Yeast prions

In higher eukaryotes PrP^{Sc} is the only known self-propagating prion phenomenon. But in yeast several non-Mendelian genetic elements have been found. They propagate in many ways like PrP^{Sc} and are therefore referred to as yeast prions (Wickner, 1994).

Normal Sup35p and Ure2p are two of possibly up to 20 proteins in yeast that can acquire the self-propagating properties proposed for mammalian prions (Sondheimer and Lindquist, 2000). [URE3] is a cytoplasmatically inherited element, which enables a strain of yeast to use ureidosuccinate despite the presence of ammonium ions, which inhibit its uptake. [URE3] is related to the URE2 gene and its product Ure2p. Propagation of [URE3] depends on the presence of Ure2p. The frequency with which a strain adopts the [URE3] phenotype can be increased greatly by overproduction of Ure2p. The same phenotype can be induced by chromosomal mutations in the gene (Wickner, 1997). As observed for mammalian prions this phenomenon can be inherited, be induced by infection, or occur spontaneously.

The same is true for the second yeast prion. The normal form of Sup35p is essential for decoding mRNA, ensuring that ribosomes terminate correctly at the end of the coding region. The functionally inactive prion form of Sup35p confers the so-called [PSI^+] phenotype on yeast cells. In such strains, Sup35p is found almost exclusively as a protease-resistant, high molecular weight aggregate.

De novo generation of self-propagating aggregates of the yeast non-Mendelian prion factor Sup35p (DePace *et al.*, 1998; King *et al.*, 1997; Santoso *et al.*, 2000) and Ure2p (Taylor *et al.*, 1999) is an important feature of these self-propagating aggregates. One recent study now unequivocally demonstrates that the establishment of the [PSI^+] phenotype in yeast *in vivo* is a direct consequence of seeding by an altered conformation of Sup35p (Sparrer *et al.*, 2000). This generation of new aggregates by inoculation with

catalytic amounts of pre-existing aggregates formed *in vitro* could so far not be performed successfully with recombinant mammalian PrP.

Human amyloidosis

Some common neurodegenerative diseases including Alzheimer's disease and amyotrophic lateral sclerosis (ALS) are similar to prion disorders in many ways. They accumulate abnormal β-sheet rich fibrillar amyloids of a normal host protein expressed in the CNS. On this basis a similar mechanism between PrP^{Sc} formation and other amyloidosis has been suggested (Gajdusek, 1988). Nevertheless, although e.g. Alzheimer disease can occur spontaneously or be inherited, none of these amyloid diseases could be shown to be infectious.

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2. STABILIZATION OF THE SCRAPIE AGENT BY FORMALDEHYDE

2.1. Introduction

Concept

The "protein only" hypothesis states that the scrapie agent is PrP^{Sc}, an abnormal, oligomeric form of the mature Prnp gene product found in tissues of TSE sufferers (Gajdusek, 1988; Griffith, 1967; Prusiner, 1991). It is believed to differ from PrP^{C} only (or mainly) conformationally and is rich in β-sheet structure (Hope *et al.*, 1986; Pan *et al.*, 1993; Stahl *et al.*, 1993; Stahl and Prusiner, 1991). By digestion with Proteinase K , the subunits of PrP^{Sc} become N-terminally truncated to the protease resistant core PrP(27-30) which is still infectious.

Upon purification, PrP^{Sc} or PrP(27-30) is found as aggregated insoluble complexes. Disaggregation and solubilization can be achieved by treatment with denaturing agents such as urea or guanidinium chloride. All these treatments, however, lead to an irreversible loss of infectivity (Prusiner *et al.*, 1993; Safar *et al.*, 1993a).

It has been shown that formaldehyde-treatment of scrapie-agent-preparations does not cause inactivation and in fact can stabilise infectivity against autoclaving procedures that inactivate non-Formol-treated prions (Brown *et al.*, 1990b; Taylor and McBride, 1987; Taylor and McConnell, 1988).

Formaldehyde is a crosslinking agent and therefore might stabilise the conformation of PrP^{Sc}. Therefore we hypothesised that it might be possible to "fix" the PrP^{Sc}-conformation by intramolecular crosslinking without intermolecular crosslinking. It might then be possible to dissociate prion aggregates with medium denaturant concentrations without loss of the conformation of the monomers and without loss of infectivity. If the monomeric, formaldehyde-crosslinked PrP^{Sc} or PrP(27-30) subunits could then be purified, it would be a proof for the existence of a small-molecular infectious unit and a possibility for structural analysis.

First, literature studies on the reactivity of formaldehyde with proteins and the action of formaldehyde on the scrapie agent were undertaken. Subsequently, crosslinking experiments of recombinant mPrP(23-231) and PrP(27-30) were performed.

Then the study on increased stability of the scrapie agent after formaldehyde treatment (Brown *et al.*, 1990b) against different inactivation methods was repeated.

In a last experiment, formaldehyde fixation of PrP(27-30) in detergent-lipid-protein-complexes (DLPCs) was performed, and the protein was repurified and solubilized by denaturing agents. Finally, the formaldehyde treated preparations of PrP(27-30) were injected into hamster brains for incubation time assays.

Literature studies

Formaldehyde in general

General effects of formaldehyde on proteins are known since about one century when it was first used as a disinfectant. Thereby it was discovered that formaldehyde has good effects on tissue fixation (for review see Fox *et al.*, 1985; Puchtler and Meloan, 1985). Further, formaldehyde has been used in the tanning process of leather, for the preparation of toxoids for vaccination (French and Edsall, 1945), and more recently for *in vitro*- and *in vivo*-crosslinking of protein complexes and proteins to nucleic acid (Orlando *et al.*, 1997).

Clarification of names and concentration

Some confusion about names and concentrations of formaldehyde solutions are found in laboratories and in the literature. Bubbling formaldehyde gas through water until no more formaldehyde is dissolved, gives a 40% (w/v) or 37% (w/w) aqueous solution. This is recently mostly called formaldehyde solution, but formerly had different names. English companies sold it as formic aldehyde, Hoechst as Formol and Schering and American companies called it Formalin. Therefore formaldehyde solution, Formalin and Formol are essentially the same.

If stored in this concentrated form formaldehyde condensates spontaneously to polyoxymethylenes. To inhibit this condensation modest amounts of alcohol (mostly methanol) are added to the stock solution.

A commonly used working dilution is 10% Formalin (= 4% formaldehyde) solution, corresponding to a concentration of 1,3 M.

Chemical reactions of formaldehyde

In aqueous solution most of the formaldehyde is hydrated to methylene glycole ($CH_2(OH)_2$) and less than 4% exists as free formaldehyde. 10% Formalin is weakly acidic with a pK of 12.8. Therefore it is normally buffered by phosphate salts.

In general the reactions of formaldehyde with proteins follow all a general two-step mechanism (French and Edsall, 1945), see Figure 1: First the addition reaction of formaldehyde to a compound containing an "active H-atom", resulting in a

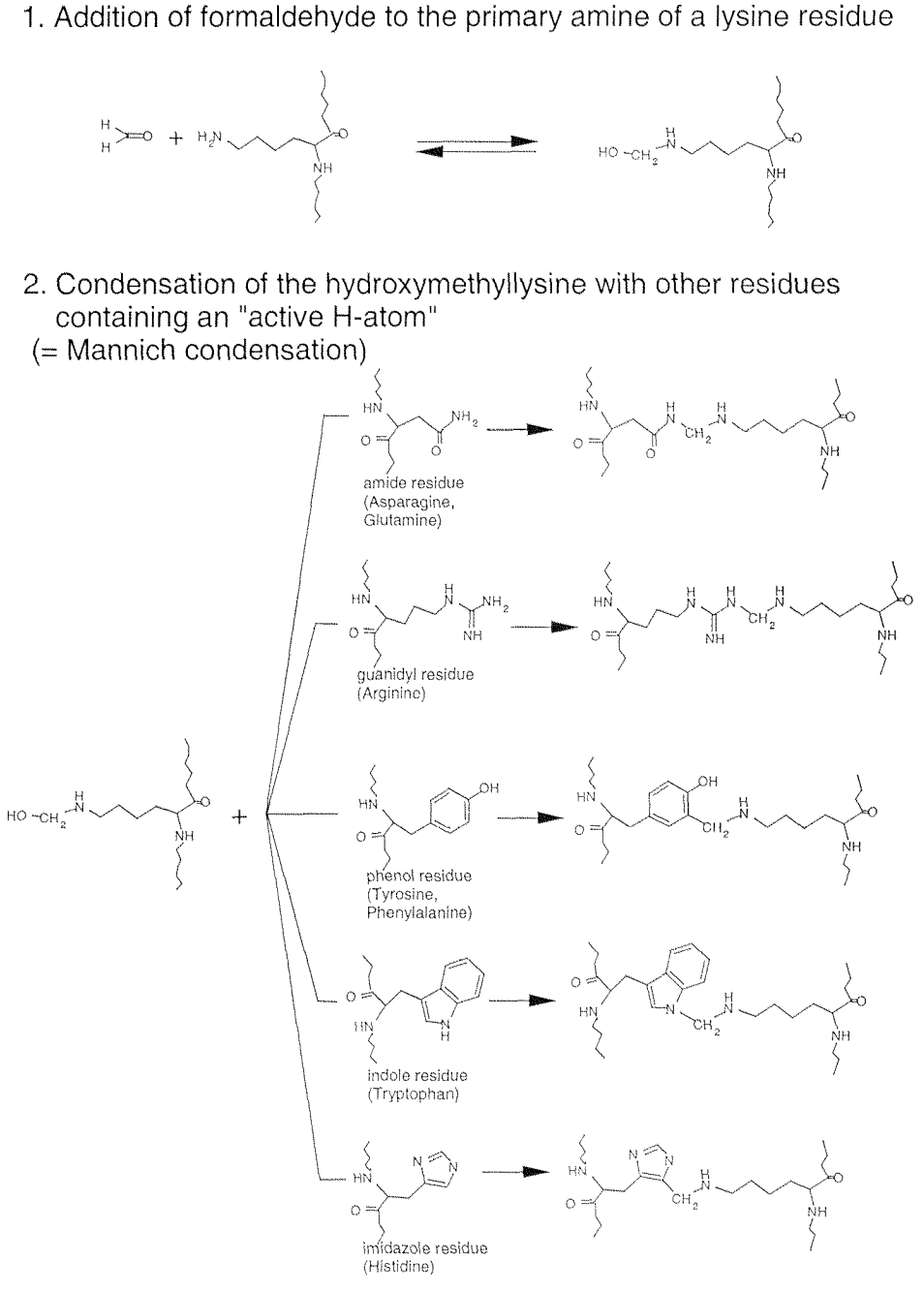

Figure 2.1.: Crosslinking by formaldehyde

The two-step mechanism of the crosslinking reactions by formaldehyde is exemplary shown for crosslinking a lysine residue with various other amino acids.

hydroxymethyl compound. This hydroxy group is reactive and can in a second step condense with another group containing an "active H-atom" (Mannich condensation), thereby forming a methylene bridge. Kinetic studies of the reaction proved this two-step process (Rott *et al.*, 1981).

The reactions can be intramolecular or intermolecular, resulting in artificial oligomers or aggregates.

The first reaction step is very fast but also readily reversible and the bound formaldehyde can easily be removed by washing. The methylene bridges formed in the second reaction step are, however, very stable and cannot be broken by high concentrations of urea or other disaggregating salts (Puchtler and Meloan, 1985; more details see below).

The amino group of lysine is most reactive to formaldehyde, however, many functional groups of amino acids contain "active hydrogen atoms" and therefore can in general react with formaldehyde. These groups include imino groups, amide groups (asparagine, glutamine), guanidyl groups (arginine), hydroxyl groups (serine, threonine), sulfhydryl groups (cystein), and aromatic rings like imidazol (histidine), indol (tryptophan) and phenol (tyrosine, phenylalanine). The peptide bond is less reactive.

For only few proteins the reactions with formaldehyde have been studied in detail. More results are known from model systems using single amino acids, as analysed by Fraenkel-Conrat (e.g. Fraenkel-Conrat *et al.*, 1945; Fraenkel-Conrat and Olcott, 1946; Fraenkel-Conrat and Olcott, 1948) and further reviewed by French and Edsall (1945), Martin *et al.* (1975) and Kitamoto *et al.* (1985).

For some proteins crosslinked adducts have been isolated. Bizzini and Raynaud (1974) report the isolation of a lysine-lysine and a ε-amino-lysine-tyrosine(C_3) adduct of diphteria-anatoxin, and Blass *et al.* (1965) the isolation of a lysine-tyrosine adduct. Formaldehyde crosslinked peptides of tryptophan-synthetase were isolated and assigned to adducts composed of asparagine-serine and glutamine-serine (Myers and Hardman, 1971).

Finally, in the presence of tetraborohydride ($NaBH_4$) formaldehyde is also used for reductive methylation of proteins (Galembeck *et al.*, 1977). The predominant reaction is the dimethylation of amino groups of lysine.

General effects of formaldehyde on proteins

The general effect of formaldehyde is assumed to be a stabilisation of the tertiary structure of proteins against unfolding by pH-changes, high ionic strength, heating, and chemical denaturation, as well as a stabilisation against digestion with proteolytic enzymes.

Toxins like diphteria toxin and tetanus toxin are inactivated by formaldehyde treatment. So-called toxoids are formed that are still immunogenic but no longer toxic (Aggerbeck and Heron, 1992; Paliwal and London, 1996). The toxoids are more stable to heat (thermal unfolding) than the toxins and are less likely subject to irreversible alterations.

The influence of the crosslinks on the distortion of the tertiary structure may be moderate, as shown by X-ray-diffraction, circular dichroism and electron microscopy of formaldehyde treated histones (Jackson, 1978). Other globular proteins have been shown to exhibit little or no protein secondary structure change after formaldehyde treatment (Mason and O'Leary, 1991), and enzymes may even retain their activity, as shown for *E. coli* tryptophane synthetase (Myers and Hardman, 1971).

It was also proposed, that formaldehyde crosslinking fixes the folded structure of proteins and prevents formation of the so-called molton globule state (Paliwal and London, 1996).

Reaction conditions

Neutral Formalin (pH 7) buffered by phosphate is mostly used for the reactions with proteins. At this pH the amino groups of lysine are sufficiently uncharged for the reaction with formaldehyde to occur, whereas in unbuffered Formalin (pH 4) the amino groups are protonated and react extremely slowly (French and Edsall, 1945). Nevertheless it was found that maximum tissue fixation, as judged by crosslinking, is achieved at pH 4-5.5 because at higher pH the increase in the amount of reversibly bound formaldehyde blocks reactive groups (Gustavson, 1956).

The required formaldehyde concentrations and reaction times vary widely dependent on the application: For tissue fixation, 4% formaldehyde at pH 7.4 for up to 48 hours is commonly used (Fox *et al.*, 1985), for toxoid preparation only about 0.2% formaldehyde for several weeks (Paliwal and London, 1996) and for intramolecular fixation of proteins a 100:1 ratio of reagent to protein at approximately 0.01% - 0.04%

formaldehyde at around pH 8 is applied overnight. Temperatures are varying between 20°C and 50°C.

The reaction can be stopped e.g. by addition of excess glycine (Orlando *et al.*, 1997), by acidification to pH < 3 (Kitamoto *et al.*, 1985) or by dialysis against Dimedone, a reagent reacting irreversibly with formaldehyde (Myers and Hardman, 1971).

Washing and dialysis (evtl. with Dimedone) reverses the formation of the initial formaldehyde adducts corresponding to the first reaction step (compare Figure 1). Some crosslinks can be reversed by boiling for at least 30 minutes in Tris-containing or denaturing buffers (Orlando *et al.*, 1997). However, methylene bridges between the ε-amino-nitrogen of lysine and carbon atoms of aromatic rings are very stable and even resist acid and alkaline hydrolysis (Bizzini and Raynaud, 1974; Fraenkel-Conrat and Olcott, 1948).

Methods used to examine formaldehyde treated proteins

The methods used to examine the reactions of proteins with formaldehyde are manifold and changed over the last century. Bound formaldehyde and the amount of release upon hydrolysis was initially measured by formaldehyde destillation (French and Edsall, 1945). Reaction products of single amino acids were examined by crystallisation (Kitamoto *et al.*, 1985). pH, guanidinium chloride, and heat stability were evaluated by fluorescence-emission-spectra (Paliwal and London, 1996). SDS-PAGE analysis showed good results for dimer and polymer formation of histones (Jackson, 1978; Hopwood, 1968), but could only give few informations for intramolecular crosslinks (Paliwal and London, 1996). Further, reactions with radioactive formaldehyde were used to measure the amount of bound formaldehyde and to analyse crosslinked amino acid residues (Bizzini and Raynaud, 1974; Galembeck *et al.*, 1977).

Formaldehyde and nucleic acids

Formaldehyde is also reacting with the bases of nucleic acids, thereby forming crosslinked dinucleotides (Feldman, 1973). Also, crosslinking of nucleic acids and nucleoproteins occurs. Formaldehyde is thus both mutagenic and a potential inhibitor of DNA replication and transcription. It is therefore used as a disinfectant that kills bacteria and viruses.

Scrapie and Formaldehyde

Resistance of TSE agents to formaldehyde

The first evidence for the resistance of the scrapie infectious agent to formaldehyde is one of the tragic stories in the history of prion diseases. In 1935, W.S. Gordon vaccinated about 45000 sheep against looping-ill virus (Gordon, 1946). The vaccine comprised a 10% saline suspension of brain, spinal cord, and spleen tissues of looping-ill-virus-infected sheep treated with 0.35% Formalin to inactivate the virus. More than two years after the vaccination, 2000 of the vaccinated sheep developed scrapie disease. It was possible to trace back that all scrapie-diseased sheep had exclusively been vaccinated with only one of three batches of vaccine. The sheep from which this batch was produced, originated from a flock with other lambs that later developed scrapie.

After this accidental demonstration of resistance of the scrapie agent to Formol, it was shown that the resistance is unusually high for a transmissible agent, withstanding up to 3% formaldehyde acting at pH 7.4 and 37°C for 13 days (Stamp *et al.*, 1959). Pattison (1965) reported a systematic analysis where he had incubated the scrapie agent with up to 12% Formol-saline for time periods between 18 hours and 28 months. He found infectivity in goats and mice in all experiments.

It also has been shown that the human CJD-agent is resistant to formaldehyde treatment and that tissue fixed for histology can be used for transmission and confirmation of the disease (Brown *et al.*, 1986; Gajdusek and Gibbs, 1976). Further, a mouse-adapted CJD-strain (Kingsbury *et al.*, 1982), hamster-adapted scrapie (Brown *et al.*, 1982) and also the BSE-agent (Fraser *et al.*, 1992) were shown to be still infectious after formaldehyde treatment. Moreover, Bernoulli *et al.* (1977) reported the transmission of CJD by use of electrodes that had been sterilised by formaldehyde vapour.

Decontamination of formaldehyde treated scrapie material

As it was known that autoclaving at high temperature reduces scrapie infectivity, this method seemed good to decontaminate Formalin-treated tissue samples (Masters *et al.*, 1985). However, the inactivation was not proven in this study (Taylor, 1986; Taylor and McBride, 1987). When autoclaved formaldehyde treated scrapie samples were examined for infectivity, formaldehyde proved to render the scrapie agent resistant to inactivation by autoclaving (Taylor and McConnell, 1988). This was further confirmed for fixed tissue samples and purified PrP^{Sc}-preparations (Brown *et al.*, 1990b).

Finally, two methods for the decontamination of Formalin fixed scrapie tissue were found: First, the use of phenolyzed Formalin (Brumback, 1988) and second the inactivation by formic acid (Brown *et al.*, 1990b; Taylor *et al.*, 1997). The second method seems to be more effective concerning the inactivation.

Formaldehyde resistance in general

In the case of all non-prion infectious agents that show formaldehyde resistance no "unlimited resistance" to high formaldehyde concentrations, as in the case of the scrapie agent, is found. A common resistance mechanism of pathogenic microorganisms, that are partially resistant to formaldehyde, is the upregulation of genes endocing alcoholdehydrogenases and thus chemical removal of formaldehyde (e.g. (Kummerle *et al.*, 1996)).

However, the described infinite resistance of the scrapie agent to formaldehyde is in fact a unique property of prions and fits into the prion concept: No nucleic acid is necessary, a protein (PrP) is an essential component of the infecious agent, and its infectious conformation can be stabilised by formaldehyde crosslinking.

Inactivation and solubilization of PrP^{Sc}

Heat inactivation

In general, heat inactivation of proteins is achieved in two steps: At intermediate temperatures denaturation occurs by thermal unfolding which often leads to aggregation and precipitation of the protein (Porter *et al.*, 1993). At higher temperatures (above 100°C) covalent bonds are disrupted: deamidation of asparagine and glutamine residues, hydrolysis of peptide bonds at aspartic acid residues, destruction of disulphide bonds, oxidation of cysteine residues and β-elimination of cystine residues (Zale and Klibanov, 1986). This leads to the irreversible disruption of the covalent protein structures (Ahern and Klibanov, 1985; Tomizawa *et al.*, 1995). Thermostable proteins are more resistant to heat and other forms of denaturation (Liao, 1993), and it has been proposed that oligomerization can be a stabilising mechanism for thermophilic systems (Merkler *et al.*, 1988). Also artificial crosslinks can stabilise enzymes against heat inactivation (Trubetskoy and Torchilin, 1985).

The infectious scrapie agent shows exceptional resistance to heat inactivation. The normally used decontamination standard for bacteria and viruses of 126°C for 20

minutes is not sufficient to render the scrapie agent non-infectious. In 1988, the UK standard was 134-138°C for 18 minutes based on porous load autoclaving, the United States' standard was 132°C for one hour for gravity displacement (Taylor and McConnell, 1988). However, as mentioned above, it was found that pre-treatment of scrapie infected brain tissue with formaldehyde abolished the inactivation effect of this high-standard autoclaving (Taylor and McConnell, 1988). The resistance was also shown for purified PrP^{Sc} (Brown *et al.*, 1990b). The reason for the high temperature resistance of the scrapie agent probably lies in its extremely stable, ordered aggregation, and formaldehyde can render further stability to the oligomer by crosslinking.

Denaturation and solubilization

As already mentioned, purification of scrapie infectivity yields aggregated PrP^{Sc} or PrP(27-30) and results in the isolation of so called prion rods (Prusiner *et al.*, 1980b). Numerous procedures were examined for their effectiveness in disrupting the prion aggregates while preserving infectivity. Salts and sonication were unsuccessful in disruption. High pH-changes and strong denaturants such as the chaotropic agents urea and guanidinium chloride at high concentrations disrupted the aggregates, but also reduced infectivity (Safar *et al.*, 1993a).

SDS is an anionic detergent that inactivates prions in a concentration-dependent manner (Prusiner *et al.*, 1980a). In this context, studies on the electrophoretic properties of the scrapie agent after SDS-treatment are interesting. Several attempts were made to isolate infectivity from a band in a SDS-gel. Only one study that could not be reproduced, claimed the recovery of the major fraction of infectivity from a SDS-gel slice containing the monomeric PrP band (Brown *et al.*, 1990a). Other studies also report of the recovery of 1-100% of infectivity from SDS-gels. However, most of the infectious agent was found in the top parts of the gel, especially the stacking gel and obviously corresponded to PrP^{Sc} oligomers not yet denatured by SDS (see Rubenstein *et al.*, 1994; Prusiner *et al.*, 1993; Lax *et al.*, 1983).

In a recent study, the SDS-treated scrapie agent was separated on a sucrose gradient (Riesner *et al.*, 1996). On the top of the gradient, spherical particles composed of 4-6 PrP-molecules with little or no infectivity were found, and at the meniscus a lipid-rich fraction composed of heterogeneous particles containing high levels of scrapie infectivity.

The only known method to solubilize PrP^{Sc} or PrP(27-30) in infectious form, as defined by remaining in the supernatant after one hour of centrifugation at 100 000 x g, is the dispersion in so called DLPCs (detergent-lipid-protein complexes, Gabizon *et al.*, 1987). Removal of the detergent by dialysis produces closed liposomes. Scrapie infectivity increases 10-100 fold after dissociation of PrP(27-30) into DLPCs. This is consistent with the "seeding model" that eventually requires breakage of PrP^{Sc} oligomers (see chapter 1: Introduction, p. 12).

Overall, two generally different kinds of infectious prion preparations can be discriminated: 1) The insoluble PrP^{Sc} aggregates, and 2) the solubilized infectious preparations, prion DLPCs and liposomes. These contain lipids by definition (Gabizon and Prusiner, 1990). From this one could principally conclude that the conformation of an isolated prion monomer is unstable and must be stabilised by aggregation or by close contact with lipids. The question of whether intramolecular crosslinks might also stabilise PrP^{Sc} so that even a PrP^{Sc} monomer would be stable in solution should be tested in the present project by formaldehyde treatment.

2.2. Materials and methods

Protein preparations

Purification of mPrP(23-231)

Full-length recombinant mPrP(23-231) was purified in its oxidized form as described (Liemann and Glockshuber, 1998).

PrP^{Sc}- and PrP(27-30)-preparation

PrP^{Sc} was isolated from Syrian hamster brains that had been infected with the 263K-strain of scrapie. Purification was done according to the protocol of Bolton *et al.* (1987). Shortly, ten scrapie infected brains were homogenized (10% (w/v)) on ice in TEND (10 mM Tris/HCl pH 8.3, 1 mM EDTA, 133 mM NaCl, 1 mM DTT) containing 10% Sarkosyl using 18 and 22 gauge needles. The homogenate was centrifuged for 30 minutes at 22000 x g (SS34 rotor, 13000 rpm, Sorvall centrifuge) at 4°C, and the supernatant transferred to ultracentrifuge tubes and was centrifuged 3 hours at 150000 x g at 4°C (in a Ti70 rotor, 38000 rpm, Beckmann ultracentrifuge). The supernatant was removed and the pellet resuspended in 1 ml TEND, 10% NaCl, 1% Zwittergent by

sonication (3 times 1 minute; Branson sonicator, 100% output) and homogenization with a Pasteur pipette. Buffer (TEND + 10% NaCl + 1% Zwittergent) was added to a final volume of 10 ml. The homogenate was centrifuged for 2 hours at 225000 x g at 20°C (Ti70 rotor, 46000 rpm, Beckmann ultracentrifuge). The supernatant was discarded and the pellet resuspended in 1 ml TNCM (10 mM Tris/HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$) by sonication (3 times 1 minute as above). RNAseA was added to 100 µg/ml and DNAseI to 20 µg/ml (about 10 U/ml), and the homogenate was incubated in a rotating vessel overnight at 4°C. The sample was then adjusted to 20 mM EDTA, 10% NaCl, 1% Zwittergent and layered on a sucrose-cushion (1 M sucrose, 100 mM NaCl, 0.5% Zwittergent). Sucrose-gradient-centrifugation was performed for 2 hours at 225000 x g at 20°C (Ti70 rotor, 46000 rpm). The supernatant was removed and the pellet resuspended in 1 ml 0.1% Sarkosyl, 10% NaCl by sonication (3 times 1 minute as above). The suspension was centrifuged in an Eppendorf centrifuge (15 minutes at 14000 x g), the supernatant was removed and the pellet resuspended in H_2O (about 1% of original homogenate volume) by sonication (3 times 1 minute as above).
To obtain the purer, N-terminally degraded form PrP(27-30), PrP^{Sc} was centrifuged and diluted into 10% NaCl, 1% Sarkosyl, 5 µg/ml Proteinase K (final volume: 4 ml) and stirred for 2 hours at 37°C. After centrifugation for 15 minutes at 14000 x g the pellet was washed with 1 mM PMSF and was subsequently washed two times with H_2O and stored in H_2O (final volume: 1% of the initial 10% (w/v) homogenate, about 1ml). A preparation contained about 9-10 log LD_{50}.

Preparation of PrP(27-30) DLPCs (detergent-lipid-protein-complexes)
This procedure corresponds to the protocol of R. Gabizon (personal communication). A small portion (20 µl) of the PrP(27-30) preparation was methanol-precipitated, dried shortly and resuspended in 1 ml 100 mM NaCl, 10 mM Hepes/NaOH, pH 7.4 by vortexing and sonication. 100 µl 20% Sarkosyl were added. The mixture was added to a Corex glass tube under argon atmosphere that contained 5 mg phosphatidylcholin dried onto the wall. The tube was closed, vortexed and sonicated in an ice bath until the solution was clear (Branson sonicator, output 100%). Then it was centrifuged for 30 min at 30000 x g. The supernatant contained the DLPCs with about 10-20 µg PrP(27-30)/ml.

Repurification of PrP(27-30) from detergent and lipids

9 volumes of cold methanol were added to the PrP(27-30)-DLPC preparation and incubated for 1 hour at –20°C. The sample was centrifuged for 30 minutes at 10000 x g at 4°C. The supernatant was removed, the pellet resuspended in 1 ml cold methanol and centrifuged for 30 minutes at 100000 x g at 4°C. The supernatant was removed and the pellet air-dried and suspended in 100 µl H_2O.

In vitro studies

Reactions of mPrP(23-231), PrP(27-30) and PrP(27-30) DLPCs with formaldehyde and ^{14}C-formaldehyde

mPrP(23-231) (5-20 µM) in 50 mM NaPi, pH 7.4, was incubated with different amounts (1 mM – 1.3 M) of formaldehyde or ^{14}C-formaldehyde for 1 to 48 hours at 22°C. The reactions were stopped either by direct application on a blotting membrane, by immediately adding SDS-sample buffer, boiling and loading on a gel, or by precipitation and washing with methanol.

Similar reactions were performed with PrP(27-30) and PrP(27-30) DLPCs. The reactions were analysed by dot blot, SDS-PAGE, Western blot, and in case of the reactions with radiolabelled formaldehyde, quantified in a scintillation counter or on a phosphor imager according to standard protocols.

Inoculation studies

Sample preparation for the first inoculation study

Aliqouots of PrP(27-30) were incubated with an equal volume of 50 mM PBS (phosphate buffered saline, pH 7.4) or 20% Formol-saline (final concentration 1.3 M formaldehyde in PBS) for 48 hours at room temperature and centrifuged for 30 minutes at 100000 x g at 4°C. The samples were diluted according to one of the following inactivation procedures: Autoclaving was performed for 30 minutes at 136°C in PBS or 10% Formol-saline; for inactivation by guanidinium chloride the protein was incubated for 3 hours in 6 M guanidinium chloride at 37°C; for SDS-solubilization, PrP(27-30)-samples were diluted with 2% SDS, boiled for 5 minutes and incubated for 3 hours at room temperature.

Prior to inoculation, all samples were methanol-precipitated and diluted in PBS.

Sample preparation for the second inoculation study

PrP(27-30) DLPCs were adjusted to 50 mM phosphate, pH 7.4 and formaldehyde was added to a final concentration of 0, 0.001% (0.13 mM), 0.01% (1.3 mM) or 0.1% (13 mM) and samples were incubated for 13 hours at 20°C. Subsequently PrP(27-30) was repurified as described above. The inactivation/solubilization-step was performed as in the first inoculation study described above.

The samples were then centrifuged at 100000 x g for 1 hour. Supernatant and pellet fractions were separated according to the soluble fraction protocol (Riesner, 1996). All samples were methanol-precipitated and diluted 1:100 into PBS.

Inoculation and infectivity assays

Incubation-time-assays were carried out in golden Syrian hamsters at the Bundesforschungsanstalt at Tübingen in collaboration with the group of Dr. M. Groschup. 30 µl of a 1:100 dilution of each sample was injected intracerebrally into groups of six hamsters. Animals were observed for decreased motor activity and ataxia as early symptoms and disorientation and coordination disturbance as later symptoms for 9 months after inoculation. Infectivity titres were calculated from the incubation time according to Prusiner *et al.* (1982), and expressed as logarithms of the mean lethal dose ($\log_{10}$ LD_{50}/30 µl inoculum volume).

Western blotting and dot blots

SDS-PAGE, dot blots and Western immunoblots were performed using standard procedures. The murine prion protein was detected in immunoblots using the polyclonal rabbit antiserum R340 that reacts with both mouse and hamster PrP. Additionally, the monoclonal antibodies 6H4 (Korth *et al.* (1997), specific for mouse and hamster), or 3F4 (Kascsak *et al.* (1987), specific for hamster only) were utilized.

2.3. Results

In vitro experiments

In order to investigate the action of formaldehyde on the prion protein, *in vitro* experiments with recombinant full-length mouse prion protein, mPrP(23-231), and formaldehyde were performed. The first goal was to establish conditions for

intramolecular crosslinking. These initial experiments were performed at protein concentrations of 5-20 µM at 22°C and pH 7.4 for 1-48 hours with varying formaldehyde concentrations (1 mM - 1.3 M). Subsequently the reactions were analysed on SDS page, assuming increased mobility of intramolecularly crosslinked PrP molecules.

The main problem at the beginning was that after reaction with high concentrations (> 50 mM) of formaldehyde the recombinant protein was no longer detectable on silver or Coomassie-stained SDS-gels, or in dot blots stained with two different antibodies (R340 and 6H4). Even glycosylated PrP^{Sc} was no longer detectable after formaldehyde-incubation when a staining kit for detection of carbohydrate residues was used. On the other hand, at low formaldehyde concentrations (-10 mM) no reaction, e.g. no significant mobility shift on the SDS-PAGE gel was visible (Figure 2).

Therefore, ^{14}C-labelled formaldehyde was used in further studies both as marker on the reacted protein and as a tool to determine the amount of the reaction. A wide spectrum of conditions with varying concentrations, incubations times and temperatures was tested. The reaction of mPrP(23-231) with ^{14}C-formaldehyde at pH 7.4 depended mainly on the ratio of the reaction components, less on the absolute concentrations, reaction time or temperature. The results can be summarised as follows: 1) The reaction became detectable when a 50-100 fold molar excess of ^{14}C-formaldehyde over mPrP(23-231) was used. A weak radioactive band of the size of mPrP(23-231) was detected and it was calculated that on average one fourth of all mPrP(23-231)-molecules were labelled with ^{14}C-crosslinks. 2) With a 500-1000 fold molar excess of ^{14}C-formaldehyde over mPrP(23-231) almost every mPrP(23-231) molecule was labelled with 1-2 ^{14}C-crosslinks, but in addition to the small band corresponding to monomeric PrP, oligomers were found that did not enter the

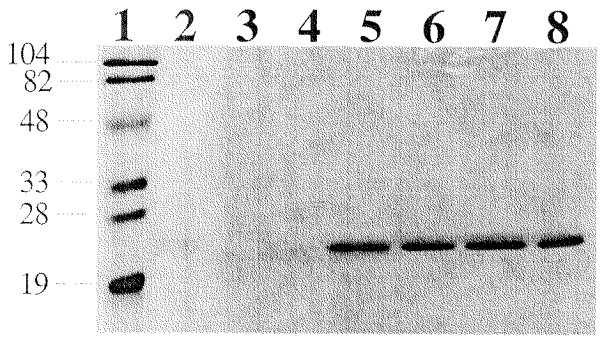

Figure 2: Coomassie-stained gel of 5 µM mPrP(23-231) samples incubated for 12 hours with decreasing amounts of formaldehyde. **1**) size marker in kDa, **2**) 1.3 M, **3**) 240 mM, **4**) 50 mM, **5**) 10 mM, **6**) 2 mM, **7**) 0.4 mM, **8**) 0 M formaldehyde.

stacking gel. 3) When more than a 5000 fold molar excess of ^{14}C-formaldehyde over mPrP(23-231) was used, only crosslinked aggregates were found that did not enter the stacking gel, and it was calculated that a mean of ten intra- and intermolecular ^{14}C-crosslinks were formed per mPrP(23-231) molecule (compare Figure 3).

The same concentration range of ^{14}C-formaldehyde was applied to PrP(27-30)-aggregates and PrP(27-30) DLPCs. It was found that the reaction was also concentration dependent, as quantified by scintillation counting. However upon analysis on SDS-PAGE, both the PrP(27-30)-aggregates and PrP(27-30) DLPCs showed no small molecular bands, but mainly aggregates. Additionally, for PrP(27-30) DLPCs part of the protein that had reacted with ^{14}C-formaldehyde gave a smear on the gel over a broad range of sizes (data not shown). We interpreted this as variable crosslinking of different glycosylated forms. As control PrP(27-30) was deglycosylated with PDGaseF and denatured. It then reacted in the same way as mPrP(23-231), and the band corresponding to monomeric PrP could be detected at low ^{14}C-formaldehyde concentrations (data not shown). Consequently the carbohydrate moieties in the PrP^{Sc} subunit might contribute to intermolecular crosslinks and could principally be responsible for the reported stabilising effect of formaldehyde on infectivity.

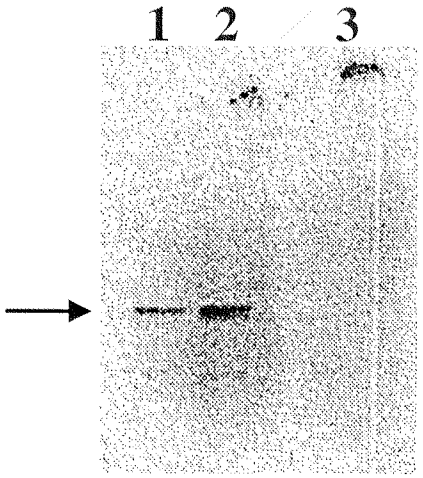

Figure 3: Autoradiography of a SDS-gel with samples of 5 µM mPrP(23-231) incubated for 12 hours with **1**) 0.13 mM, **2**) 1.3 mM and **3**) 13 mM ^{14}C-formaldehyde. The arrow indicates the size of monomeric mPrP(23-231).

Inoculation studies

Test of stabilisation of PrP(27-30) by formaldehyde

To verify the stabilising effect of formaldehyde on purified, infectious PrP(27-30) preparations against autoclaving and to test if stabilisation of PrP(27-30) can also be achieved against denaturation, an inoculation study in hamsters was undertaken in

collaboration with Dr. M.Groschup, Bundesforschungsanstalt, Tübingen. Purified PrP(27-30) was treated with 1.3 M formaldehyde as in the study by Brown *et al.* (1990b), and subsequently different inactivation procedures were applied: autoclaving at 136°C for 30 minutes, treatment with 6 M guanidinium chloride for 3 hours at 37°C or treatment with 2% SDS, boiled for 5 minutes and incubated at room temperature for 3 hours. As controls, non-Formol-treated samples were used. All samples were injected into hamster brains for incubation time assays.

The results are summarised in Table 1. In the control experiments, the autoclaving procedure caused a reduction of infectivity of more than 5 log ID_{50}-units, treatment with

	number of infected/ inoculated animals	incubation time	log ID_{50}-units
1. PrP(27-30) control	6/6	101.6 +/- 6.6	5.3
2. PrP(27-30) autoclaved	0/6	-	0
3. PrP(27-30) + 6 M Gdn/HCl	5/6	136, 136, 150, 171, 192 (157 +/- 24.2)	<0.6
4. PrP(27-30) + 2% SDS	6/6	110.5 +/- 3.3	4.2
5. PrP(27-30) + 1.3 M formaldehyde	6/6	120.5 +/- 5.5	3.2
6. PrP(27-30) + 1.3 M formaldehyde autoclaved*	2/6	150, 171 (160.5 +/- 14.8)	<0.5
7. PrP(27-30) + 1.3 M formaldehyde autoclaved*	0/6	-	0
8. PrP(27-30) + 1.3 M formaldehyde + 6M Gdn/HCl	5/6	129, 129, 133, 168, 175 (146.8 +/- 22.7)	<1.2
9. PrP(27-30) + 1.3 M formaldehyde + 2% SDS	3/6	126, 129, 133 (129.3 +/- 3.5)	<1.5

*6.) was autoclaved in the presence of formaldehyde, 7.) after the unreacted formaldehyde has been washed away

Table 1: Incubation times and infectivity titres for the test of stabilisation of PrP(27-30) by formaldehyde against inactivation

6 M guanidinium chloride resulted in around 4.5 log reduction and treatment with 2% SDS only in a 1 log reduction.

The formaldehyde treatment by itself caused a reduced titre of 2 log ID_{50}-units and was further decreased about 2-3 log by autoclaving. Also, denaturation by guanidinium chloride or SDS after formaldehyde-treatment caused a further inactivation, so that the titre was at least 4 log ID_{50}-units reduced compared to the untreated control. These results differ strongly from those published by Brown *et al.* (1990b). Possible reasons for this discrepancy will be discussed in the next section.

Figure 4 shows a dot blot (A) and a Western blot (B) of the samples used for inoculation. All formaldehyde-treated samples are at the limit of detectability. Also the

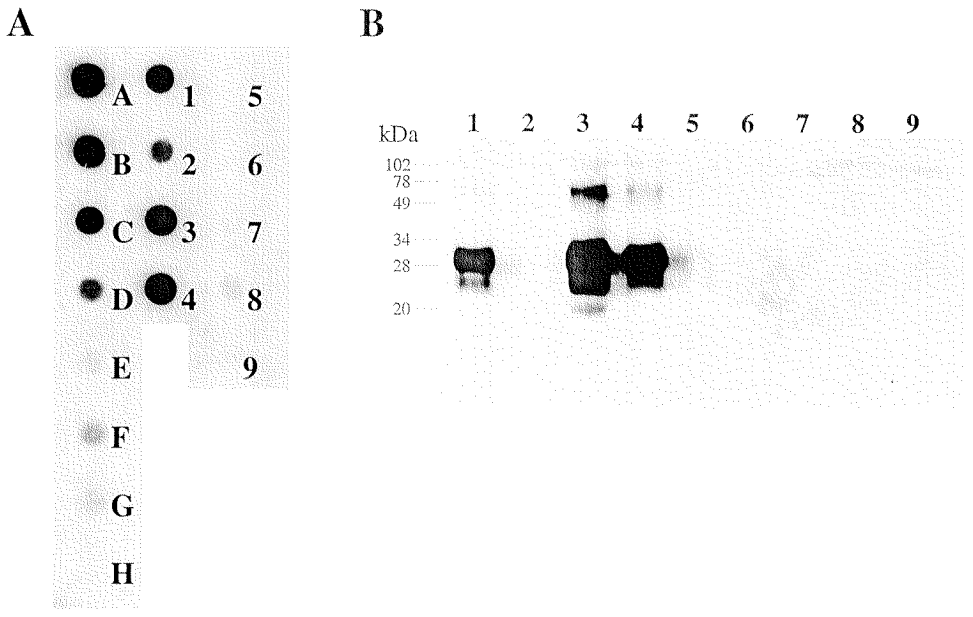

Figure 4: Dot blot analysis of the PrP(27-30)-samples used for the first infectivity study, immunospecific staining was performed with anti-PrP-antibody R340. Sample **1**) control; **2**) autoclaved; **3**) + 6 M GdnHCl; **4**) +2% SDS; **5**) + 4% formaldehyde; **6**) + 4% formaldehyde, autoclaved; 7) + 4% formaldehyde, autoclaved in the presence of formaldehyde; **8**) + 4% formaldehyde, + 6 M Gdn/HCl; **9**) + 4% formaldehyde, + 2% SDS.

A: Dot blot: the samples A-H represent a concentration standard of mPrP(23-231) with A) 1 µg, B) 0.2 µg, C) 40 ng, D) 8 ng, E) 1.6 ng, F) 0.3 ng, G) 0.06 ng and H) 0. Samples 1-9 each represent a 1 µl-aliquot of the injected sample (before the 1:100 dilution).

B: Western blot analysis of injected samples of PrP(27-30), immunostaining with anti-PrP-antibody R340. Numbers 1-9 indicate the same samples as described in A.

autoclaved sample is weaker than the controls in the dot blot and invisible in the Western blot. Possibly, the autoclaving procedure disrupts the protein chemically. In fact, this sample does not show infectivity any more, whereas for the formaldehyde treated samples up to 3.2 log LD_{50}-units were found. In addition, the guanidinium chloride denatured sample can well be detected on the Western blot but shows only vanishing amounts of infectivity. Therefore detectability of PrP(27-30) with anti-PrP antibodies might not correlate with infectivity.

Study on infectivity of solubilized crosslinked PrP(27-30)

The goal of a second infectivity study was to test if infectious PrP(27-30) can be obtained in a soluble form after formaldehyde crosslinking. For this purpose, PrP(27-30) was first dispersed into DLPCs and then reacted with the concentration of formaldehyde (0.01% = 1.3 mM), that gave the best intramolecular crosslinking for recombinant mPrP(23-231) (Figure 3). Additionally, a ten times higher and a ten times lower concentration of formaldehyde was utilised. Subsequently, PrP(27-30) was separated from detergents and lipids and it was solubilised with 6 M guanidinium chloride or 2% SDS. Supernatant and pellet were separated according to the "soluble-fraction-protocol" (Riesner *et al.*, 1996), and diluted aliquots were injected into hamster brains.

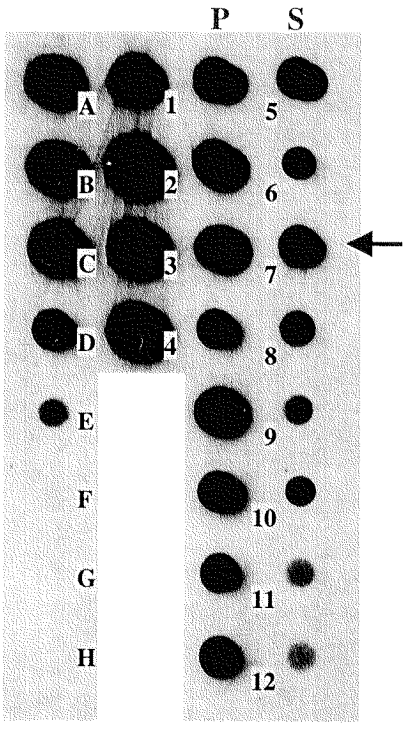

Figure 5: Immuno-dot blot analysis of aliquots of the samples used in the second infectivity study. A-H represent a concentration standard of mPrP(23-231) with A) 1 µg, B) 0.2 µg, C) 40 ng, D) 8 ng, E) 1.6 ng, F) 0.3 ng, G) 0.06 ng and H) 0.
1-4 represents the controls of formaldehyde treated samples after repurification of PrP(27-30) from DLPCs. **5-8** are the samples treated with 2% SDS; **9-12** are treated with 6 M Gdn/HCl (pellet (P) and supernatant (S) fractions are indicated at the top).
Samples **1**, **5**, **9**: control without formaldehyde; **2**, **6**, **10**: 0.1% formaldehyde; **3**, **7**, **11**: 0.01% formaldehyde; **4**, **8**, **12**: 0.001% formaldehyde.
The arrow indicates the only supernatant fraction that contained infectivity.

Figure 5 shows a dot blot of aliquots of the samples used for inoculation. The protein is detectable in all cases as only relatively low concentrations of formaldehyde (0.13-13 mM, corresponding to 0.001-0.1%) were utilised. Repurification of the protein after dispersion into DLPCs and fixation with formaldehyde, was very effective, as can be seen in the controls (Figure 5, 1-4). 0.2-1 µg/µl were obtained in all cases. Solubilization was attempted with 6 M guanidinium chloride and 2% SDS. The samples were then separated into a soluble and an insoluble fraction by ultracentrifugation at 100000 x g for one hour. As judged from the blot for all samples most of the protein remained insoluble, whereby the SDS-solubilization was slightly more effective than treatment with guanidinium chloride. Overall, however, no increase in solubility through formaldehyde treatment prior to guanidinium chloride or SDS incubation could be detected.

The infectivity data are summarised in Table 2. The controls show higher infectivity than those of the first infectivity study. This improves the quantification of the analysis, as infectivity titres can be calculated more exactly when the incubation times are not close to the limit, where no disease occurs.

The data in Table 2 show further that after denaturant treatment the infectivity decreases strongly (at least 3 log ID_{50}-units). For guanidinium chloride treated samples no infectivity is found in the soluble fractions and only very small amounts for the pellets (0.3-1.1 corresponding to a reduction of 6 log ID_{50}-units) when the protein was treated with Formol. The control in the absence of formaldehyde is completely inactivated. The pellets of the SDS-samples retained 2-3 log ID_{50}-units of infectivity. This corresponds

	Control after DLPC repurification	SDS-solubilization		Gdn/HCl-solubilization	
		pellet	supernatant	pellet	Supernatant
1) Control	7.4	3.6	0	0	0
2) 0.1% Formol	7.2	2.3	0	1.1	0
3) 0.01% Formol	7.5	1.9	**4.2**	<0.5	0
4) 0.001% Formol	8.8	~2.5(?)	0	0.3	0

Table 2: Infectivity titres for the study on infectivity of solubilized, crosslinked PrP(27-30) (log ID_{50} – units after inoculation into hamsters)

to a decrease of approximately 4-5 logs compared to the controls. Only the solubilized fraction treated with 0.01% formaldehyde retained a higher amount of infectivity (4.2 log ID_{50}-units) after SDS denaturation. This represents the largest amount of infectivity found for all the denaturant treated samples, and it is the only soluble fraction containing detectable infectivity.

2.4. Discussion

The aim of this project was to study the reaction of PrP^{Sc} with formaldehyde and to find conditions where PrP^{Sc} could be crosslinked mainly intramolecularly by formaldehyde so that soluble PrP^{Sc} might be obtained after dissociation of formaldehyde-treated PrP^{Sc} with a denaturing agent. This experiment would principally show if a small molecular infectious unit can exist which might also be suitable for structural analysis.

The widely unspecific crosslinking agent formaldehyde was used, as it was known since long, that it does not inactivate scrapie infectivity (Gordon, 1946; Pattison, 1965; Stamp *et al.*, 1959).

In a first series of *in vitro* experiments, conditions were established to crosslink recombinant mPrP(23-231) predominantly intramolecularly. Recombinant mPrP(23-231), which has the same amino acid sequence as mouse PrP^{Sc}, appeared to be the best model for setting up crosslinking conditions as it could be produced in sufficient amounts. The additional residues 23-89 in mPrP(23-231) compared to PrP(27-30) are unlikely to have influenced the crosslinking result because the rate of formaldehyde crosslinking is mainly concentration dependent and for different proteins widely similar and model reactions seem adequate, as outlined in the introduction.

Applying these conditions to PrP(27-30) DLPCs, PrP(27-30) samples were obtained that showed a mixture of high-molecular weight aggregates and various sizes of small-molecular crosslinked proteins on SDS-PAGE gels. PrP(27-30) DLPCs are a detergent solubilised infectious form of the scrapie agent, that is also used to prepare PrP(27-30)-liposomes. For these liposomes it was calculated that they contain on average 2-4 molecules of PrP(27-30) per liposome (Gabizon *et al.*, 1987). One would therefore expect to obtain primarily crosslinked dimers, trimers and tetramers. This is, in fact, difficult to judge from a gel with differently glycosylated forms and could result in smearing as observed.

subunits in PrP(27-30) DLPCs. These relatively low amounts did not affect the infectivity of the samples compared to a non-Formol-treated control (compare Table 2), but had proven to be optimum for introduction of only intramolecular crosslinks (Figure 3). After denaturation and solubilization most of the samples had no or only negligible amounts of infectivity. Only the SDS-solubilized sample after 0.01% formaldehyde fixation retained 4.2 log LD_{50}-units of infectivity. This sample was treated with the optimal concentration for intramolecular crosslinks and therefore a PrP(27-30) monomer or small oligomer might have been stabilised in the infectious conformation. However, a contamination can also not be excluded, and a repeat of the experiment would in any case be necessary. Further, the data still means a reduction of infectivity of more than a thousand fold compared to the control. If one assumes that in this case only 1/1000 of the protein retained the "infectious conformation" it is clearly not possible to analyse such a small fraction with the protein analysis techniques available today.

If one compares the first and the second inoculation study, a striking fact is, that the titres of the controls differ by a factor of 100 (2 log ID_{50}-units, compare Table1 and 2), although similar amounts of protein were injected into hamster brains. Interestingly, the infectivity for the PrP(27-30) repurified from DLPCs is higher than for the original preparation. Previously, Gabizon *et al.* (1987) showed that dissociation of PrP(27-30) into DLPCs or liposomes increased the titre. It is not clear if this is an effect of disaggregation and dissociation or an effect of the lipids themselves. In our case PrP(27-30) was again aggregated during the repurification procedure. But perhaps it was less aggregated than in the original preparation. Yet it was not tested if the lipids were separated quantitatively or still could have an influence. Thus we can only state that after DLPC-solubilization the infectivity increased.

For the two infectivity studies, very different concentrations of formaldehyde were utilised (1.3 M compared to 0.13-13 mM). Only the high formaldehyde concentrations decreased the infectivity titre, whereas in both cases no strong increase of stability against inactivation procedures was found and the titre after formaldehyde fixation and denaturation was always lowered.

Formaldehyde is an unspecific reagent that crosslinks not only proteins but all molecules containing "active H-atoms", i.e. also nucleic acids. Therefore, formaldehyde-induced molecular crosslinking that would preserve transcriptional capability is not conceivable, a strong argument against a virus-theorie for scrapie.

Formaldehyde by itself does eventually lead to a reduction but not to inactivation of TSE-agents.

As mentioned in the introduction part, in theory the previously reported reactions of formaldehyde fit to the prion concept. Fixation should not alter infectivity, but stabilise it against other inactivation procedures. However, our experiments give slightly different data. The observed 2 log reduction of infectivity by treatment with 1.3 M formaldehyde could be explained by too much crosslinking, thereby decreasing the number of aggregates and creating less infectious entities. The number of infectious units is not an absolute physical quantity, related to the number of discrete uniform infectious particles, but is instead a variable quantity that can be reversibly altered.

The lack of stabilisation, in particular against autoclaving, is more difficult to explain. One possibility is certainly that the chosen conditions for inactivation were too harsh.

In summary, it must be stated that different prerequisites for the project that had been reported in the literature were not given. Our findings show that the results of (Brown *et al.*, 1990b) could not be reproduced, and crosslinking of PrP(27-30) proved difficult. However, in one case we succeeded in generating infectivity in soluble form after formaldehyde treatment, what might indicate the existence of a small molecular infectious unit.

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3. Cu^{2+}-CONVERSION OF mPrP(23-231)

3.1. Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that are characterized by accumulation of an abnormally folded protease resistant form (PrP^{Sc}) of the cellular prion protein in the brains of diseased animals (Griffith, 1967; Prusiner, 1991). The inability to separate PrP^{Sc} from scrapie infectivity has made it the prime candidate for the putative protein agent of TSEs and the "protein only" hypothesis states that PrP^{Sc} is the main or only component of the infectious prion. Until today this hypothesis is not proven. The decisive experiment would be to produce *in vitro* infectious material out of previously non-infectious PrP^{C}. This experiment has so far only been performed successfully with the yeast prion Sup35 (Sparrer *et al.*, 2000). Many attempts to generate PrP^{Sc} *in vitro* have been made and the so called *in-vitro*-conversion showed that radioactively marked PrP^{C} can be converted into PrP^{res} (protease resistant PrP) if it is incubated in a specific reaction with PrP^{Sc} (Kocisko *et al.*, 1994). However, always an excess of PrP^{Sc} over PrP^{C} was required. Therefore the problem of this experiment is the detection of new infectivity as infectivity assays show variabilities of over one log. Consequently, a two fold increase in infectivity, which would at the most be produced by the *in-vitro*-conversion cannot be detected.

When PrP^{Sc} is completely denatured with high amounts of denaturant (urea or guanidinium chloride), it irretrievably looses Proteinase K (PK) resistance as well as infectivity (Prusiner *et al.*, 1993). But when it is only partially denatured with 2-3 M guanidinium chloride, PK-resistance and infectivity are only lost transiently and can be regained after diluting to about 0.5 M guanidinium chloride (Kocisko *et al.*, 1996). Exactly this is done during the *in-vitro*-conversion, where PrP^{C} is added in the renaturation step and is then converted to PrP^{res} (Bessen *et al.*, 1995; Caughey *et al.*, 1995; Kocisko *et al.*, 1994).

Recently, several observations have suggested a possible role for PrP^{C} in Cu^{2+} metabolism. First, it was found that purified recombinant PrP, as well as synthetic peptides corresponding to the N-terminal region of the prion protein, bind Cu^{2+} ions (Brown *et al.*, 1997; Hornshaw *et al.*, 1995; Stockel *et al.*, 1998; Viles *et al.*, 1999). Binding is specific for Cu^{2+} over other transition metals (Stockel *et al.*, 1998). Additionally, it was shown that Cu^{2+} binding causes conformational changes in the

octapeptide repeats, raising the possibility that the metal may trigger some functional alteration in PrP^C (Hornshaw *et al.*, 1995; Miura *et al.*, 1996). A second major observation is that neuronal membranes from PrP knockout mice contain a 10-15 fold reduced content of Cu^{2+}, but not of several other metals, in comparison to wild type controls (Brown *et al.*, 1997). These results suggest that PrP^C could be a major Cu^{2+}-binding protein in the brain.

Concerning the *in-vitro*-conversion experiment towards PrP^{Sc}, it was shown that after guanidinium chloride denaturation the renaturation to PK-resistant and infectious PrP^{Sc} is enhanced by the addition of Cu^{2+} (McKenzie *et al.*, 1998).

Here it was found, that in a reaction similar to the *in-vitro*-conversion, but in the absence of PrP^{Sc} and in the presence of Cu^{2+} in the reaction buffer, PK-resistant oligomers could be produced from recombinant mPrP(23-231). The reaction was dependent on the concentrations of Cu^{2+}, guanidinium chloride and cetylpyridinium chloride. Cleavage of the oligomers by PK occurred at several sites, one corresponding to the site where PrP^{Sc} is cleaved. However, electron microscopy revealed aggregates of globular shape different from the PrP^{Sc}-fibrils, and no infectivity was produced.

3.2. Materials and methods

Purification of mPrP(23-231)

Full-length recombinant mPrP(23-231) was purified in its oxidized form as described (Liemann & Glockshuber, 1998).

"Conversion" reaction

100 µl of 20 µM mPrP(23-231) was incubated in 3 M guanidinium chloride at 37°C overnight. For the conversion reaction, 3 volumes of 50 mM acetic acid/NaOH, pH 6.0, 130 mM NaCl, containing various amounts of $CuSO_4$ and cetylpyridinium chlorid (CPC) were added and incubated at 37°C for 48 hours. Subsequently, the samples were centrifuged for 10 min at 14000 x g. The pellet was washed with 100 µl ddH_2O and suspended in 20 µl ddH_2O.

Proteinase K-digestion

To 5 µl portions of the conversion reaction 1 µl 60 µg/ml proteinase K in 600 mM Tris/HCl, pH 8.0, 300 mM NaCl (final concentrations: 10 µg/ml proteinase K, 100 mM

Tris/HCl, pH 8.0, 50 mM NaCl) was added and incubated at 37°C for 60 min. The reaction was stopped by addition of 5 µl PMSF (10 mg/ml).

SDS-polyacrylamide-gelelectrophoresis

For SDS-PAGE 12,5% acrylamide gels were prepared. Gel electrophoresis was performed according to standard methods.

Immunoblotting

SDS-PAGE-separated protein bands were blotted onto a nitrocellulose membrane (Millipore, HAW P304P0) using standard methods. The membrane was blocked using 5% nonfat dry milk in TBST (0.05% Tween20, 150 mM NaCl, 10 mM Tris/HCl, pH 8.0) and was incubated with a polyclonal rabbit antibody serum against mouse prion protein (R340, 1:5000 in TBST + 1% milk) for one hour at room temperature. After washing an anti-rabbit-immunoglobulin-horseradish-peroxidase-conjugate was added (1% milk in TBST) and the membrane was incubated for 1 hour. The membrane was then washed and developed with a ECL chemiluminescence detection kit (Amersham).

Protein sequencing

Edman sequencing of PK-resistant PrP-fragments was performed by Dr. Peter Hunziker (Institute of Biochemistry, University of Zürich) and Dr. Rene Brunisholz (Institute of Molecular Biology and Biophysics, ETH Zürich).

Electron microscopy

Electron microscopy was done by Peter Tittmann (Institute of cell biology, ETH Zürich). Samples (5 µl) of the protein suspension (aggregated mPrP(23–231)) were adsorbed to glow discharged carbon–coated Cu^{2+} grids. These were washed twice with deionized water, negatively stained with 2% (wt/vol) uranyl acetate, and air–dried after removal of excess liquid. The specimens were examined in a Philips CM12 transmission electron microscope at 100 kV, and images were recorded with a Gatan 694 slow scan CCD camera.

Inoculations

Samples (20 µl) were diluted to 0.2 ml by addition of PBS buffer containing 5% BSA and were sonicated (Branson sonicator). 30 µl of each sample was injected intracerebrally into each of four Tga20 indicator mice (Fischer *et al.*, 1996) while in deep anesthesia. Animals were monitored for onset of clinical TSE symptoms every two days.

3.3. Results

In this study, it was found that in a specific denaturation-renaturation reaction in the presence of Cu^{2+}-ions mPrP(23-231) forms PK resistant aggregates. The reactions were performed using similar conditions as those described for the *in-vitro*-conversion (Bossers *et al.*, 1997; Caughey *et al.*, 1995; Kocisko *et al.*, 1994; Kocisko *et al.*, 1995; Raymond *et al.*, 1997) but without PrP^{Sc}. Instead, Cu^{2+} was added to the buffer, as a study had shown that this improved reconstitution of guanidinium chloride denatured PrP^{Sc} to PK-resistant, infectious prions (McKenzie *et al.*, 1998). Here, the denaturation was performed in 3 M guanidinium chloride and the renaturation in conversion buffer containing 0.75 M guanidinium chloride, 50 mM sodium acetate pH 6.0, 0.2 mM CPC, 10 mM $CuSO_4$. The denaturant guanidinium chloride, the surface active quarternary

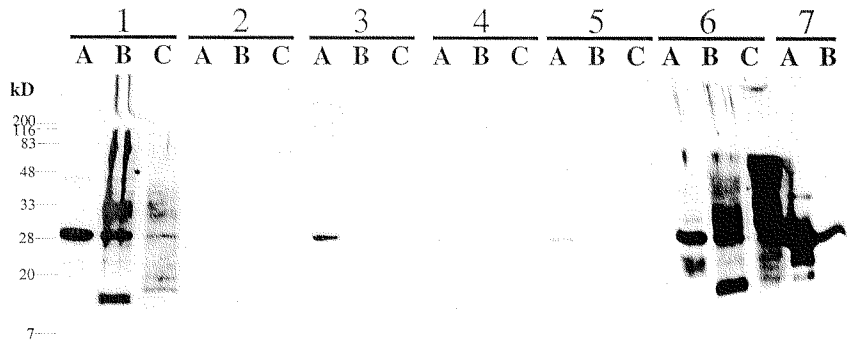

Figure 1:
Western blot analysis of converted mPrP(23-231). A) aggregates, B) aggregates after PK-digest and C) aggregates after denaturation with SDS and PK-digest. 1) Control: denaturation in 3 M Gdn/HCl, renaturation in 50 mM acetic acid/NaOH pH 6.0, 0.75 Gdn/HCl, 130 mM NaCl, 10 mM $CuSO_4$, 2 mM CPC; 2) no Gdn/HCl in all steps; 3) no $CuSO_4$; 4) 6 M Gdn/HCl in all steps; 5) no CPC; 6) no NaCl; 7) mPrP(23-231) without conversion.

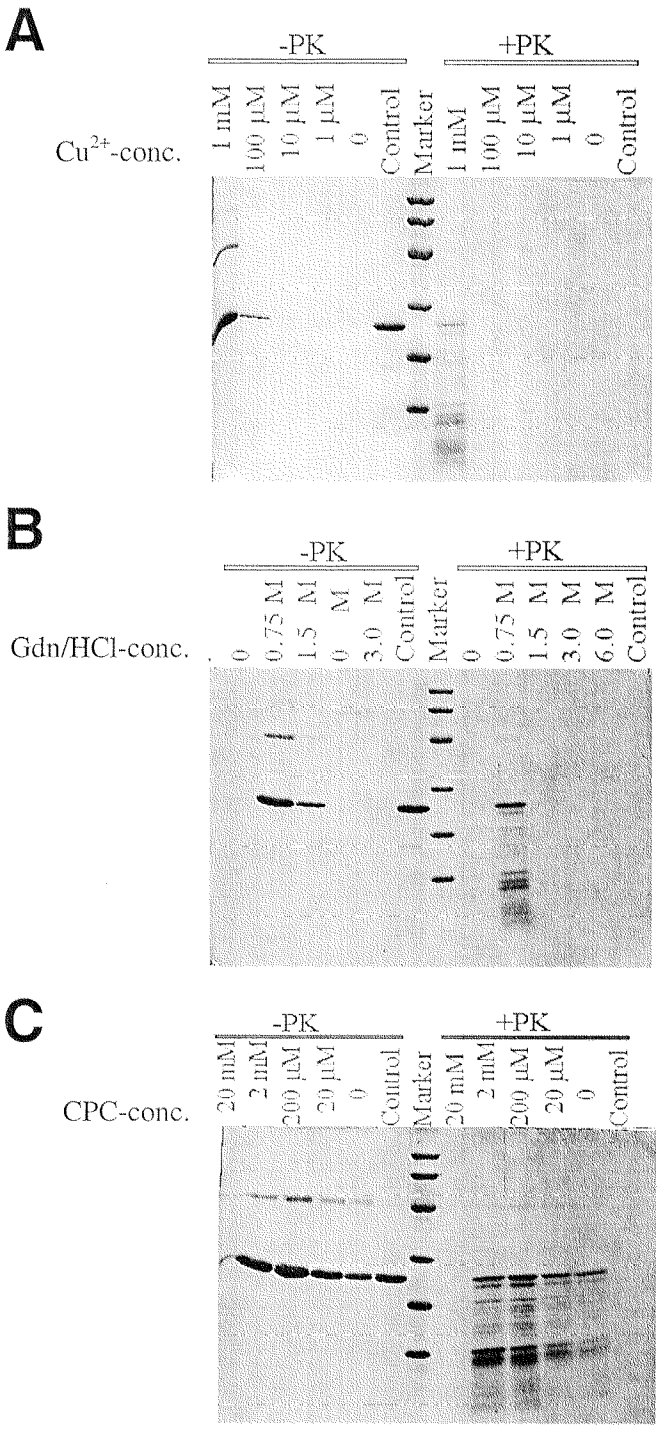

Figure 2:
Dependence of the aggregation reaction of mPrP(23-231) at pH 6.0 on A) Cu^{2+}-, B) Gdn/HCl- and C) CPC-concentrations. Coomassie-stained SDS-PAGE gels are shown of insoluble fractions. The respective concentrations are indicated above each line. –PK means undigested, +PK stands for Proteinase K digested protein. The marker shows the sizes 97, 66, 45, 31, 21 and 14 kDa.

ammonium salt cetylpyridinium chloride (CPC) and bivalent Cu^{2+} ions are necessary for aggregation and PK-resistance, see Figure 1.

Incubation time, Cu^{2+}-, CPC- and guanidinium chloride concentrations were tested to define the optimal conditions for generation of PK-resistant aggregates. In fact, a separate denaturation step before the addition of the conversion buffer is not necessary, but aggregation does neither occur without guanidinium chloride or in the presence of 6 M guanidinium chloride. This means that a low partial denaturation of the protein may be necessary (compare Figure 2B). The reaction proceeds very weakly without CPC. More and more aggregation is found with increasing amounts up to 2 mM CPC, but CPC at a concentration of 20 mM inhibits the reaction completely (see figure 2C). CPC is a detergent-like molecule and supposedly induces the aggregation by exposing hydrophobic parts of the prion protein. For the reported "conversion" reaction Cu^{2+} was necessary at concentrations of 1 mM (McKenzie *et al.*, 1998). Here, at 100 µM Cu^{2+}, first aggregates can be detected, but no PK-resistance (see figure 2A). Other bivalent metal ions (Mn^{2+}, Mg^{2+}, Ca^{2+}) had no effect (data not shown). Upon short-time incubation (< 1 hour), the aggregates dissolve if washed with H_2O. In a second step (about 1-6 hours), the aggregates resist washing and show PK-resistance, but this resistance is lost after treatment with 50 mM EDTA. In a third stage, after 48 hours of incubation, the aggregates even show PK-resistance in the presence of 50 mM EDTA (data not shown).

Upon PK-digestion of the Cu^{2+}-induced mPrP(23-231)-aggregates, several PK-resistant bands are detectable. They correspond to fragments of the prion protein starting at

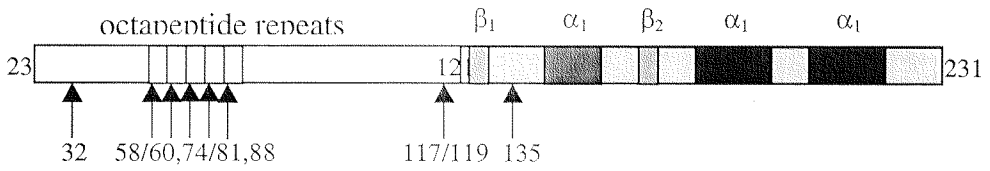

Figure 3:
Scheme of the primary structure of mPrP(23-231) with the detected PK-cleavage sites of the aggregates indicated below by arrows. The protein is cleaved N-terminal to the indicated amino acids. The N-terminal, unfolded tail is shown in white. The C-terminal domain is shown in grey, regular secondary structure elements derived from the NMR-structure are represented by dark grey (β-sheets) and black (α-helices) bars.

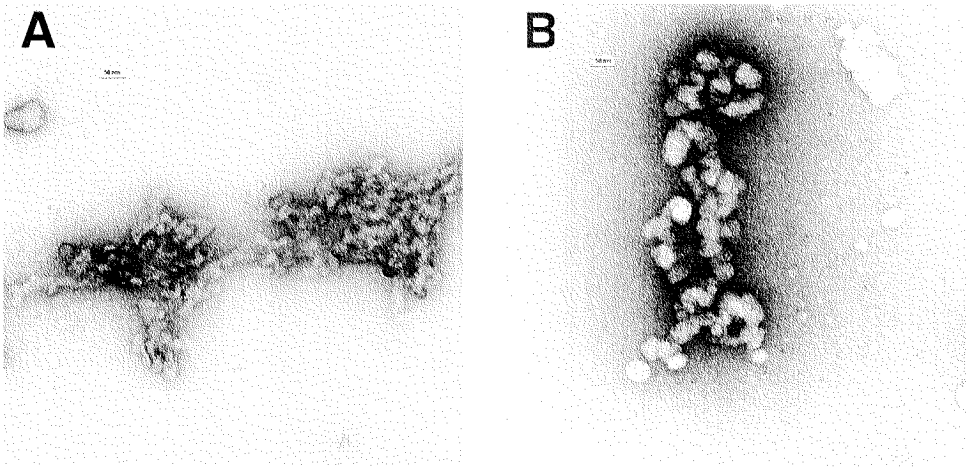

Figure 4:
Negative stain electron microscopy of Cu^{2+}induced aggregates of mPrP(23-231). **A**) undigested, **B**) PK-digested. Bar = 50 nm.

various sites between the N-terminus and the beginning of the C-terminal domain. To examine the digestion sites in detail N-terminal sequencing of the bands was performed and revealed that the protein is cleaved N-terminal to amino acid 32, within the octarepeats N-terminal to amino acid 58/60, 74/81 and 88 (see Figure 3). This last digestion site corresponds to the beginning of PrP(27-30), the protease resistant core of PrP^{Sc} (Prusiner *et al.*, 1984). Further cleavage sites are at the N-terminus of amino acids 117/119 and 135. These numerous digestion sites are in contrast to a PK digest of PrP^{Sc}, where the protein is only cleaved at one site. Further, the partial resistance of the aggregated mPrP(23-231) found here refers to a concentration of 10 µg/ml PK for one hour at 37°C and is significantly lower than the protease resistence of PrP^{Sc} that withstands 50 µg/ml PK for one hour at 37°C.

Electron micrographs of the produced aggregates of mPrP(23-231) in the presence of Cu^{2+} are shown in Figure 4. The undigested protein forms globules of about 10-20 nm in diameter, that adhere further together. After PK-digestion a similar picture is found, but the globules have a larger diameter of 30-50 nm. Comparing these pictures to the fibrillar, amyloid-like structures found for PrP^{Sc} (Merz *et al.*, 1981) it becomes evident that these Cu^{2+}-induced aggregates have no morphological similarity with fibrils of PrP^{Sc} or PrP(27-30).

To analyse if any infectivity has been produced in the Cu^{2+} conversion of mPrP(23-231), aggregates were injected into four Tga20 indicator mice (Fischer *et al.*, 1996). The animals stayed healthy for 200 days incubation time, whereas control animals inoculated with a 1% scrapie-infected brain homogenate died after a mean incubation time of 65 days. These data provide further evidence against the hypothesis that Cu^{2+}-induced aggregates are similar to PrP^{Sc}. Consequently, Cu^{2+}-induced aggregates of mPrP(23-231) are not very likely to be relevant for prion diseases.

3.4. Discussion

Within the framework of the "protein only" hypothesis the conversion of PrP^{C} to PrP^{Sc} is the central molecular event underlying scrapie propagation. Many data support the "protein only" hypothesis, but to prove it, a purified soluble form of the prion protein must be converted to an infectious agent *in vitro* (Kaneko *et al.*, 1995; Kocisko *et al.*, 1994; Raeber *et al.*, 1992). Several attempts to produce PrP^{Sc} *in vitro* have been made with recombinant PrP^{C} produced in bacteria or cell culture as starting substance. Raeber *et al.* (1992) tested various approaches to convert the cellular prion protein into the scrapie isoform in cell free systems without success. First amyloid formation was realised with PrP peptides (Come *et al.*, 1993). Kaneko *et al.* (1995) report aggregation of recombinant PrP by mixing it with peptides. Finally, *in-vitro*-conversion of soluble protease sensitive PrP^{C} to aggregated protease resistant PrP^{res} succeded by mixing PrP^{C} with PrP^{Sc} after partial denaturation (Kocisko *et al.*, 1994). This reaction shows several similarities to PrP^{Sc} formation, and reproduces strain specificity (Bessen *et al.*, 1995) and species specificity (Bossers *et al.*, 1997; Kocisko *et al.*, 1995). However, it so far has not been possible to prove generation of infectivity in this cell free system (Hill *et al.*, 1999).

Here an aggregation reaction of recombinant mPrP(23-231) with Cu^{2+} was established, whereby the resulting complexes show some similarity to PrP^{Sc}. However, the resulting PK-resistance differs from the PrP^{Sc} typical protease resistance, and the structures of the oligomers, as seen by electron microscopy also differ from infectivity preparations. No infectivity was found for the "converted" recombinant mPrP(23-231).

Cu^{2+} binding to PrP has been shown by several groups (Bush, 2000; Kretzschmar, 1999; Prince & Gunson, 1998; Sayre *et al.*, 1999; Waggoner *et al.*, 1999) and Cu^{2+}-ions seem to be essential for some function of the prion protein in oxidative stress that is still not

elucidated in detail (Brown *et al.*, 1997). Only few studies have been performed on a possible role of Cu^{2+} in prion disease (McKenzie *et al.*, 1998; Wadsworth *et al.*, 1999; Waggoner *et al.*, 1999). The formation of PK-resistant aggregates of mPrP(23-231) with Cu^{2+} and CPC, described here, shows a further reaction of prion protein with Cu^{2+} and, for the first time, that Cu^{2+} can play an essential role in aggregating PrP. Still the reaction is not determined by Cu^{2+} alone but needs further components, the denaturing agent guanidinium chloride and the surface active quarternary ammonium salt CPC.

The electron microscopical pictures show globular-shaped aggregates of mPrP(23-231) reminiscent of the spherical particles, found after SDS-denaturation of PrP^{Sc} and sucrose gradient fractionation (Riesner *et al.*, 1996). Similar pictures were also found for aggregates of recombinant PrP obtained after SDS (0.2%) denaturation and dilution to 0.01% SDS (Post *et al.*, 1998). This kind of structure seems not as densely packed as PrP^{Sc} and always proved to be non-infectious. Shaked *et al.* (1999) also showed that PK-resistance is not inevitably linked with infectivity by generation of PK-resistant detergent-insoluble non-infectious PrP aggregates with amorphous structures from infectious PrP(27-30).

McKenzie *et al.* (1998) found partially denatured PrP^{Sc} samples, that were no more infectious and that did not renature by simple dilution, but they could be reconstituted to infectious PK-resistant PrP^{Sc} by addition of Cu^{2+} to the renaturation reaction. Therefore it was a challenge to try if the addition of Cu^{2+} could also induce PrP^{Sc}-formation in denatured recombinant prion protein.

The "conversion" reaction of recombinant mPrP(23-231) described here requires a partial denaturation with about 0.75 M guanidinium chloride. The effect of this low denaturation is not completely clear, as in the folding transition of mPrP(23-231), one finds almost 100% folded molecules under these conditions (Liemann & Glockshuber, 1998). However, CPC may reduce the stability of the protein under the applied conditions.

So far *in vitro* generation of PrP^{Sc} has only been observed for PrP molecules that were denatured from purified PrP^{Sc}. Several studies on this reaction emphasise that only a partial, but not a full denaturation of PrP^{Sc} allows the renaturation to PrP^{Sc} and infectivity (Caughey *et al.*, 1995; McKenzie *et al.*, 1998). Hence, the hypothesis was made that "some native species", like a seed that is retained during partial PrP^{Sc}

denaturation, is necessary. This can be a reason why in our studies no infectivity was obtained, as an initial seed was missing.

In fact, the "conversion" reaction of mPrP(23-231) described here generates partially PK resistant aggregates. After digestion (Figure 2) part of the full length protein in addition to numerous cleavage products is seen. Therefore, the polymeric nature of the aggregates, which strongly reduces the average protease accessible surface area per subunit, could be responsible for the observed PK-resistance (Kocisko *et al.*, 1996).

Overall, while some properties of the produced mPrP(23-231) complexes resemble those of PrP^{Sc}, such as insolubility and protease resistance, these forms are clearly not equivalent. The "protein only" hypothesis in its strict form says that the infectious agent contains only PrP^{Sc}. However, there could be further components like lipids (Klein *et al.*, 1998), a carbohydrate scaffold (Appel *et al.*, 1999) or still undetected minor constituents. Even the requirement of a second protein like the hypothetical protein X (Telling *et al.*, 1995) must be considered. A further possibility is that a component like a molecular chaperone (Saborio *et al.*, 1999) is necessary for prion formation, but not part of the infectious particle, which could render an *in-vitro*-conversion of PrP^{C} to infectious PrP^{Sc} impossible in its absence.

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4. CATALYZED REDUCTION OF THE BURIED DISULFIDE BOND OF THE MURINE PRION PROTEIN AND GENERATION OF AN AMYLOID STATE

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4.1. Abstract

During the conversion of the cellular prion protein PrP^{C} into its amyloid isoform PrP^{Sc}, the supposed causative agent of transmissible spongiform encephalopathies, the protein turns from an α–helical monomer into an ordered oligomer with increased β–sheet content that accumulated in acidic endosomes of scrapie infected cells. Both PrP^{C} and the subunits of PrP^{Sc} contain a single disulfide bridge. Reduction of the disulfide bond in recombinant murine PrP^{C}, mPrP(23–231), induces a β–sheet rich structure and oligomerization into fibrils at acidic pH and moderate ionic strength, suggesting that a transient reduction of PrP may occur during formation of PrP^{Sc} *in vivo*. At physiolgical pH, the native fold strongly protects the disulfide bond in mPrP(23-231) from reduction by dithiothreitol (DTT), while only moderate protection is observed at acidic pH. As PrP^{Sc} accumulates in acidic endosomes of scrapie-infected cells, we further tested whether the reduction of PrP at acidic pH can be catalyzed by enzymes. We show that the bacterial disulfide oxidoreductases DsbA, DsbC and thioredoxin indeed catalyze the reduction and subsequent fibril formation of mPrP(23-231). However, Proteinase K digests of the fibrils with and without prior reoxidation of protein subunits with diamide failed to generate a PrP^{Sc}-typical resistance pattern. Our data indicate that a catalyzed, transient reduction of the disulfide bond in the PrP^{C} may indeed occur in the reducing environment of endosomes, but the relevance of the fibrillar aggregates of reduced PrP for the mechanism of prion propagation still remains to be established.

Keywords

prion protein, disulfide bond, amyloid formation, thiol-disulfide oxidoreductases

4.2. Introduction

Transmissible spongiform encephalopathies (TSE's) are fatal degenerative neurological disorders with long incubation times. They include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, as well as Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI) and kuru in man. The diseases are manifested as infectious, genetic and sporadic disorders (Weissmann, 1991; Weissmann, 1996). The "protein-only" hypothesis states that the infectious agent of TSEs, the prion, is devoid of nucleic acid and composed mainly, if not entirely, of PrP^{Sc}, an abnormal isoform of the host-encoded cellular prion protein PrP^{C} (Gajdusek, 1988; Griffith, 1967; Prusiner, 1991). Prion replication *in vivo* is accompanied by the conversion of PrP^{C} into PrP^{Sc}, where PrP^{Sc} may act as a template for the conformational change (Prusiner *et al.*, 1990). Studies on scrapie–infected cultured cells showed that PrP^{Sc} is derived from PrP^{C} by a posttranslational mechanism after PrP^{C} has reached the cell surface (Caughey & Raymond, 1991), and that PrP^{Sc} eventually accumulates in acidic endosomes.

Mammalian PrP^{C} is a cell surface protein of 209 amino acids (residues 23–231, amino acid numbering according to human PrP) that is attached to the membrane via a glycosyl phosphatidyl inositol (GPI) anchor at Ser231 (Schätzl *et al.*, 1995; Wopfner *et al.*, 1999). In addition, PrP^{C} bears two N-glycosylation sites at Asn181 and Asn196 and a single disulfide bond connecting Cys179 and Cys214 (Hope *et al.*, 1986; Stahl *et al.*, 1993; Stahl & Prusiner, 1991). No differences between the covalent structures of PrP^{C} and PrP^{Sc} have been observed (Hope *et al.*, 1986; Stahl *et al.*, 1993; Stahl & Prusiner, 1991), suggesting that the difference between both PrP isoforms is exclusively based on different protein conformations. Indeed, cirular dichroism (CD) and infrared spectroscopy data showed that PrP^{C} is rich in α-helix (Baldwin *et al.*, 1994; Pan *et al.*, 1993; Pergami *et al.*, 1996) while PrP^{Sc} has high β-sheet content (Caughey & Raymond, 1991; Gasset *et al.*, 1993; Pan *et al.*, 1993; Safar *et al.*, 1993). In addition, PrP^{C} is a monomer, detergent-soluble and sensitive to proteases, while PrP^{Sc} aggregates into detergent–insoluble amyloid. Treatment of the PrP^{Sc} amyloid with proteinase K yields the protease-resistant core PrP27–30 that retains infectivity. The subunits in PrP27–30

are generally uniformly N-terminally truncated and comprise ~ residues 90–231 (Meyer *et al.*, 1986), demonstrating that PrP^{Sc} is an ordered oligomer.

During the last years, high resolution nuclear magnetic resonance (NMR) structures of the murine (Riek *et al.*, 1996; Riek *et al.*, 1997), hamster (James *et al.*, 1997) and human prion protein (Zahn *et al.*, 2000) have been determined. All these proteins were recombinantly produced in *Escherichia coli* and, except for the single disulfide bond, lack all posttranslational modifications of mammalian PrP^{C}. The recombinant proteins from the different species show very similar structures and are composed of two distinct moieties. While the C–terminal residues 125–231 form a globular domain with three α–helices and a two-stranded antiparallel β–sheet, the N-terminal segment 23–124 is flexibly disordered. These results are entirely consistent with the available spectroscopic data on PrP^{C} isolated from mammalian cells, indicating that the tertiary structure of the recombinant prion proteins obtained from *E. coli* is identical to that of PrP^{C}.

Recombinant prion proteins produced in Escherichia coli have been shown to possess high structural plasticity depending upon pH, redox conditions, denaturant concentrations, and presence of detergents (Jackson *et al.*, 1999a; Mehlhorn *et al.*, 1996; Post *et al.*, 1998; Swietnicki *et al.*, 1997; Zhang *et al.*, 1997). Regarding the requirement of the Cys 179-214 disulfide bond for structure and stability of PrP^{C}, the following oberservations have been reported: After reduction of the disulfide bond, both recombinant hamster PrP(90–231) (Mehlhorn *et al.*, 1996; Zhang *et al.*, 1997) and human PrP(90–231) (Jackson *et al.*, 1999b) adopt a β–sheet rich structure. Jackson *et al.* have additionally shown that, at pH 4 and high ionic strength, reduced human PrP(90–231) spontaneously forms amyloid fibrils which are reminiscent of PrP^{Sc} . This raised the possibility that, although both PrP^{C} and the subunits of PrP^{Sc} possess the disulfide bond (Turk *et al.*, 1988), there may be a reduced form of PrP that represents an intermediate state during the conversion from PrP^{C} to PrP^{Sc}. In other words, there is a principal possibility that redox processes might participate in the mechanism of formation of PrP^{Sc}.

The aim of this study was to characterise the stability of the disulfide bond of mPrP(23–231) against reduction, and the influence of the disulfide bond on the structure of amyloidogenic properties of the murine prion protein. We first examined the structure

of reduced and oxidized full length mouse PrP(23–231) as well as the thermodynamic stabilities of both forms. Our data shows that oxidized mPrP(23–231) is α–helical whereas reduced mPrP(23–231) shows predominantly β–sheet–like structure. However, the structures and thermodynamic stabilities of the reduced isoforms alternate strongly with changing pH.

Subsequently, conditions for the reduction of the disulfide bond in folded and unfolded state at pH 4.0–9.0 were investigated. This revealed that the disulfide bond is extremely stable and inaccessible in the folded molecule. At slightly increased ionic strength the reduction of mPrP(23–231) at pH 4.0 led to the formation of amyloid aggregates, and the development of these fibrils was accelerated by oxidoreductases. We also demonstrate reoxidation of the disulfide bond of reduced mPrP(23–231) in both its soluble and aggregated form. However, in spite of the β-sheet-like structure and the fibrillar aggregation the protein, the fibrils only show very limited Proteinase K resistance. Hence, although we demonstrate that catalyzed reductive amyloid formation of mPrP(23-231) can indeed occur, the role of the disulfide bond for PrP^{Sc} maturation and propagation of prion infectivity still remains to be shown.

4.3. Materials and methods

Enzymes

DsbA, DsbC, and thioredoxin were purified as described previously (Maskos, 1995; Jonda *et al.* 1999).

Purification of mPrP(23–231)

Full–length mouse mPrP(23–231) was purified in its oxidized form as described (Liemann & Glockshuber, 1998).

To obtain reduced mPrP(23–231), the purified protein was first denatured and reduced in 10 mM Tris/HCl, pH 8.0, 50 mM DTT, 8 M urea for one hour at 37°C. The protein was refolded by extensive dialysis against 10 mM formic acid/NaOH, pH 4.0, 1 mM EDTA at 4°C, either containing or lacking DTT (1 mM) (see below). All buffers were

degased and flushed with nitrogen to avoid air oxidation. In DTT–free buffer the amount of free thiols was probed with Ellman's reagent (Ellman, 1959).

Protein concentrations

Protein concentrations were determined by the specific absorbance of the proteins: ($A_{280\ nm,\ 1\ mg/ml,\ 1\ cm}$) = 2,7 for mPrP(23–231) (Hornemann *et al.*, 1997), ($A_{280\ nm,\ 1\ mg/ml,\ 1\ cm}$) = 1.1 for DsbA (Wunderlich & Glockshuber, 1993), ($A_{280\ nm,\ 1\ mg/ml,\ 1\ cm}$) = 1.2 for Trx (Mossner *et al.*, 1998) and ($A_{280\ nm,\ 1\ mg/ml,\ 1\ cm}$) = 0.78 for DsbC (Maskos, 1995).

Circular dichroism spectra

Far–UV circular dichroism (CD) spectra were measured between pH 2 and 6 for reduced mPrP(23–231) and between pH 4 and 9 for oxidized mPrP(23–231) at a constant ionic strength of 182 mM at 22°C on a Jasco 710 CD spectropolarimeter in 0.1 cm quartz cuvettes, accumulated for 16 or 32 times and corrected for the buffer. Protein samples with 17–20 µM mPrP(23–231) were centrifuged (15 min, 14000 x g) prior to the measurements in order to remove possible aggregates. The following buffers were used in the range of pH 2–9 (constant ionic strength of 200 mM): 50 mM sodium phosphate pH 2, 76 mM NaCl; 50 mM formic acid/NaOH pH 3, 174 mM NaCl; 50 mM formic acid/NaOH, pH 4.0, 150 mM NaCl; 50 mM acetic acid/NaOH, pH 5.0, 150 mM NaCl; 10 mM MES/NaOH, pH 6.0, 175 mM NaCl; 50 mM MOPS/NaOH, pH 7.4, 150 mM NaCl; 50 mM Tris/HCl, pH 8.0, 150 mM NaCl; 50 mM boric acid/NaOH, pH 9, 162 mM NaCl. All buffers contained 1 mM EDTA, and samples of reduced mPrP(23–231) additionally contained 0.1 mM DTT.

pH–induced transition

To examine the structural changes of reduced mPrP(23–231) over the pH range 1.0–6.0, 20 µM protein was incubated in 50 mM phosphate, pH 1.0–2.6, 50 mM formic acid/NaOH, pH 2.9–4.1, acetic acid/NaOH, pH 4.4–5.3, 10 mM Mes/NaOH, pH 5.6–6.5. 150 mM NaCl, 1 mM EDTA, and 0.1 mM DTT were included in all buffers. Mean residue ellipticities were recorded at 210 nm for 30 seconds on a Jasco 710 CD

spectropolarimeter in 0.1 cm quartz cuvettes. The averaged ellipticities were corrected for the buffers.

Urea–induced equilibrium transitions

For refolding experiments, the protein was first denatured for one hour at room temperature in 10 M urea, 50 mM formic acid/NaOH, pH 4.0, 150 mM NaCl, 1 mM EDTA or 10 M urea, 50 mM acetic acid/NaOH, pH 5.5, 140 mM NaCl, 1 mM EDTA or 50 mM MOPS, pH 7.4, 150 mM NaCl, 1 mM EDTA.. Then the stock solutions were diluted 1:11 with the same buffers containing different urea concentrations, yielding a final protein concentration of 15–20 μM mPrP(23–231). For transitions of reduced mPrP(23–231), 0.1 mM DTT was included The equilibrium transitions were monitored at 22°C by recording the far-UV CD signal at 222 nm for 30 seconds on a Jasco 710 spectropolarimeter in 0.1 cm cuvettes. The averaged ellipticities were corrected for the buffer. The urea concentrations were determined by refractometry. The transitions at pH 7.4 were evaluated according to a two state equilibrium with a six parameter fit as described (Santoro & Bolen, 1988).

Aggregation

Either oxidized or reduced mPrP(23–231) was incubated at a concentration of 20 μM in 50 mM formic acid/NaOH, pH 4.0, 1 mM EDTA plus NaCl concentrations ranging from 0–1 M. The reactions were incubated at 37°C for 1 hour or 24 hours, respectively, and centrifuged for 10 minutes at 14000 g. The amount of protein in the supernatant was analyzed by SDS–PAGE, and the Coomassie–stained gels were scanned and quantified by a Elscript 400–densitometer (Hirschmann), and the percentage of aggregated material was calculated.

To examine aggregation during reduction, 20 μM mPrP(23–231) was incubated at 37°C in 50 mM formic acid/NaOH, pH 4.0, 0.2 M NaCl, 1 mM EDTA and 50 mM DTT with or without 2 μM DsbA. At various time points between 0 and 48 hours, aliquots were taken and centrifuged for 10 minutes at 14000 g. The amount of soluble protein in the supernatant was analysed as above.

Electron microscopy

Samples (5 µl) of the protein suspension (aggregated mPrP(23–231)) were adsorbed to glow discharged carbon–coated copper grids. These were washed twice with deionized water, negatively stained with 2% (wt/vol) uranyl acetate, and air–dried after removal of excess liquid. The specimens were examined in a Philips CM12 transmission electron microscope at 100 kV, and images were recorded with a Gatan 694 slow scan CCD camera.

Reduction of mPrP(23–231) and HPLC– analysis

Reduction of native mPrP(23–231) (20 µM) by 50 mM DTT was performed between pH 4.0 and 9.0 at 37°C and constant ionic strength of 182 mM in the following buffers: 50 mM formic acid/NaOH, pH 4.O, 150 mM NaCl; 50 mM acetic acid/NaOH, pH 5.0, 150 mM NaCl; 10 mM MES/NaOH, pH 6.0, 175 mM NaCl; 50 mM MOPS/NaOH, pH 7.4, 150 mM NaCl; 50 mM Tris/HCl, pH 8.0, 150 mM NaCl; 50 mM Boric acid/NaOH, pH 9, 162 mM NaCl. EDTA (1 mM) was included in all reactions.

For enzymatic reduction of mPrP(23-231) catalytic amounts (1/10 molar amount of protein corresponding to 2 µM) of the oxidoreductases Trx, DsbA or DsbC were included.

To quench the reduction after various time points between 0 and 8 hours 9 volumes of 10% formic acid were added to the samples, resulting in a final pH below 2.

Reduction of denatured mPrP(23–231) was performed in 8 M urea with the same buffers as above with DTT concentrations ranging from 0.022 mM to 150 mM for a constant time (10 minutes or 100 minutes). The reaction was stopped by adding 9 volumes 10% formic acid, 6 M Gdn/HCl.

Reduced and oxidized mPrP(23–231) were separated on an analytical HPLC column (VYDAC, C18, 4.6 x 250 mm) at 55°C. A gradient of 35 % (v/v) acetonitrile to 37 % (v/v) acetonitrile in 0.12 % (v/v) trifluoracetic acid was used, at a flow rate of 0.8 ml/min.

Reoxidation

To determine reoxidation of reduced mPrP(23–231) by oxidized DsbA, both proteins were mixed at a concentration of 20 µM. Reactions were performed at 37°C at pH 4.0 in 50 mM formic acid/NaOH containing 1 mM EDTA. All buffers were degassed and flushed with nitrogen. The mixture was incubated for 18 hours, 9 volumes 10% formic acid were added and the reaction products were analyzed by HPLC as described above for the *Reduction of mPrP(23–231).*

Reoxidation of reduced mPrP(23–231) (20µM) by diamide (1 mM) was performed at 37°C in 50 mM formic acid/NaOH, pH 4.0, 1 mM EDTA. When aggregates of reduced mPrP(23–231) were used 0.2 M NaCl was included. Reaction times varied between 0 and 8 hours and the reactions were quenched by addition of 9 volumes 10% formic acid. Reaction products were analyzed by HPLC as described above.

Modification of free sulfhydryl groups with iodoacetamide

Reduced mPrP(23–231) (20 µM) was incubated in 50 mM Tris/HCl, pH 8.0, 8 M urea, or 50 mM formic acid/NaOH, pH 4.0, 8 M urea, or only 50 mM formic acid/NaOH, pH 4.0. All buffers additionally contained 1 mM EDTA and 1 mM iodoacetamide for 8 hours at room temperature. To quench the reactions, 9 volumes of 10% formic acid were added and the reaction products were separated on an analytical HPLC column (VYDAC, C18, 4.6 x 250 mm) at 55°C. A gradient of 35 % (v/v) acetonitrile to 37 % (v/v) acetonitrile in 0.12 % (v/v) trifluoracetic acid was used at a flow rate 0.8 ml/min. Individual fractions were further analysed by mass spectrometry.

Proteinase K digestion

mPrP(23–231) (20 µM) was digested in 100 mM Tris/HCl, pH 8.0, 1 mM EDTA (and additionally 300 mM NaCl for samples with aggregated protein) containing various concentrations of Proteinase K (0–50 µg/ml) for one hour at 37°C. The reaction was terminated by adding Pefabloc (Boehringer) to a final concentration of 5 mM. Samples were heated in SDS loading buffer to 95°C for 10 min. and protein fragments were separated by SDS–PAGE. Gels were Coomassie–stained.

4.4. Results

Reduced mPrP(23–231) shows β–sheet–like far–UV CD spectra at acidic pH and aggregates at pH values above 6.0.

To follow the contents of secondary structure of oxidized and reduced mPrP(23–231) at various pH, far-UV circular dichroism (CD) spectra were recorded at pH 4.0 and 7.4 for oxidized mPrP(23–231) and at pH values between 1.0 and 6.0 for reduced mPrP(23–231). In the absence of denaturants, the oxidized form adopts its α-helical structure, independent of pH, with minima in the far-UV CD spectra at 208 nm and 222 nm (Fig. 1A).

The spectra of reduced mPrP(23–231) between pH 1 and 6 exhibit a single minimum around 215 nm, strongly indicative of β–sheet–like structure. However, the shapes of the spectra significantly change with pH (Fig. 1B). Therefore we examined the pH-dependence of the CD-signal at 210 nm. The mean residue ellipticities of reduced mPrP(23–231) show the lowest value at pH 4.7 and increase with both lower and higher pH (Fig. 1C). This indicates stongly fluctuating conformational changes over the acidic pH range.

At pH values above 6, reduced mPrP(23–231) aggregates quantitatively and irreversibly. In order to obtain information on the structure of the protein at higher pH, it was possible to measure a spectrum of the protein at medium urea concentrations that maintain solubility. The CD–spectrum of reduced mPrP(23–231) at pH 7.4 in 3 M urea has a more α–helical character, but the intensity of the signal is much lower than that of oxidized mPrP(23–231) (Fig. 1D). Obviously, a major structural change occurs in reduced mPP(23-231) by shifting the pH from 6.0 to 7.4. The same was observed for reduced human PrP(90–231) (Jackson *et al.*, 1999b). However in that case the protein did not aggregate at high pH.

Thermodynamic stabilities of reduced mPrP(23–231) are strongly pH-dependent

To determine thermodynamic stabilities of reduced mPrP(23-231) at different pH values urea induced folding equilibriums of oxidized and reduced mPrP(23–231) were determined at pH 4.0, pH 5.5 and pH 7.4 by monitoring the far UV–CD signal at 222

nm as function of the urea concentration (Fig. 2). For the reduced form at pH 7.4 only refolding from 10 M urea to ≥ 3 M urea was measured as at lower urea concentrations the protein aggregated non-specifically.

At pH 7.4, both redox forms of mPrP(23–231) show cooperative one–step transitions. Analysis according to the two–state model of folding (Santoro & Bolen, 1988) yielded similar stabilities for oxidized and reduced mPrP(23–231) with a free energy of folding of –26.7 and –23.7 $kJ \cdot mol^{-1}$, respectively (Table I). The ΔΔG–value between the oxidized and the reduced form is therefore only 3.0 $kJ \cdot mol^{-1}$.

However, at pH 5.5 where the two isoforms display completely different structures according to the CD-spectra. The reduced protein has only half of the stability (–10.7 $kJ \cdot mol^{-1}$) of the oxidized protein with –20.3 $kJ \cdot mol^{-1}$. The equivalent values of cooperativity around 4.3 $kJ \cdot mol^{-1}M^{-1}$ indicate that a similar sized portion of the protein is folded.

At pH 4.0, both redox forms of mPrP(23–231) show a broad transition that is reminiscent of a three-state equilibrium with a folding intermediate. The proteins unfold at very similar denaturant concentrations. The intermediate seems to be only partially populated and could also be an oligomeric form. The differences between oxidized and reduced protein are small.

In summary, at neutral pH, reduced mPrP(23–231) shows α-helix-like CD spectra that clearly differ from those of the oxidized protein, but shows almost the same thermodynamic stability as the oxidized form. At pH 5.5, the structure of the reduced protein is β-sheet-like and the stability is half of the oxidized form. At pH 4.0 both isoforms show similar three-state transitions.

Reduced mPrP(23–231) forms fibrillar aggregates at acidic pH and ionic strength above 200 mM

The aggregation behaviour of the oxidized and the reduced isoform of mPrP(23–231) was tested by incubation with increasing NaCl-concentrations for 1 or 24 hours at pH 4.0 and following precipitation by centrifugation at 14000 x g (Fig. 3A). Oxidized mPrP(23–231) aggregates only at very high ionic strength. The aggregation process occurs slowly, as can be seen in Figure 3A. 1 hour after addition of NaCl, aggregates

are only seen at concentrations above 0.5 M NaCl, whereas after 24 hours in the sample with 0.4 M NaCl more than 80% of the protein is in the precipitate. Electron microscopy showed, that these aggregates of oxidized mPrP(23–231) consisted of amorphous material (data not shown).

Reduced mPrP(23–231) aggregates readily at pH 4.0 and ionic strength above 200 mM. Already after one hour, the reaction is complete. Ultrastructural studies by electron microscopy revealed that the protein forms amyloid aggregates that were found as dense mashes of fibrils of about 5 nm in diameter (see Fig. 3B).

As mentioned before, at pH above > 6 reduced mPrP(23–231) instantly aggregates, even at low ionic strength. The aggregates show undefined amorphous structure (data not shown). Oxidized mPrP(23–231) stays soluble under these conditions.

Reduction of the disulfide bridge of native mPrP(23–231) is extremely slow

To investigate reduction of mPrP(23–231) at pH 4.0–9.0 the protein was mixed with 50 mM DTT, the reaction was stopped after different times by adding formic acid (final pH < 2) and the acid–quenched samples were separated by HPLC. The rate constants for the reactions were determined (see Table II). At all pH values tested (pH 4.0–9.0) the reduction is extremely slow, consistent with complete unfolding of mPrP(23–231) prior to reduction of the disulfide bond. Noteworthy, the reduction at pH 4.0 was with a second order rate constant of $7.4 \cdot 10^{-5}$ $s^{-1}M^{-1}$ about 2–3 times faster than at pH 5.0–8.0 (see Fig 4A, table II). Only at pH 9 the rate constant increased over this to $9.4 \cdot 10^{-5}$ $s^{-1}M^{-1}$. The finding was surprising because disulfide exchange is expected to be ten times slower per decreasing pH unit (Shaked *et al.*, 1980). This suggests that an increased rate of unfolding of mPrP(23–231) strongly facilitates reduction of the disulfide bond at acidic pH.

We also investigated the reduction of mPrP(23–231) in the denatured state for the same pH–range as above. In 8 M urea, the reaction increased with pH below the pK_a of DTT (Fig 4B). The logarithmic plot of pH against the second order rate constants shows the linear increase of the reaction rate with increasing pH, giving a slope of 0.77. The discrepancy from the expected value 1.0 can be a result of charged residues in the vicinity of the SS–bond, as there are Asp178, Asn181, Thr216 and Gln212.

Additionally local residual structure is retained around the disulfide bond in the unfolded state (Hosszu *et al.*, 1999).

Thiol-disulfide oxidoreductases catalyze the reduction of mPrP(23–231)

We next examined the kinetics of the reaction catalyzed by the oxidoreductases DsbA, DsbC and thioredoxin (Fig 5). The enzymes were used in catalytic amounts, i.e., 0.1 molar equivalents relative to mPrP(23–231) at the thiol disulfide level. At pH 4.0 and 7.4, the reaction rates of the reduction of mPrP(23–231) by DTT increase up to 40–fold by catalysis (Table II). At pH 4.0, both thioredoxin and DsbA give only a moderate rate increase of 2.9-fold, while DsbC denatures at acidic pH. However, at pH 7.4 the acceleration by DsbA is 6.1-fold, that of Trx 23.9-fold and that of DsbC 41.7-fold.

Catalyzed formation of aggregates

After having shown that the reduced protein aggregates to amyloid and that mPrP(23-231) can be reduced under appropriate conditions we investigated amyloid formation during the reduction of the protein..

Reduction with 50 mM DTT was performed as before but in the presence of $\geq$ 200 mM ionic strength to favour aggregation. Samples were taken after different reaction times and the fraction of aggregated mPrP(23–231) was separated by centrifugation and quantified. Under all conditions investigated, we observed immediate aggregation upon reduction (Fig. 6). Addition of catalytic amounts of DsbA to the reaction at pH 4.0 gives a significant increase of the reaction rate and acceleration of the formation of aggregates. The aggregates consisted of fibrillar structures similar to those produced from fully reduced mPrP(23-231) (Fig. 3B).

Reoxidation of reduced mPrP(23–231)

Having established conditions for reduction and amyloid formation of mPrP(23-231) at acidic pH, we wanted to test if the disulfide bond could be reformed by oxidation. In a first experiment equal molar amounts of reduced mPrP(23–231) and oxidized DsbA were incubated at pH 4.0 and the reaction was analysed after 18 hours. No oxidation of mPrP(23–231) had occurred (Fig. 7A) although DsbA is the most efficient dithiol oxidant known so far. However it was possible to modify the SH–groups of reduced

mPrP(23–231) by carboxamidomethylation with iodoacetamide in the control reactions in 8M urea at pH 8.0 and pH 4.0 and in the native state at pH 4.0 (Fig. 7B). Those results suggest that in the structure of reduced mPrP(23–231) the SH–groups are only accessible to a small molecule like iodoacetamide, but not for DsbA. The reason could be that they are buried inside of the molecule or cannot be accessed because of oligomerization of the molecules.

Reoxidation of reduced mPrP(23–231) was successful with diamide, a low molecular weight thiol specific oxidant (Kosower & Kosower, 1995) (Fig. 8). At pH 4.0 the kinetic measurement gave a second order rate constant of 0.16 $M^{-1}s^{-1}$ for the non–aggregated protein and about the same, 0.15 $M^{-1}s^{-1}$, for the salt–aggregated protein. The disulfide bridge was exclusively formed intramolecularly and the reaction reached yields of up to 90%.

The aggregated protein was not solubilized through formation of the disulfide bond, demonstrating that the original conformation of the oxidized mPrP(23–231) could not be re-established.

Also at pH 7.4 where the reduced protein aggregates instantly reoxidation with diamide occurred.

Proteinase K resistance

Oxidized, reduced and reoxidized mPrP(23–231) were digested with increasing amounts of Proteinase K (Fig.9). Native oxidized mPrP(23–231) is very sensitive to digestion, whereas the reduced and reoxidized form show a slight resistance to low concentrations (up to 0.5mg/ml) of the protease. This resistance is identical for the soluble and the aggregated protein. The pattern of the proteolytic fragments is in all cases the same. However, the resistance found here is at least a factor of 100 lower than the Proteinase K resistance of PrP^{Sc}, and the generation of a PrP^{Sc}-specific fragment comprising residues 90-231 was not observed.

4.5. Discussion

The prion concept relies on the "protein only" hypothesis which states that infectious prions are composed largely, if not entirely, of PrP^{Sc} molecules (Prusiner, 1991). The underlying event in the propagation of prions and the pathogenesis of prion diseases seems to be a profound conformational change in PrP^{C} as it is converted into PrP^{Sc} (Gasset *et al.*, 1993; Pan *et al.*, 1993; Safar *et al.*, 1993).

Several spectroscopic measurements have shown that the conversion of PrP^{C} into PrP^{Sc} is accompanied by a reduction in the α–helical content of PrP and an increase in β–sheet structure (Pan *et al.*, 1993; Prusiner *et al.*, 1983). Consequently PrP can exist in at least two reasonably stable conformations. Therefore it is evident that diverse studies on structural, biophysical and biochemical properties of prion proteins are undertaken. Recombinant PrP is generally used as model system for these studies due to the necessity to obtain sufficient amounts of protein can be achieved by overexpression of the protein in bacterial systems (Hornemann & Glockshuber, 1996; Hornemann *et al.*, 1997; Swietnicki *et al.*, 1997; Zahn *et al.*, 1997; Zhang *et al.*, 1997). Recombinant PrP produced in *E. coli* lacks the N–linked glycosylation and the GPI membrane anchor, but these posttranslational modifications seem not necessary for PrP^{Sc} formation (Rogers *et al.*, 1993; Taraboulos *et al.*, 1990).

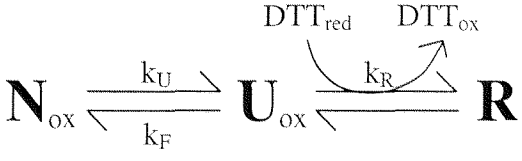
Here, we worked with full length mouse PrP(23–231). The structure of this protein in oxidised form has been determined by NMR (Riek *et al.*, 1997). The C–terminal segment 125-231 forms a stable domain which is mainly α–helical, whereas the N-terminal segment 23-124 is flexibly disordered. Far-UV CD–spectra of oxidised mPrP(23-231) confirm the α–helical character of its C-terminal domain, whereas the reduced form shows β–sheet–like spectra. This finding is remarkable as, in principle, the structure of a protein should unequivocally be encoded by the amino acid sequence, and disulfide bridges are only assumed to be stabilising elements.

The thermodynamic stability of the reduced mPrP(23–231) depends strongly upon pH. At pH 7.4 it is almost as high as that of oxidised mPrP(23–231). At pH 5.5, the reduced protein is much less stable. This seems to be an effect of the pH-dependent structural changes occurring in reduced mPrP(23-231). In case of the oxidised protein the structure of the monomer is certainly stabilised by the disulfide bond that connects the

C-terminal α-helices 2 and 3. Whereas the reduced protein could acquire part of its stability from oligomerization: By light scattering experiments we found that reduced mPrP(23–231) in its soluble form at acidic pH is oligomeric (data not shown). However, Jackson *et al.* reported for reduced human PrP(90–231) a monomeric β–sheet form (Jackson *et al.*, 1999b). For hamster PrP(90–231), the reduced protein was again reported to form oligomers of 6–7 PrP molecules (Zhang *et al.*, 1997). At the moment, these differences cannot be clearly explained and must be attributed to the different species origin of the proteins and the different length of the molecules.

Remarkably, while oxidised mPrP(23–231) is stable and soluble over a wide pH–range, reduced mPrP(23–231) starts to precipitate at pH–values > 6. At acidic pH the reduced form aggregates at much lower ionic strength than oxidised mPrP(23–231).

The disulfide bridge of mPrP(23-231) proved to be extremely stable against thiol–disulfide interchange reactions with the strong reductant DTT. The stability of the disulfide bond is best explained by the following model which assumes a two-state folding mechanism of mPrP(23-231) and a requirement for total unfolding prior to attack of the disulfide bond by DTT.



N_{ox} represents the native oxidised folded mPrP(23–231), U_{ox} the unfolded oxidised mPrP(23-231)and R the reduced protein. k_U, k_F and k_R represent the rate constants of unfolding, folding and reduction, respectively. The oxidised protein was indeed shown to be in a very fast equilibrium between N and U (Wildegger *et al.*, 1999). Surprisingly, the apparent reduction rate increases at low pH and the protein is more easily reduced, although less of the attacking thiol is present in the ionized form. This is clearly due to the significantly lower stability of oxidised mPrP(23-231) at acidic pH. Catalysis of mPrP(23-231) reduction may principally have two different origins. On the one hand,

the enzymes certainly all accelerate disulfide reduction itself. On the other hand, however, they may also selectively bind to the unfolded state and thereby increase its lifetime for subsequent attack of the disulfide bond. Such a peptide binding activity has particularly been suggested for DsbC, which indeed proved to be the most efficient reduction catalyst (Darby *et al.*, 1998).

The observation that PrP^C can be reduced by the aid of catalysts, oxidoreductases as they exist also in eukaryotes, leads to some speculation. Under physiological conditions PrP^C is endocytosed (Borchelt *et al.*, 1992; Caughey & Raymond, 1991) and in the reducing environment of the endosomes it could indeed be reduced, change its structure, and become susceptible to fibrillar aggregation. In fact, protein disulfide isomerase, a eukaryotic homologue of DsbA and DsbC was found in endosomes (Noiva, 1999). Other, endosome-specific disulfide oxidoreductases are also very plausible candidates for PrP reduction catalysts. The first representative of endosome-specific oxidoreductases has recently been described. It is the c-interferon-inducible thiol reductase (GILT) which indeed has its pH-optimum at acidic pH and appears to be involved in reductive antigen processing (Arunachalam *et al.*, 2000).

The disulfide bridge is very stable against reduction with $t_{1/2}$ of up to one week, even against DTT–concentrations of 50 mM. Therefore it can also be speculated that the PrP molecule could, after internalization reach for the cytoplasm and stay oxidised in this reducing environment. In view of the still unknown function of PrP^C, the protein with its unstructured N–terminal part could then exert a signalling or binding function.

We have performed the reduction of mPrP(23–231) *in vitro* and analysed the kinetics of the reaction directly by HPLC, and also examined the simultaneous aggregation. The uncatalyzed reduction at pH 4.0 has a $t_{1/2}$ of 52 hours (Table II). After this time about 30% of the protein is aggregated (Fig. 6). In case of the DsbA–catalyzed reaction the $t_{1/2}$ is 17.5 hours, whereby at this time point about 35% of mPrP(23–231) is aggregated. As we chose an ionic strength where exclusively the reduced protein aggregates, about 60–70% of the reduced mPrP(23–231) should be aggregated and this is what we found. Therefore, the reduction was the rate limiting step under our conditions, and aggregation proceeded immediately. Thus, fast aggregation of reduced PrP will presumably also occur at lower protein concentrations than 20 μM both *in vivo* and *in vitro*.

Data on the reduction of the disulfide bond in PrP^{Sc} preparations are ambiguous. In one study, neither DTT nor 2–mercaptoethanol at high concentrations altered the infectivity of prion rods (Prusiner *et al.*, 1980). On the other hand, report showed, that in the presence of SDS exposure of PrP^{Sc} to 2–mercaptoethanol reduced infectivity (Somerville *et al.*, 1980). This raises the question about accessibility of the disulfide bond in the aggregated PrP^{Sc}–molecules. The in vitro reaction of protease sensitive PrP to PK–resistant PrP–res in the presence of catalytic amounts of PrP^{Sc} was inhibited by the disulfide reducing agent DTT (Herrmann & Caughey, 1998). In none of these studies reoxidation of the disulfide bridge was included, which might be an essential step as PrP^{Sc} is oxidized (Turk *et al.*, 1988).

We showed that the protein subunits in aggregated fibrils of reduced PrP can indeed be reoxidised by small oxidants such as diamide and possibly also by molecular oxygen, while the cysteins are obviously not accessible for larger oxidants like DsbA. Whether the SS-bond in PrP^{Sc} is indeed required for infectivity remains to be shown. One possible scenario could be that reoxidation of PrP fibrils formed in endosomes occurs after cell lysis and liberation of the fibrils into the oxidising environment of the extracellular space.

Overall, we have studied the shift of recombinant prion protein into an oligomeric β-sheet form to analyse one of the possible mechanisms of PrP^{C} to PrP^{Sc} conversion. We showed that reduced mPrP(23-231) forms amyloid fibrils at slightly elevated ionic strength and acidic pH. In these aggregates the disulfide bond can be reoxidized while the protein remains in aggregated form. However, both kinds of aggregates do not show the typical Proteinase K–resistance that is a hallmark of PrP^{Sc}. Therefore the process of PrP^{Sc}-formation cannot solely consist of oxidative processes of the disulfide bond but must involve further steps that remain to be established.

4.6. References

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4.7. Tables

Table I. Two-state analysis of the equilibrium unfolding transitions of oxidized and reduced mPrP(23–231) at 22°C and a constant ionic strength of 180 mM.

		ΔG [a] ($kJ \cdot mol^{-1}$)	m [b] ($kJ \cdot mol^{-1} \cdot M^{-1}$)	$urea_{1/2}$ [c] (M)
pH 7.4	ox. PrP(23–231)	-26.7 ± 0.9	4.4 ± 0.1	6.0
	red. PrP(23–231)	-23.7 ± 2.1	4.6 ± 0.4	5.1
pH 5.5	ox. PrP(23–231)	-20.3 ± 1.1	4.2 ± 0.2	4.8
	red. PrP(23–231)	-10.7 ± 1.6	4.3 ± 0.4	2.5

[a] free energy of folding in the absence of denaturant
[b] cooperativity of the transition
[c] transition midpoint

Table II. Rate constants of the reduction of mPrP(23-231) by DTT at 37°C at a constant ionic strength of 180 mM

Conditions	k_1 (s^{-1}) [3]	$t_{1/2}$ (h)	k_2 (s^{-1} M^{-1}) [4]	Acceleration factor [5]
50 mM DTT, pH 4.0 [1]	$3.7 \cdot 10^{-6}$	52.0	$7.4 \cdot 10^{-5}$	
50 mM DTT, pH 5.0 [1]	$1.1 \cdot 10^{-6}$	175.0	$2.2 \cdot 10^{-5}$	
50 mM DTT, pH 6.0 [1]	$1.5 \cdot 10^{-6}$	128.4	$3.0 \cdot 10^{-5}$	
50 mM DTT, pH 7.4 [1]	$1.8 \cdot 10^{-6}$	107.0	$3.6 \cdot 10^{-5}$	
50 mM DTT, pH 8.0 [1]	$2.2 \cdot 10^{-6}$	87.5	$4.4 \cdot 10^{-5}$	
50 mM DTT, pH 9.0 [1]	$4.7 \cdot 10^{-6}$	41.0	$9.4 \cdot 10^{-5}$	
50 mM DTT, Trx, pH 4.0 [1]	$1.1 \cdot 10^{-5}$	17.5	$2.2 \cdot 10^{-4}$	2.9
50 mM DTT, Trx, pH 7.4 [1]	$4.3 \cdot 10^{-5}$	4.5	$8.6 \cdot 10^{-4}$	23.9
50 mM DTT, DsbA, pH 4.0 [1]	$1.1 \cdot 10^{-5}$	17.5	$2.2 \cdot 10^{-4}$	2.9
50 mM DTT, DsbA, pH 7.4 [1]	$1.1 \cdot 10^{-5}$	17.5	$2.2 \cdot 10^{-4}$	6.1
50 mM DTT, DsbC pH 7.4 [1]	$7.5 \cdot 10^{-5}$	2.6	$1.5 \cdot 10^{-3}$	41.7
8 M urea, 5.5–150 mM DTT, pH 4.0 [2]			$2.9 \cdot 10^{-3}$	39
8 M urea, 1.8 – 50 mM DTT, pH 5.0 [2]			$1.7 \cdot 10^{-2}$	770
8 M urea, 0.6 – 50 mM DTT, pH 6.0 [2]			$1.5 \cdot 10^{-1}$	5000
8 M urea, 0.2 – 50 mM DTT, pH 7.4 [2]			$8.6 \cdot 10^{-1}$	24000
8 M urea, 0.2 – 16 mM DTT, pH 8.0 [2]			3.5	80000
8 M urea, 0.02–0.6 mM DTT, pH 9.0 [2]			20.3	220000

[1] Reactions were performed in the appropriate buffers (see Methods). Where indicated 1/10 molar equivalent of oxidoreductase (Trx, DsbA, DsbC) was included.
[2] Reactions were performed for a constant time of 10 min or 100 min
[3] k_1: pseudo first order rate constant
[4] k_2: second order rate constant
[5] Acceleration relative to the second order rate constant of the uncatalyzed reduction by DTT in the absence of denaturant

4.8. Legends to Figures

Figure 1

Far–UV circular dichroism spectra of **A**) oxidized and **B**) reduced mPrP(23–231) at various pH values measured at 22°C and 182 mM ionic strength. **C**) pH–dependence of the CD signal at 210 nm of reduced mPrP(23–231) at 22°C. **D**) Far–UV circular dichroism spectra of oxidized and reduced mPrP(23–231) at pH 7.4 in 3 M urea compared to the spectrum of reduced mPrP(23-231) at pH 6.0, 0 M urea.

Figure 2

Urea–induced refolding transitions of oxidized and reduced mPrP(23–231) at **A**) pH 7.4 **B**) pH 5.5 and **C**) pH 4.0. The mean residue ellipticities at 222 nm were measured at 22°C, protein concentrations of 17-20 μM and constant ionic strength of 182 mM. The solid lines result from fitting the data according to a two state model (**A** and **B**) or according to a three state model (**C**).

Figure 3

mPrP(23–231) aggregates at pH 4.0 and high ionic strength.

A) Aggregation was induced by increasing NaCl concentrations, aggregated material was separated from the soluble fraction by centrifugation (10 min, 14000 x g) and quantified by densitometric analysis of Coomassie-stained SDS-PAGE gels.

B) Negative stain electron microscopy of fibrillar aggregates of reduced mPrP(23–231) formed at pH 4.0 and 0.3 M NaCl.

Figure 4

Reduction of mPrP(23–231)

A) HPLC analysis of the reduction of mPrP(23–231) with 50 mM DTT at pH 4.0–9.0 for 8 hours at 37°C. **B**) pH-dependence of the rate constants of reduction of native (□) and urea-unfolded (○) mPrP(23-231) by DTT at 37°C and 180 mM ionic strength and **C**) protection factors for the reduction of the disulphide bond in native compared to unfolded mPrP(23-231)

Figure 5

HPLC analysis of the uncatalysed and catalysed reduction of mPrP(23–231) (20 μM) with 50 mM DTT at 37°C and **A**) pH 4.0 or **B**) pH 7.4. The disulphide oxidoreductases thioredoxin (Trx), DsbA and DsbC were added at a concentration of 2 μM, corresponding to 0.1 molar equivalents at the thiol/disulphide level.

Figure 6

DsbA-catalysed reduction and fibril formation of mPrP(23-231)

mPrP(23–231) aggregates at pH 4.0 and 0.2 M NaCl (232 mM ionic strength) during reduction at 37°C. Soluble and aggregated material were separated by centrifugation (10 min, 14000g) after various reaction times. Reduction was performed by 50 mM DTT with or without catalytic amounts (0.1 molar equivalents) of DsbA.

Figure 7

Accessibility of the free SH–groups of reduced mPrP(23–231) at pH 4.0.

A) No reoxidation was achieved by equimolar amounts of oxidised DsbA. Shown is the HPLC analysis of 1) oxidised mPrP(23–231) (control 1), 2) reduced mPrP(23–231) (control 2) and 3) the reaction mixture of reduced mPrP(23–231) with oxidised DsbA (DsbA is eluted at the end of the gradient on the HPLC and is not shown.).

B) The free SH–groups of mPrP(23–231) can be carboxamidomethylated by 1 mM iodoacetamide (IAM). Shown is the HPLC analysis of 1) reduced mPrP(23–231), 2) IAM–modification at pH 8, 8 M urea, 3) IAM–modification at pH 4, 8 M urea and 4) IAM–modification at pH 4; A_2 corresponds to the doubly alkylated form of mPrP(23-231).

Figure 8

HPLC-analysis of the reoxidation of reduced mPrP(23–231) by 1 mM diamide at pH 4.0 and 37°C in its **A**) soluble and **B**) aggregated (+ 200 mM NaCl) state.

Figure 9

Coomassie stained gels of Proteinase K digests of **A**) oxidised, **B**) reduced and **C**) reoxidised mPrP(23-231). The concentrations of PK are indicated above the lanes. Both the soluble and the aggregated, fibrillar form of reduced mPrP(23-231) at pH 4.0 were used for reoxidation with diamide and subsequent Proteinase K resistance assays.

4.9. Figures

Figure 1

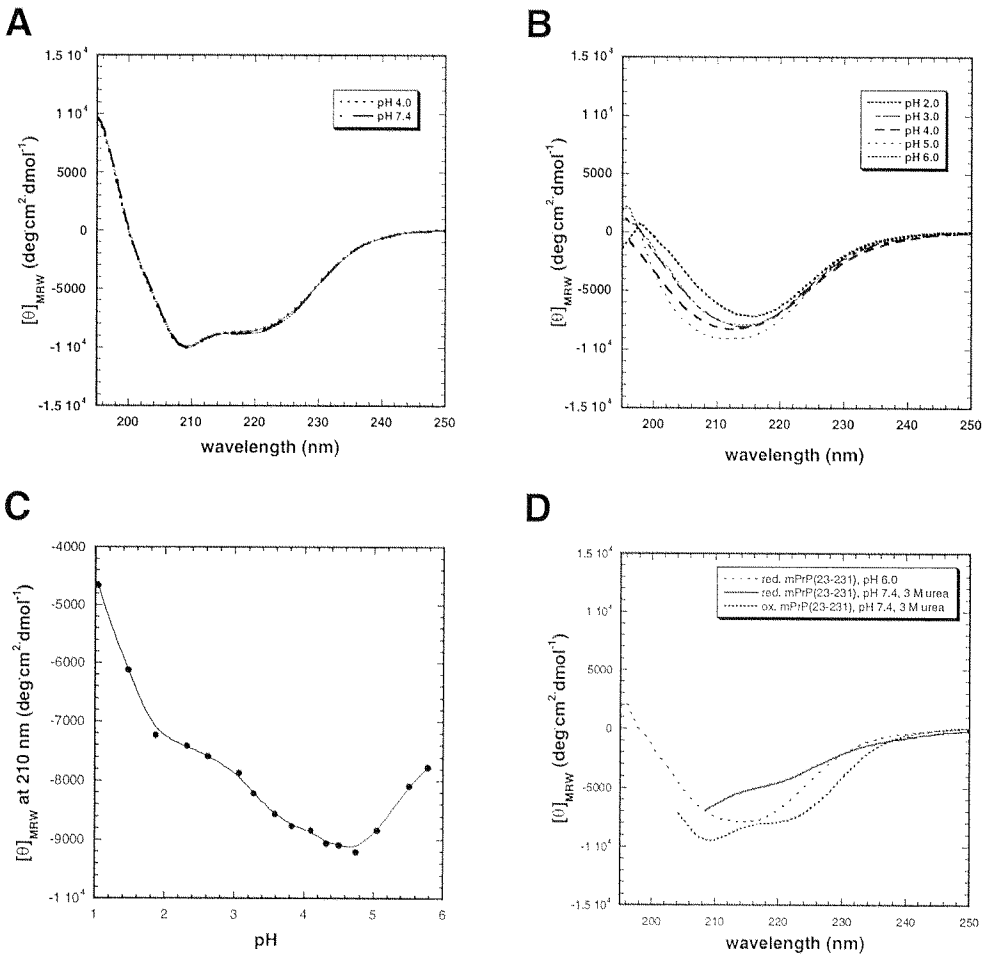


Figure 2

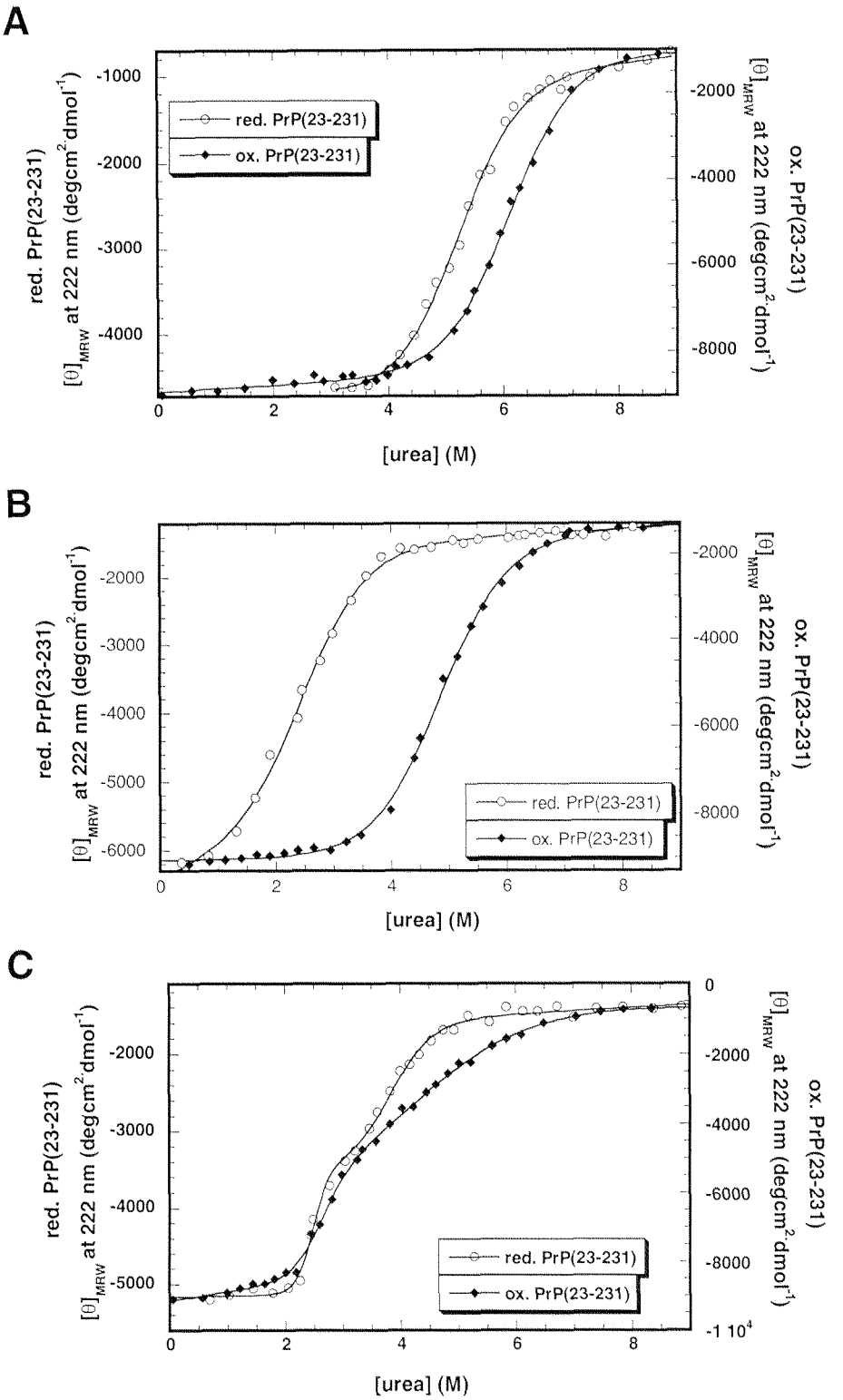
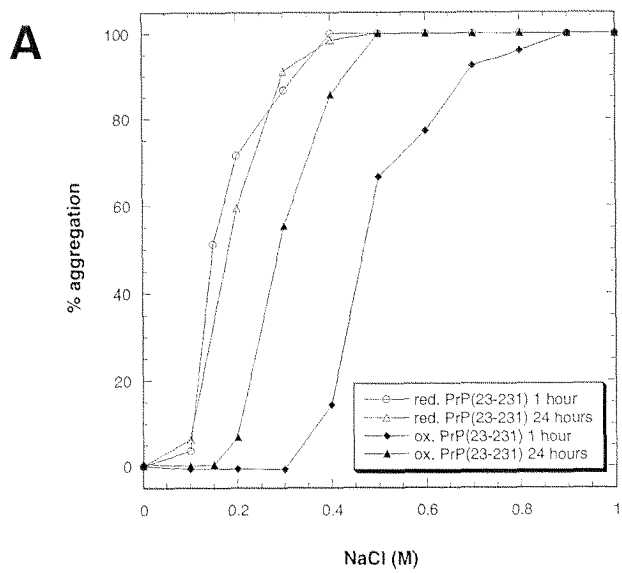

A
red. PrP(23-231)
ox. PrP(23-231)
red. PrP(23-231) [θ]MRW at 222 nm (degcm2dmol-1)
ox. PrP(23-231) [θ]MRW at 222 nm (degcm2dmol-1)
[urea] (M)
B
red. PrP(23-231)
ox. PrP(23-231)
red. PrP(23-231) [θ]MRW at 222 nm (degcm2dmol-1)
ox. PrP(23-231) [θ]MRW at 222 nm (degcm2dmol-1)
[urea] (M)
C
red. PrP(23-231)
ox. PrP(23-231)
red. PrP(23-231) [θ]MRW at 222 nm (degcm2dmol-1)
ox. PrP(23-231) [θ]MRW at 222 nm (degcm2dmol-1)
[urea] (M)

Figure 3


A
100
80
60
40
20
0
% aggregation
0
0.2
0.4
0.6
0.8
1
NaCl (M)
red. PrP(23-231) 1 hour
red. PrP(23-231) 24 hours
ox. PrP(23-231) 1 hour
ox. PrP(23-231) 24 hours

B

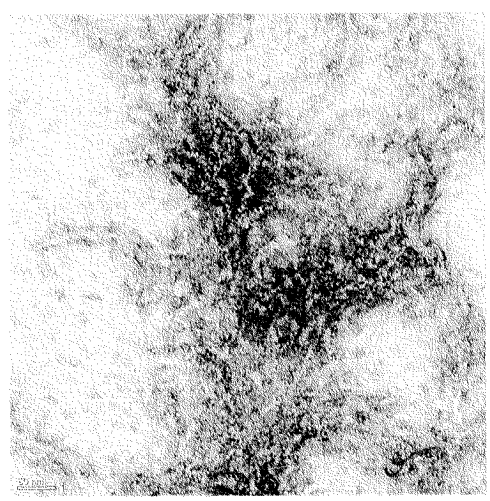


Figure 4

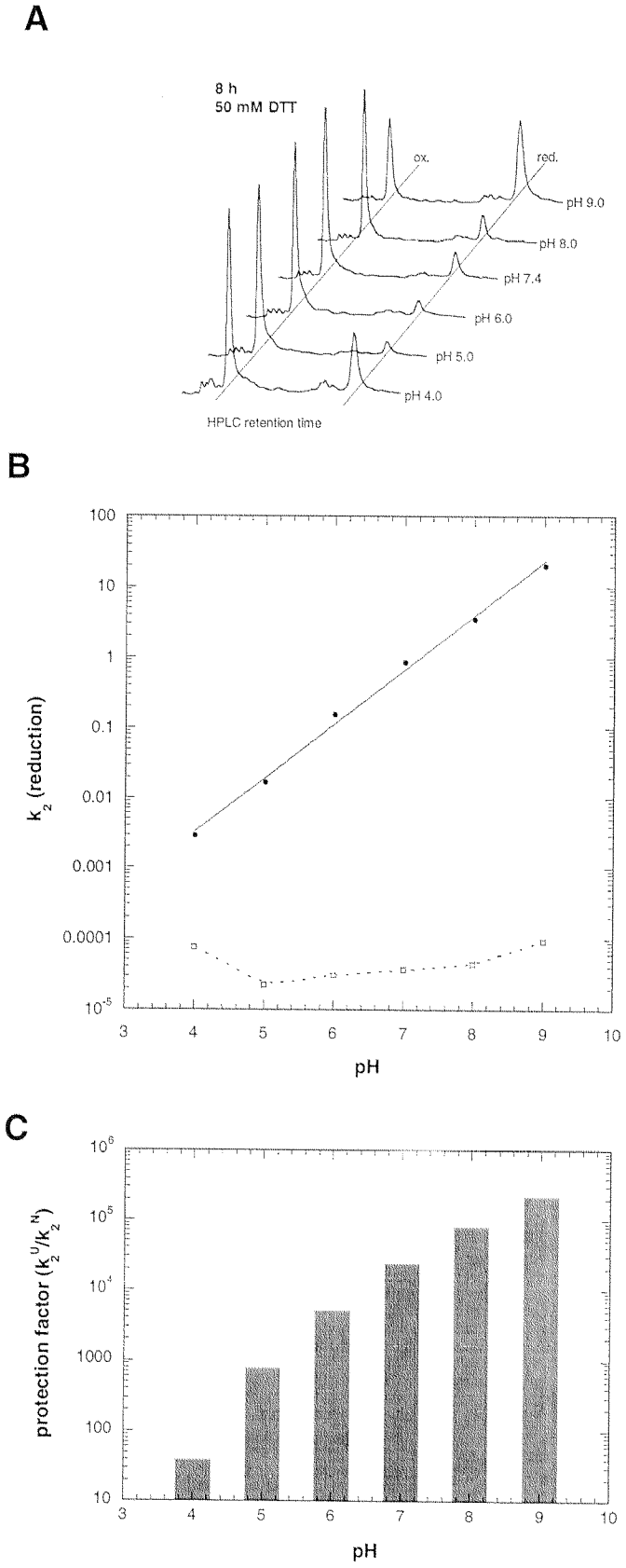
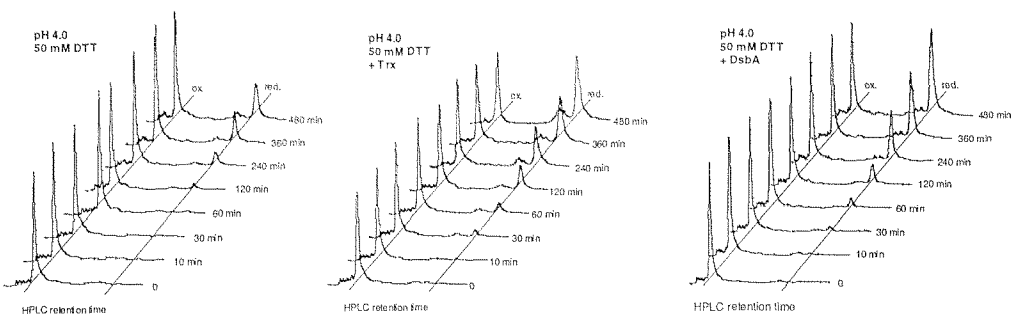
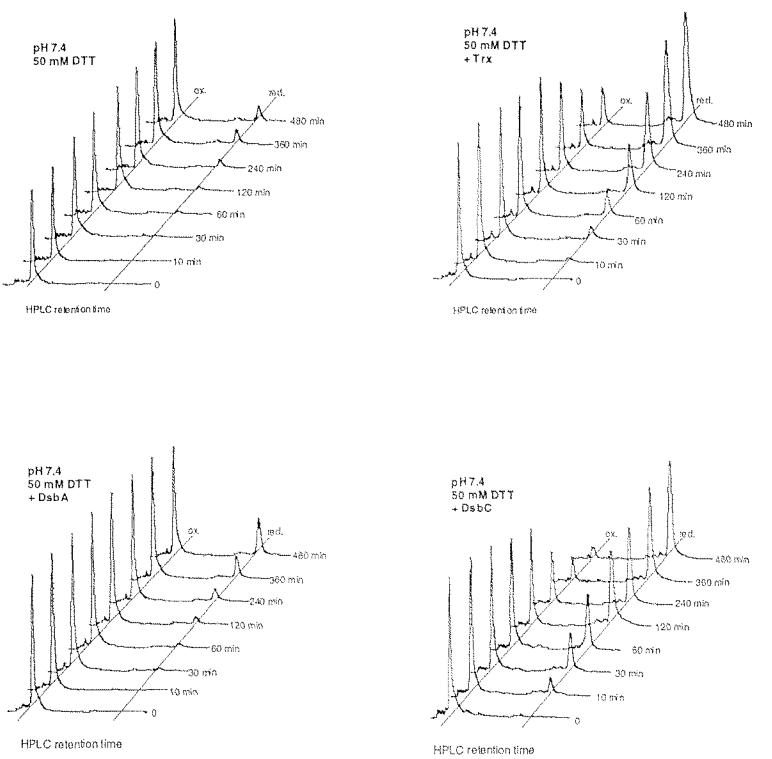

A
8 h
50 mM DTT
ox.
red.
pH 9.0
pH 8.0
pH 7.4
pH 6.0
pH 5.0
pH 4.0
HPLC retention time
B
k2 (reduction)
100
10
1
0.1
0.01
0.001
0.0001
10-5
3
4
5
6
7
8
9
10
pH
C
protection factor (k2U/k2N)
10^6
10^5
10^4
1000
100
10
3
4
5
6
7
8
9
10
pH

Figure 5

A


pH 4.0
50 mM DTT
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time
pH 4.0
50 mM DTT
+ Trx
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time
pH 4.0
50 mM DTT
+ DsbA
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time

B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50 mM DTT
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time
pH 7.4
50 mM DTT
+ Trx
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time
pH 7.4
50 mM DTT
+ DsbA
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time
pH 7.4
50 mM DTT
+ DsbC
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time

Figure 6

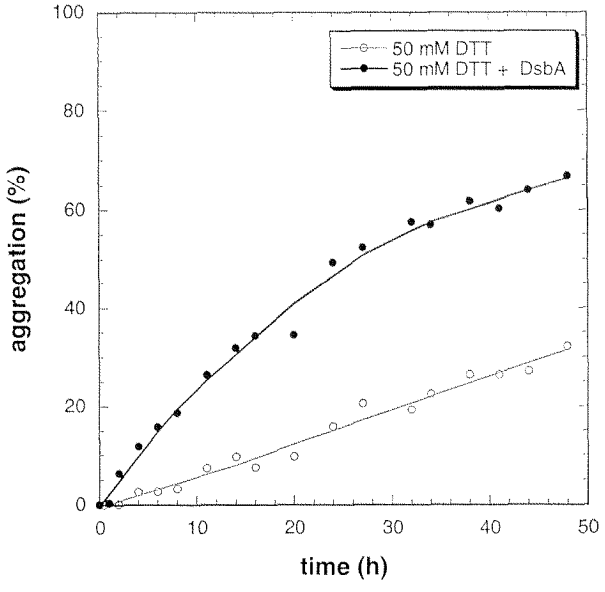
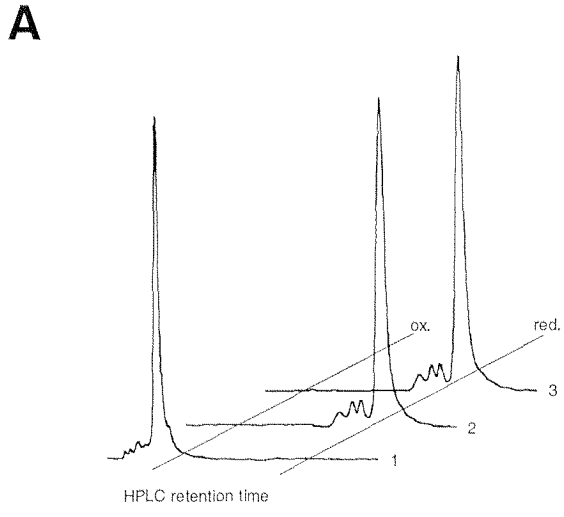

100
80
60
40
20
0
aggregation (%)
0
10
20
30
40
50
time (h)
50 mM DTT
50 mM DTT + DsbA

Figure 7


A
ox.
red.
3
2
1
HPLC retention time

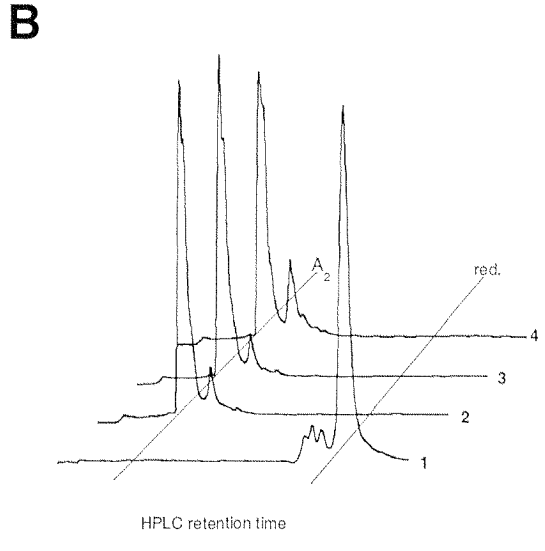
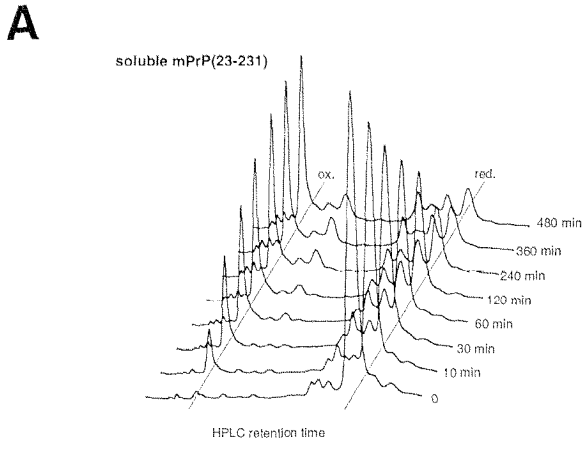

B
A2
red.
4
3
2
1
HPLC retention time

Figure 8


A
soluble mPrP(23-231)
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time

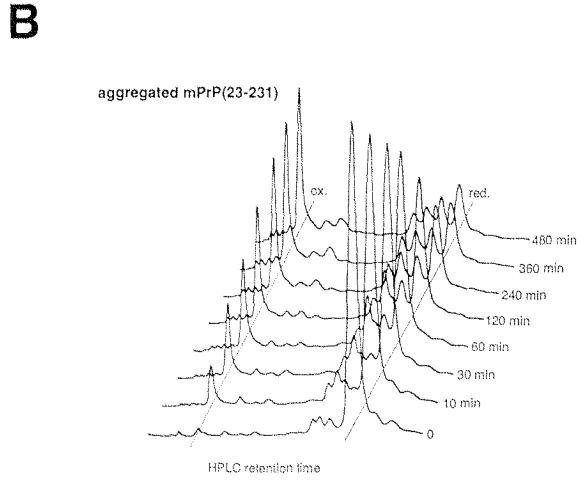
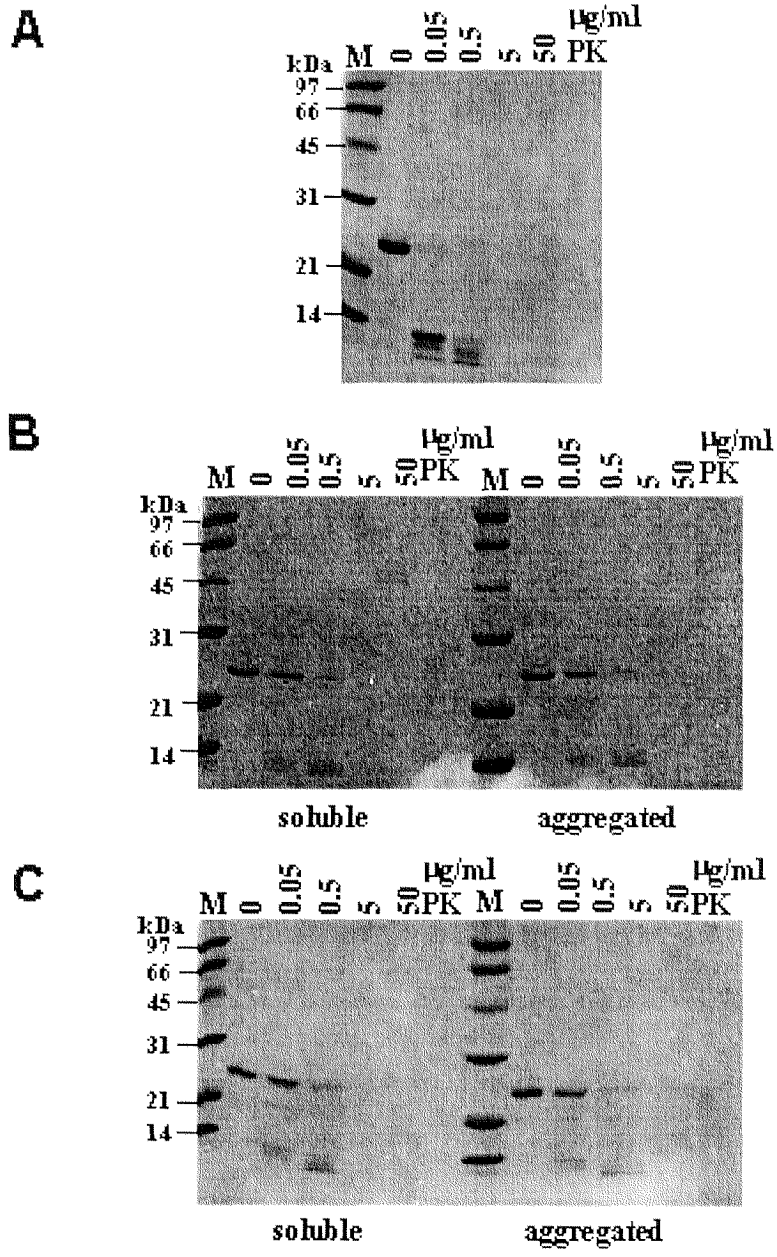

B
aggregated mPrP(23-231)
ox.
red.
480 min
360 min
240 min
120 min
60 min
30 min
10 min
0
HPLC retention time

Figure 9


A
kDa
M
0
0.05
0.5
5
50
µg/ml PK
97
66
45
31
21
14
B
soluble
aggregated
C
soluble
aggregated

5. CHARACTERIZATION OF REDUCED mPrP(121-231)

5.1. Introduction

During prion disease, the cellular prion protein PrP^{C} is converted into PrP^{Sc}. This change is only conformationally as the monomeric, soluble α-helical form PrP^{C} is converted into an oligomeric, aggregated state, PrP^{Sc}, that displays high β-sheet content (Weissmann, 1996). No covalent modifications have been observed.

Upon reduction of recombinant prion proteins a similar reaction can be observed (Jackson *et al.*, 1999a; Jackson *et al.*, 1999b; Mehlhorn *et al.*, 1996; Zhang *et al.*, 1997). The α-helical soluble protein transforms into β-sheet-like structure, is prone to oligomerization, and forms fibrils upon increase of ionic strength. This reaction has first been observed for hamster PrP(90-231) (Mehlhorn *et al.*, 1996; Zhang *et al.*, 1997) and was also described for human PrP(91-231) (Jackson *et al.*, 1999a; Jackson *et al.*, 1999b). We have examined the reduction for mPrP(23-231) with similar results (see previous chapter).

The oxidized full-length prion protein consists of an unfolded tail (residues 23-124) and a C-terminal domain (residues 125-231) that is mainly α-helical (Riek *et al.*, 1997). The single disulphide bond links helix 2 and helix 3 by Cys179 and Cys214 in this folded domain. No detailed structure of any reduced isoform of PrP is known. To examine the role of the C-terminal domain in reduced PrP, we studied reduction of isolated mPrP(121-231).

5.2. Materials and methods

Purification of mPrP(121-231) and preparation of reduced mPrP(121-231)

mPrP(121-231) was purified in its oxidized form from the *E.coli* periplasm, as described (Liemann 98). Oxidized mPrP(121-231) was denatured in 8 M urea, 10 mM Tris/HCl, pH 8.0. 50 mM DTT was added and the solution was incubated for one hour at room temperature. 10 volumes of 8 M urea in the appropriate buffer (50 mM formic acid/NaOH, pH 4.0 or 50 mM MOPS/NaOH, pH 7.4) were then added and the samples were concentrated to the original volume in centricon 10 tubes (Amicon). This dilution/concentration procedure was repeated.

CD-measurements

For circular dichroism (CD)-measurements the stock solution of reduced mPrP(121-231) was diluted ~1:10 with the same buffers (50 mM formic acid/NaOH, pH 4.0 or 50 mM MOPS/NaOH, pH 7.4) containing different urea concentrations, yielding a final concentration of 20 µM mPrP(121-231). Protein samples were centrifuged (10 minutes, 14.000 g) prior to the measurements to minimize light scattering.

Refolding transitions were monitored at 22°C by recording the far-UV CD signal at 222 nm for 30 seconds on a Jasco 710 spectropolarimeter in 0.1 cm cuvettes. The averaged ellipticities were corrected for the buffer. The urea concentration was determined by refractometry. The transition at pH 4.0 was evaluated according to a two-state model of folding with a six parameter fit (Santoro and Bolen, 88), only fixing the signal of the native protein at 0 M urea.

For selected samples, far-UV CD spectra were measured between 180 and 250 nm at 22°C, accumulated 16 times and corrected for the buffer.

5.3. Results

When mPrP(121-231) was reduced, already the first experiments showed that there is no soluble form of reduced mPrP(121-231) in the absence of denaturants at any pH

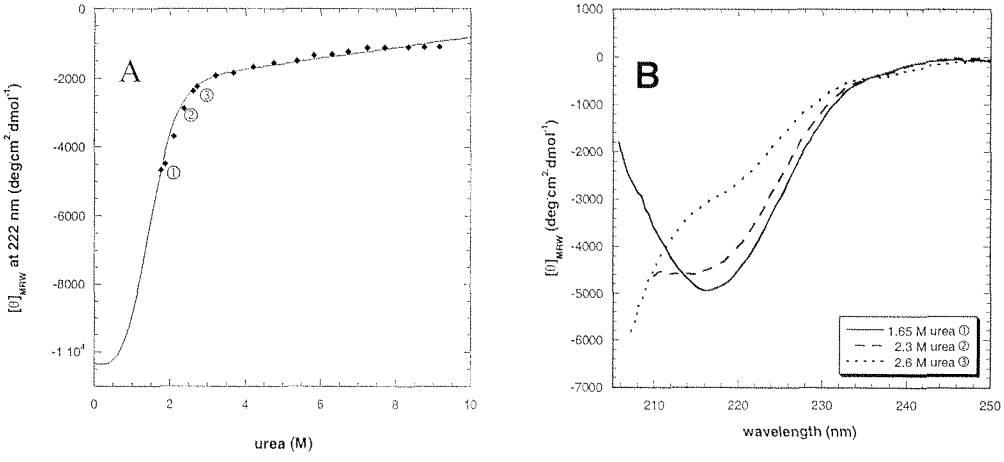


Figure 1:
A) Urea-induced refolding transition of reduced mPrP(121-231) at pH 4.0. The mean residue ellipticities at 222 nm were measured at 22 °C. The solid line results from fitting the data according to a two-state model (see text). **B**) Far-UV CD spectra of samples from the refolding transition as indicated in **A**.

between 4 and 9. The protein immediately aggregated upon dilution or dialysis from 8 M urea under all conditions tested.

Therefore, only refolding transitions could be measured to investigate the stability of reduced mPrP(121-231). They are shown for pH 4.0 and 7.4 in Figure 1A and Figure 2. The protein aggregates below 1.7 or 4 M urea respectively, so that at both pH values no complete transitions could be measured. However, at pH 4.0, the protein adopts β-sheet like structure when the urea concentration is decreased below 2.6 M (see Figure 1B). The spectra show a minimum at 215 nm. This partial transition was superimposable to the full transition of reduced mPrP(23-231) at pH 4.0 (data not shown) and, by fixing the signal of the native protein at 0 M urea to the value expected for the isolated C-terminal part of mPrP(23-231), an interpolation could be done, and the stability be estimated to be –9.9 ± 2.7 $kJ \cdot mol^{-1} \cdot M^{-1}$, with a cooperativity of 6.8 ± 1.1 $kJ \cdot M^{-1}$ and a midpoint of transition at 1.4 M urea. The data are only an estimation under the prerequisite that the segment 121-231 in reduced mPrP(23-231) and in reduced mPrP(121-231) have the same mean residue ellipticity at 222 nm in the absence of denaturant.

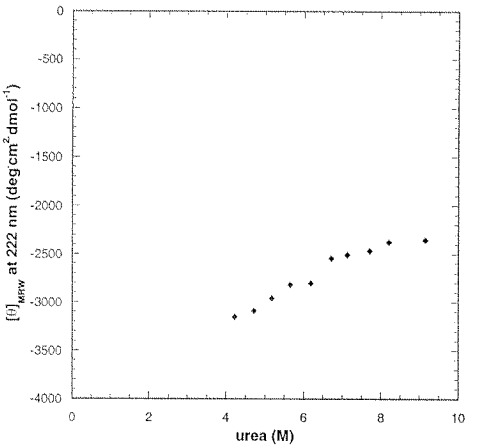


Figure 2:

Urea-induced changes in the CD-signal at 222 nm of reduced mPrP(121-231) at pH 7.4. The mean residue ellipticities at 222 nm were measured at 22 °C.

At pH 7.4 the CD signal at 222 nm decreases slightly upon decrease of urea, but no cooperative transition is observable (Figure 2). From the CD spectra no regular secondary structure could be determined (data not shown).

5.4. Discussion

Upon reduction of the disulphide bond the recombinant full-lenght prion protein, mPrP(23-231) folds completely differently compared to the oxidized form. Here we studied reduced mPrP(121-231), the segment corresponding to the folded domain of oxidized mPrP. At acidic pH in the presence of 2 M urea β-sheet-like CD-spectra are displayed. At lower denaturant concentrations the reduced mPrP(121-231) precipitated. Similarly, reduced full-length mPrP(23-231) shows also β-sheet-like structure at pH 4.0, but this reduced prion protein does not aggregate in the absence of urea. Therefore, the N-terminal part seems to influence the solubility of reduced mPrP at low pH. In the literature it was described that reduced human PrP(91-231) (Jackson *et al.*, 1999b) and hamster PrP(90-231) (Zhang *et al.*, 1997) are soluble. Therefore, we speculate that the amino acids 90-120 are responsible for the solubility.

The C-terminal domain mPrP(121-231) that mainly contains α-helices in the oxidized state (Riek *et al.*, 1996) forms a clear β-sheet like structure upon reduction. Therefore, we suppose that the α-helices are transformed into β-sheets in the reduced protein, and that this occurs as well in mPrP(23-231). Our data also suggest that it is very likely that the segment that forms the β-sheet structure in reduced mPrP(23-231) is again restricted to residues 121-231, the part of defined secondary structure in the oxidized protein.

At pH 7.4, mPrP(121-231) and mPrP(23-231) behave similar inasmuch as both aggregate at low denaturant conditions and no clearly defined structure can be determined in the presence of limited urea concentrations.

5.5. References

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Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R. and Wuthrich, K. (1996) NMR structure of the mouse prion protein domain PrP(121-321). *Nature*, **382**, 180-2.

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Weissmann, C. (1996) The Ninth Datta Lecture. Molecular biology of transmissible spongiform encephalopathies. *FEBS Lett*, **389**, 3-11.

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6. INFECTIVITY OF SCRAPIE PRIONS BOUND TO A STAINLESS STEEL SURFACE

E. Zobeley, E. Flechsig, A. Cozzio, M. Enari, & Ch. Weissmann (1999)

Molecular Medicine 5, 240-243

Molecular Medicine 5: 240-243, 1999

Molecular Medicine

Infectivity of Scrapie Prions Bound to a Stainless Steel Surface

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Abstract

Background: The transmissible agent of Creutzfeldt-Jakob disease (CJD) is not readily destroyed by conventional sterilization and transmissions by surgical instruments have been reported. Decontamination studies have been carried out thus far on solutions or suspensions of the agent and may not reflect the behavior of surface-bound infectivity.

Materials and Methods: As a model for contaminated surgical instruments, thin stainless-steel wire segments were exposed to scrapie agent, washed exhaustively with or without treatment with 10% formaldehyde, and implanted into the brains of indicator mice. Infectivity was estimated from the time elapsing to terminal disease.

Results: Stainless steel wire (0.15 × 5 mm) exposed to scrapie-infected mouse brain homogenate and washed extensively with PBS retained the equivalent of about 10^5 LD_{50} units per segment. Treatment with 10% formaldehyde for 1 hr reduced this value by only about 30-fold.

Conclusions: The model system we have devised confirms the anecdotal reports that steel instruments can retain CJD infectivity even after formaldehyde treatment. It lends itself to a systematic study of the conditions required to effectively inactivate CJD, bovine spongiform encephalopathy, and scrapie agent adsorbed to stainless steel surfaces such as those of surgical instruments.

Introduction

The agent responsible for transmissible spongiform encephalopathies, the prion, is far more resistant to physical and chemical inactivation than conventional pathogens. These properties are reflected in the difficulties encountered in sterilizing prion-containing material by conventional procedures, in particular heat sterilization and formaldehyde treatment (1–5). More than 100 cases of proven or suspected iatrogenic transmissions to humans have been catalogued (6).

A particularly well-documented and disturbing report concerns an electrode that had been inserted into the cortex of a patient with unrecognized Creutzfeldt-Jakob disease (CJD) and, following a decontamination procedure involving treatment with benzene, 70% ethanol, and formaldehyde vapor after each use, was employed in succession on two additional patients who subsequently came down with CJD. Following these events, the tip of the electrode was implanted into the brain of a chimpanzee where it again caused lethal spongiform encephalopa-

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thy (7,8). The electrodes in question had a complex structure: a steel shaft of about 6 mm in diameter, with multiple silver contacts separated by rings of insulating plastic, allowing for the existence of crevices into which infectious material might have penetrated and become inaccessible to mechanical cleansing.

Instruments used for tonsillectomy or appendectomy on unrecognized nvCJD sufferers could become contaminated with the agent (9,10), so the question of how surgical instruments could be sterilized effectively without being damaged has assumed increased importance. In view of the report that scrapie-infected tissue dried onto surfaces is more resistant to inactivation than a suspension of the same material (quoted in ref. 4), extrapolation from available data on sterilization of suspensions may be unreliable. It thus became of interest to generate a model system for the sterilization of stainless steel instruments. We here show that mouse-adapted scrapie prions can firmly bind to stainless steel wire and give rise to infection when implanted into the brain of indicator mice, even after treatment with 10% formaldehyde.

Materials and Methods

Scrapie Infection and Diagnosis

RML is a mouse-adapted scrapie isolate (11) that was passaged in Swiss CD-1 mice (Charles River Laboratories, Wilmington, MA). Inoculum stock was prepared as a 10% (w/v) homogenate of RML scrapie-infected CD-1 mouse brains in 0.32 M sucrose; RML4.1 had a titer of 8.7 log LD_{50} units/ml and 20 mg/ml total protein. Inoculated mice were checked for the development of scrapie symptoms every other day and, once they developed disease, every day (12). Apparent prion titers were estimated from incubation times (13) using the parameters derived for *Tga20* indicator mice (14).

Scrapie Agent–Exposed Wire

Stainless steel wire segments (diameter, 0.15 mm; length, 5 mm; stainless steel suture monofilament wire, Art. Nr. 01614037, USP 4/0, B. Braun Melsungen AG, Melsungen, Germany; batch 1/7502) were incubated with 10% homogenate of RML scrapie-infected CD-1 mouse brain in phosphate-buffered saline (PBS) for 16 hr, washed five times for 10 min in 50 ml PBS, all at room temperature. The wire segments were air dried and stored at room temperature for 1 day, and one segment was inserted into the brain of each of four *Tga20* mice, while in deep anesthesia, by pushing it through a 25-gauge injection needle.

To determine the amount of protein bound to a wire segment, ten 5-mm wire segments were treated with brain homogenate, washed and dried as above. They were then incubated in 0.1 ml 2 M NaOH for 1 hr at 20°C and the eluate was diluted with 0.3 ml water. Protein was determined by the Micro BCA Protein assay (Pierce, Rockford, IL), using bovine serum albumin (BSA) dilutions as standards.

Results

We exposed segments of thin stainless-steel wire to a 10% homogenate of freshly prepared scrapie-infected mouse brain, washed them exhaustively with only PBS or with PBS followed by exposure to 10% formaldehyde for 1 hr, and inserted them into the brains of indicator mice. Aliquots (30 μl) of a standard 1% brain homogenate (RML4.1) and of the last PBS wash of the wires were inoculated intracerebrally. The effective infectious dose was estimated by the incubation time method (13) using the parameters determined for *Tga20* indicator mice (14).

As shown in Table 1, prion-exposed wire segments that had been washed exhaustively with PBS caused terminal disease after 72 ± 3 days; this is equivalent to a dose of about 5.2 ± 0.4 log LD_{50} units administered as brain homogenate. After formaldehyde treatment, terminal disease occurred after 87 ± 9 days (equivalent to a dose of about 3.5 ± 1 log LD_{50} units). Thirty microliters of a 1% brain homogenate (RML4.1) produced disease after 65 ± 1 days (a dose of 6 ± 0.1 log LD_{50} units) whereas 30 μl of the fifth PBS wash did not cause disease.

Ten segments of 0.5-cm wire segment, exposed to scrapie-infected brain homogenate and exhaustively washed with PBS, were eluted with 0.1 ml 2 M NaOH. No protein was detected in the eluate; the limit of detection was about 50 ng per segment.

Discussion

Stainless steel wire exposed to scrapie-infected brain homogenate and washed exhaustively with PBS retained a high level of infectivity,

Table 1. Infectivity of prion-coated wire

Treatment[a]	Incubation Time to Terminal Disease (no. mice sick/total)	Log LD_{50} Units[b]
Wire[c]	72 ± 3 (4/4)	8 ± 0.3
Wire, formaldehyde-treated[d]	87 ± 9 (6/6)	5.5 ± 1
1% scrapie-infected brain homogenate[e]	68 ± 1 (4/4)	6 ± 0.1
PBS wash[f]	>120 (0/4)	<1

[a]Wire segments were inserted into the brain of four Tga20 mice in deep anesthesia. Liquid samples (30 μl) were injected intracerebrally.

[b]Apparent infectivity titers were estimated from incubation times using the formula $\log LD_{50} = 13 - 0.13 \times$ (incubation time in days) (14).

[c]Stainless steel wire (diameter about 0.15 mm; 8 mm length) exposed to scrapie brain homogenate and washed five times with PBS, as described in Materials and Methods.

[d]Wire exposed to scrapie brain homogenate, washed four times with 50 ml PBS, and incubated in 10% formaldehyde for 1 hr at room temperature.

[e]Thirty microliters of RML9.1 diluted 10-fold in PBS containing 5% BSA.

[f]Thirty microliters of the 5th 50-ml PBS wash of wire segments exposed to scrapie homogenate.

which was diminished about 30-fold but not abolished by formaldehyde treatment.

Our experiments show that the anecdotal clinical findings of Bernoulli et al. (7) could be reproduced under defined laboratory settings, both in regard to the tight binding of prion infectivity to a stainless steel surface and to the considerable resistance of the bound infectivity to formaldehyde treatment. Failure to elute detectable amounts of protein from the exposed wires means that ≤50 ng per wire segment was bound and/or that the binding is partly or entirely irreversible under our experimental conditions.

The approach we report here enables the performance of sterilization experiments that more closely mimic real-life circumstances than experiments with solutions, suspensions, and homogenates. It is advisable that not only scrapie but also BSE and CJD prions be used in such tests, for which appropriate indicator mice exist (15–17).

Our findings also raise the interesting question as to whether the wire-bound prions desorb from the wire when inserted into the brain or whether they initiate infection from the bound state.

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