Acid inactivation of prions: efficient at elevated temperature or high acid concentration

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Scrapie prion rods isolated from hamster and non-infectious aggregates of the corresponding recombinant protein rPrP(90–231) were incubated with hydrochloric acid. The amount of PrP and of infectivity that survived incubation in HCl at varying times, acid concentrations and temperatures was quantified by Western blot densitometry and bioassays, respectively. Prion rods and rPrP aggregates showed similar HCl hydrolysis kinetics of PrP, indicating structural homology. For 1 M HCl and 25 °C, the rate of PrP hydrolysis follows first-order kinetics at 0.014 h⁻¹; the rate of infectivity inactivation is 0.54 h⁻¹. Hydrolysis for 1 h at 25 °C was only slightly proportional to HCl concentration up to 5 M, but complete loss of infectivity and PrP reduction to < 2 % was observed at 8 M HCl. The temperature dependence of unhydrolysed PrP, as well as infectivity at 1 M HCl for 1 h, showed a slight decrease up to 45 °C, but a sigmoidal decrease by several orders of magnitude at higher temperatures. The slow hydrolysis of PrP and inactivation of infectivity by acid treatment at room temperature are attributed to solvent inaccessibility of prion rods and rPrP aggregates, respectively. The more effective hydrolysis and inactivation at temperatures above 45 °C are interpreted as thermally induced disaggregation with an activation energy of 50–60 kJ mol⁻¹. Most importantly, infectivity was always inactivated faster or to a higher extent than PrP was hydrolysed at several incubation times, HCl concentrations and temperatures.

INTRODUCTION

Prions are the causative agents of transmissible spongiform encephalopathies (TSEs), such as Creutzfeldt–Jakob disease of man, bovine spongiform encephalopathy (BSE) and scrapie of sheep (Prusiner, 1982; reviewed by Prusiner, 1998, 2001; Weissmann, 1991; Collinge, 2001; Hörlinmann et al., 2001; Riesner, 2001; Aguzzi & Heikenwalder, 2003; Priola et al., 2003). We are aware of other molecular models for TSEs (Chesbro, 1991), but we use only the prion model as the basis for our studies. Prions are proteinaceous, infectious particles and, so far, the host-encoded prion protein (PrP) is the only component reported to correlate with infectivity. In the infected organism, PrP is present as both the cellular isoform PrPc and an abnormal, scrapie isoform, PrPsc. Upon purification using detergents and digestion by proteinase K (PK), PrPsc is transformed into an N-terminally truncated, but still infectious, form of 27–30 kDa, designated PrP 27–30 (McKinley et al., 1991). PrP 27–30 is found aggregated into fibrils (prion rods), which are fairly regular structures with a diameter of 10–20 nm and a length of 75–200 nm. Prion rods possess the ultrastructural and Congo red-staining properties of amyloid (Prusiner et al., 1983). Recently, fibrils were formed from mouse recombinant PrP(89–230) corresponding to the N-terminally truncated mouse prions, and these synthetic fibrils were shown to be infectious (Legname et al., 2004).

Prion rods were isolated from brains of terminally scrapie-sick hamsters. Scrapie prion isolates, particularly in the form of prion rods, are characterized by an unusual resistance to thermal or chemical treatments used to inactivate conventional agents of infectious diseases (reviewed by Darbord, 1999; Taylor, 2000). The presence of large amounts of external lipids protects scrapie prions against heat hydrolysis of PrP (Appel et al., 2001). Conditions for complete inactivation of infectivity have been described in several reports (reviewed by Danner, 1991; Ernst & Race, 1993; Taylor et al., 1994; Budka et al., 1995; Manuelidis, 1997; Oberthür, 2001). Most widely used are treatment with 2.5–5 % sodium hypochlorite solution or 1 M NaOH at room temperature for 1–2 h, and autoclaving at 134 °C for 1–4 h. Thus, prion infectivity is inactivated efficiently under alkaline conditions. Inactivation factors >10⁶–8, i.e. reductions of
log_{10}(ID_{50}) by more than 6.8, were determined in 1 M NaOH (1 h, 25 °C) for brain homogenate (Brown et al., 1986) or 3-1 log_{10}(ID_{50}) reduction in 0.3 M NaOH (1 h, 30 °C) for partially purified scrapie isolates (Prusiner et al., 1981). In contrast, acidic conditions were reported as not affecting the level of infectivity in brain homogenates (Brown et al., 1986) or partially purified scrapie isolates (Prusiner et al., 1981). However, acidic as well as alkaline conditions are known to hydrolyse monomeric proteins to a comparable extent (Kellner et al., 1999). It is also known that suppressed gastric acidity in mice leads to higher oral infection rates with low doses of the scrapie agent (Martinsen et al., 2002).

To elucidate the supposedly high acid resistance of prions, infectious hamster scrapie prion rods and non-infectious β-sheet-rich aggregates (later called β-aggregates) of the corresponding recombinant protein rPrP(90–231) (Jansen et al., 2001) were treated with hydrochloric acid. High acid resistance of both samples was confirmed. Efficient hydrolysis of prion rods and rPrP β-aggregates was observed only at elevated temperatures (85 °C, 1 M HCl) or high HCl concentrations (8 M HCl, 25 °C). The experimental data can be interpreted by a three-step mechanism of inactivation. Partial inactivation, which is possibly surface denaturation of prion rods, precedes the disaggregation of the rods that is concomitant with the major loss of infectivity; disaggregation facilitates hydrolysis of PrP, totally destroying the infectivity.

**METHODS**

**Biological safety.** Prion material was handled in a BSL2 biocontainment laboratory. Infectious samples were manipulated in biosafety hoods by using two layers of disposable gloves and over-sleeves, with the upper glove being discarded whenever leaving the biosafety hood. Waste was decontaminated by autoclaving at 134 °C for 4 h or by treatment with 1 M NaOH for 1 h at room temperature, if high temperatures were not feasible.

**Prion protein samples.** Recombinant prion protein rPrP(90–231) is based on the Syrian hamster sequence, but lacks Asn-linked glycosylation and the C-terminal glycosylphosphatidylinositol anchor. Samples of rPrP(90–231) were kindly provided by Dr Stanley B. Prusiner and Ana Serban (University of California, San Francisco, CA, USA). They were prepared as described by Mehlihorn et al. (1996) and stored at a concentration of 10 mg protein ml⁻¹ in 20 mM sodium acetate buffer, pH 5.5, with 0.005% thimerosal as an antimicrobial agent.

Hamster scrapie prion rods (batch SF) were kindly provided by Dr Stanley B. Prusiner and Ana Serban (University of California, San Francisco, CA, USA). Isolation of prion rods from brains of terminally scrapie-sick Syrian hamsters (strain 263K) has been described by Prusiner et al. (1982a, 1983).

Another batch of Hamster scrapie prion rods (batch BE) was prepared at the Robert Koch-Institut (Berlin, Germany) according to a procedure described previously (Diringer et al., 1997). Throughout this work, scrapie infectivity was always in the form of prion rods.

**PrP-specific antibody 3F4.** mAb 3F4 (IgG2A, ascites fluid, 6 mg protein ml⁻¹) was purchased from Senetek.

**Preparation of aggregated rPrP(90–231) samples.** Samples of rPrP(90–231) at a concentration of 10 mg protein ml⁻¹ [20 mM sodium acetate buffer (pH 5.5), 0.005% thimerosal] were applied into the upper compartment of 10 kDa Centricon tubes (Amicon) and centrifuged to remove the acetate buffer. The protein was retained on the membrane. The buffer was removed from the lower compartment and 10 mM sodium phosphate buffer (pH 7.2) containing 0.2% SDS was added to the upper compartment, incubated for 10 min at 37 °C, centrifuged and the filtrate was collected. This elution step was repeated three times. The combined eluates contained rPrP(90–231) solution at approximately 2 mg ml⁻¹, as measured by A_{386}. In order to induce β-aggregation (Jansen et al., 2001), the samples were further diluted 1:20 with 10 mM sodium phosphate buffer (pH 7.2) (final SDS concentration, 0.01%). Aliquots of 10 µl were taken immediately and incubated overnight at 37 °C. Thus, each sample contained the same amount of aggregated rPrP(90–231) (approx. 1 µg).

**Preparation of scrapie prion rod samples.** A high-titre preparation of scrapie prion rods containing 180 µg PrP 27–30 was suspended in 1-8 ml deionized water and homogenized by sonication for 1 min at 50–70 W (beaker sonicator, Labsonic U; B. Braun). From the suspension, aliquots of 10 µl for PrP hydrolysis experiments and of 100–300 µl for bioassays were transferred into separate polymer tubes. Each sample for hydrolysis experiments contained the same amount of PrP 27–30 (approx. 1 µg).

**Hydrolysis/inactivation experiments.** To samples prepared as above, aqueous HCl (or water for controls) was added to achieve a final HCl concentration of 1–8 M in a volume of 100 µl. Individual samples were kept at room temperature for 1–160 h or incubated for 1 h at various temperatures. Acid inactivation/degradation was stopped by adding a stoichiometric amount of 1 M NaOH (5 M NaOH in the case of high HCl concentrations). The final pH was checked by applying a 2 µl aliquot onto an indicator stick (Neutralit pH 5–10; Merck) and was found to be between pH 6.5 and 8.0 in all cases.

**Quantification of PrP by Western blot.** The method has been described in detail previously (Appel et al., 2001). The samples were mixed with loading buffer, heated and applied onto SDS/polyacrylamide gels (acylamide/bisacylamide, 30:1; acrylamide concentration in loading/resolving gel, 4/12%; 1.5 mm wide). After electrophoresis, the protein was electroblotted onto a PVDF membrane. PrP was detected by using the mAb 3F4 (Kascak et al., 1987) and enhanced chemiluminescence. The sensitivity threshold of this method was 10 ng PrP. Developed films were scanned and PrP concentrations were determined by densitometric comparison with at least three standards of rPrP or PrP 27–30 (hamster scrapie isolate) on the same gel.

**Determination of remaining infectivity by animal bioassays.** PBS (pH 7.4) was added to the neutralized samples to a final volume of 1 ml or 500 µl. Samples were quick-frozen by immersion in liquid nitrogen and stored at −80 °C. Bioassays of infectivity of prion rods were performed in Syrian hamsters by an incubation-time interval procedure (Prusiner et al., 1982b). For each determination of remaining infectivity, six hamsters were inoculated intracerebrally with a 30 or 50 µl aliquot of the sample. As bioassays were terminated up to a limit of 200 days, 2·5 log_{10}(ID_{50}) or more could be detected. Samples containing 6·5–8·5 log_{10}(ID_{50}) before acid inactivation were diluted 30-fold or 10-fold for the purpose of serial inoculations [30 µl out of 1000 µl or 50 µl out of 500 µl, i.e. −1·5 or −1·0 log_{10}(ID_{50})]. The difference between starting infectivity [8·5 or 6·5 log_{10}(ID_{50})], dilution [1·5 or 1 log_{10}(ID_{50})] and detection limit of the bioassay [2·5 log_{10}(ID_{50})] is 3·0–4·5 log_{10}(ID_{50}). Thus, the detection window for infectivity inactivation was 3·4–5·
RESULTS

In order to investigate the molecular basis of high acid resistance of infectivity (Mould et al., 1965; Prusiner et al., 1981; Brown et al., 1986), scrapie prion rod isolates from hamster brain and in vitro-generated β-aggregates (Jansen et al., 2001) of the corresponding recombinant prion protein rPrP(90–231) were incubated with hydrochloric acid. The amount of PrP remaining intact after various incubation times, acid concentrations and temperatures was quantified by gel electrophoresis and Western blot. Remaining infectivity was tested in selected samples by incubation-time bioassays in Syrian hamsters. Table 1 lists the results of bioassays and the corresponding values of PrP. Relative remaining infectivity was always lower than the relative amount of remaining PrP.

Fig. 1 presents an example of a Western blot for densitometric determination of PrP after acid hydrolysis. On the right side are four lanes with known amounts of untreated prion rod samples in order to establish a calibration curve. On the left side are three prion rod samples acid-treated for 20, 130 and 160 h. Different starting amounts of PrP were chosen in order to bring the amount of remaining PrP into the calibration range. As lane 3 (900 ng prion rods, acid-degraded for 160 h at 25°C with 1 M HCl) presented a densitometric signal similar to lane 6 (40 ng untreated prion rods), a hydrolysis factor of 900/40, i.e. 22.5, can be derived.

A small deviation in molecular mass (approx. 2 kDa) of the acid-treated samples was observed. This might be explained by acid cleavage of a small PrP amino acid stretch, most probably from the N terminus. An acid-induced decomposition of amino acid side chains, preferably Trp, is also possible. The glycosylation pattern (signals at ~28, ~23 and ~18 kDa), the PrP dimer band (~55 kDa) and the 3F4 antibody-recognition site are not changed significantly.

Time course of hydrolysis/inactivation

Fig. 2 details the time course of hydrolysis of PrP and inactivation of prion infectivity by acid treatment of samples from aggregated rPrP and scrapie prion rods. Remaining PrP and remaining infectivity are plotted over incubation time (1 M HCl at 25°C). Within the error limits of Western blot densitometry (10%), the data points of rPrP in β-aggregates and of PrP 27–30 in scrapie prion rods (sample SF) overlap (solid line). However, infectivity of the prion rods is inactivated much faster than PrP is hydrolysed (dotted line). When the experiment was repeated with prion rods (sample BE), less inactivation was obtained. This difference is possibly a consequence of the mild inactivation conditions (see Discussion). As a control experiment, rPrP was kept first in the soluble state at pH 4.5 in the absence of SDS. Then, the solution conditions were altered to 1 M HCl, the sample was divided into two aliquots and both aliquots were incubated for 8 h, one at 25°C and the other at 37°C. In both cases, rPrP in 1 M HCl was aggregated completely. Therefore, we can conclude that rPrP in 1 M HCl is always in the aggregated state and a control experiment on the hydrolysis kinetics of soluble rPrP cannot be performed.

The plot of the data in Fig. 2(b) indicates an exponential decay of PrP, i.e. first-order reaction kinetics with respect to PrP concentration:

$$\ln\left(\frac{C_{PrP}(t)}{C_{PrP}^0}\right) = -k_1 \times t$$

with rate constant $k_1$, a starting concentration of PrP $C_{PrP}^0$ and the concentration of PrP after the reaction time $t$, $C_{PrP}(t)$.

The plot according to equation (1) shows the linear relationship in the time interval of 0–115 h. After 115 h, the amount of remaining PrP drops below 10% and determination of PrP by Western blot becomes less reliable. However, all points after 115 h are either at or below the regression curve, indicating that acid hydrolysis of PrP follows a uniform mechanism.

In conclusion, the hydrolysis of scrapie prion rods and rPrP β-aggregates by 1 M HCl at 25°C is first order with respect to PrP concentration. The slope of the regression line yields a rate constant of 0.014 h⁻¹. As already seen in Fig. 2(a), inactivation of infectivity is much faster than PrP hydrolysis, with a rate constant of 0.54 h⁻¹, the value of which is, however, much less accurate.

Although the influence of acid treatment on the PK resistance of prions was not within the scope of this study, we tested PK resistance (4 µg PK in 100 µl, 1 h incubation) at a single time point, i.e. 27 h, 1 M HCl, sample (BE) (data not shown). Whilst the infectivity had dropped to 18%, the PK resistance was not significantly different from the PrP hydrolysis, i.e. above 50% (see Fig. 2). Recombinant PrP in the β-aggregated form showed no PK resistance, with or without acid treatment.

HCl concentration dependence of hydrolysis/inactivation

Fig. 3 depicts the logarithmic dependence of PrP hydrolysis ($C_{PrP}/C_{PrP}^0$) and the inactivation of prion infectivity ($ID_{50}/ID_{50}^0$) on the concentration of HCl. A linear relationship in Fig. 3 would be in accordance with a mechanism in which the proton from HCl is a reaction partner in the rate-determining step and is present in large excess over PrP. Pseudo-first-order kinetics would be obtained.

A linear but weak dependence of $\ln(C_{PrP}/C_{PrP}^0)$ upon HCl concentration was observed in the range of 1–5 M HCl and a much stronger dependence above 5 M HCl. Thus, at high HCl concentrations, a different and more efficient mechanism of PrP hydrolysis must be assumed. Upon treatment
Table 1. Infectivity and PrP after treatment of prion rods with hydrochloric acid

Remaining infectivity is always smaller than remaining PrP (compare last two columns).

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<tr>
<th>Conditions</th>
<th>PrP per sample (µg)*</th>
<th>Individual incubation times (days)</th>
<th>Mean incubation time ± SD (days)</th>
<th>Infectivity [log,ID_{50}] per sample</th>
<th>Remaining infectivity (%)</th>
<th>Remaining PrP (%)</th>
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<th>Infectivity [log(ID50) per sample]</th>
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*Scrapie prion samples prepared in San Francisco (SF) or in Berlin (BE).
†Animal found dead within first week after inoculation.

Fig. 1. Analysis of the extent of acid hydrolysis of hamster scrapie prion rods (SF) for 20, 130 or 160 h. Western blot of prion protein PrP27–30 remaining after acid hydrolysis (25 °C, 1 M HCl) (left) and of non-treated calibration standards (right). Different starting amounts of PrP27–30 (shown below each lane) were chosen to allow quantification of remaining PrP in the calibration range. The heavy band around 28 kDa was evaluated by densitometry. Molecular mass of all three PrP signals is reduced by ~2 kDa after acid treatment.

with different concentrations of HCl, prion infectivity was always inactivated to a higher extent than PrP hydrolysis. In this aspect, Fig. 3 confirms the general trend from Fig. 2. A more quantitative conclusion cannot be drawn. Definitely, inactivation at 8 M HCl is complete, i.e. no measurable infectivity is left.

Temperature dependence of hydrolysis/inactivation

Fig. 4 illustrates the temperature dependence of acid hydrolysis/inactivation. Whilst little decrease of intact PrP and less than an order of magnitude of infectivity were observed between 4 and 45 °C (1 M HCl, 1 h), increasing the temperature to 65 °C yielded a strong decrease of intact PrP and infectivity, which was complete at 85 °C. The solid line is a mean (spline fit) of all PrP data points – linear with a low slope until 45 °C, followed by sigmoidal decline of PrP above 45 °C. The broken line connects the infectivity data points above 45 °C. The relative remaining infectivity is always...
Fig. 2. Dependence of the amount of PrP and of infectivity upon the time of incubation in 1 M HCl (25 °C). Samples were β-aggregates of recombinant PrP(90–231) (○) and prions prepared from terminally scrapie-sick hamster brains (●, □, ■). Data points ● and □ were obtained with prion rods (sample SF); data points ■ were obtained with prion rods (sample BE) (see Methods). PrP was determined by Western blot densitometry (Fig. 1) and infectivity by incubation-time bioassays (Table 1). (a) Linear presentation of PrP and infectivity. The solid line is a spline fit of all PrP data points, indicating similar hydrolysis kinetics in both samples. The broken line connects infectivity data points (sample SF), intended to guide the eye. (b) Logarithmic presentation of the ratios C_prP/C_prP and ID50/ID50. The solid line is a linear fit of all PrP data points, resulting in first-order hydrolysis kinetics with a rate constant of 0-014 h⁻¹. The broken line is an interpretation in a linear fit of infectivity data points from prion rods (sample SF), resulting in a rate constant of 0-54 h⁻¹.

lower than the relative remaining PrP. If we regard the infectivities on a logarithmic scale, we might interpret them in a biphasic mode. In the range of <45 °C, about 1 log(ID50) is inactivated. This can be concluded not only from the single point at 25 °C (1 h, 1 M HCl), but also from 25 °C (3 h, 1 M HCl) or 25 °C (1 h, 3 M HCl). The infectivities at temperatures >45 °C are also depicted, with a ten times-enlarged scale. On this scale, it becomes obvious that the decrease of infectivity is in parallel with or even steeper than the sigmoidal decrease of PrP hydrolysis.

The temperature dependence of remaining PrP or infectivity can be interpreted by the temperature dependence of rate constants, given by the Arrhenius equation:

\[
\frac{\partial \ln(k/\partial) \ln(1/T)}{R} = -\frac{E_A}{R} \quad \text{(equation 2)}
\]

with \(E_A\) being the activation energy for the hydrolysis/inactivation process and \(R\) the gas constant.

Because \(\ln\left[\frac{C_{PrP}}{C_{PrP}(1h)}\right]\) is proportional to the rate constant \(k\) (see equation 1), a plot

\[
\ln\left[\frac{C_{PrP}}{C_{PrP}(1h)}\right] = -\frac{E_A}{R} \times \left(\frac{1}{T}\right) + \text{const.}
\]

(equation 3)

is presented in Fig. 4.

A linear relationship is observed in the ‘transition range’ between 50 and 75 °C for PrP and between 50 and 65 °C for infectivity. The activation energy of acid hydrolysis of aggregated rPrP and of scrapie prion rods is about 51 (±4.4) kJ mol⁻¹ (1 M HCl) and the corresponding value for infectivity inactivation is 57 (±2.8) kJ mol⁻¹.

**DISCUSSION**

At elevated temperatures (85 °C, 1 M HCl, 1 h) or high HCl concentrations (8 M HCl, 25 °C, 1 h), efficient hydrolysis of PrP in scrapie prion rods and rPrP β-aggregates was observed. In all experiments with prion rods, infectivity was inactivated faster and more efficiently than PrP was hydrolysed (see Table 1).
Temperature dependence of acid hydrolysis/inactivation in 1 M HCl for 1 h. (a) Remaining PrP and remaining infectivity are plotted against temperature. The solid line is a spline fit of all data points from aggregated recombinant PrP and prion rods (sample SF). The broken line connects infectivity data points from prion rods (sample SF), intended to guide the eye. The dotted line connects infectivity data points drawn in tenfold-expanded scale; the infectivity (%) values $\times 10$ are given in parentheses. (b) Double-logarithmic plot of the ratios $C_{\text{PrP}}/C_{\text{PrP}}^0$ and $\text{ID}_{50}/\text{ID}_{50}^0$ versus reciprocal temperature. From the linear relationship of PrP hydrolysis between 50 and 75 °C, a slope of 6130 (±530) K, equivalent to an activation energy of 51 (±4.4) kJ mol$^{-1}$, was evaluated. From the linear relationship of prion inactivation between 50 and 65 °C, a slope of 6910 (±340) K, equivalent to an activation energy of 57 (±2.8) kJ mol$^{-1}$, was evaluated.

In infectious samples, the prion protein PrP is present in the form of diffuse aggregates, fibrils, amyloid plaques or rods, depending upon the host, the disease, the strain and the preparation protocol (DeArmond & Prusiner, 2003). The present study was carried out with scrapie prion rods, which are defined most precisely by the purification protocols; they contain only the N-terminally truncated prion protein PrP 27–30, together with about 1% host sphingolipids (Klein et al., 1998) and 10–15% of a glycogen-like polysaccharide (Appel et al., 1999) as secondary components. Under several inactivation conditions, prion infectivity is inactivated when proteins are destroyed (Prusiner, 1982; Diener et al., 1982). A number of reports from different groups show that prion infectivity is proportional to the amount of intact PrP (McKinley et al., 1983; Gabizon et al., 1989; Brown et al., 1990; Beekes et al., 1996). In conclusion, if a chemical or physical treatment destroys PrP, it also destroys prion infectivity. We do not challenge this conclusion. However, our acid hydrolysis/inactivation data indicate that the opposite is not true. The prion infectivity seems to be dependent not only on intact monomeric PrP, but also on intact prion structure, including the secondary components. Prion infectivity was used in this work in the form of prion rods, because prion rods represent a particularly stable and reproducible form of prion infectivity and are therefore well-suited to quantitative studies. The more customary form of spiking material for validation studies has been homogenates or macerates of infected brain (Kimberlin et al., 1983; Ernst & Race, 1993; Taylor et al., 1994; Somerville et al., 2002). However, Brown et al. (1990) demonstrated that, after being subjected to steam autoclaving at 134 °C for 30 min, reduction in infectivity of prion-rod preparations was less than that of whole-brain tissue. Consequently, results from inactivation studies on prion rods are particularly reliable under the aspect of product safety.

Some authors reported that PrP (Manuelidis et al., 1987) or the birefringent structure of prion rods (Wille et al., 2000) can be separated from infectivity by detergent treatment or through differential effects of heat treatment (Kimberlin et al., 1983; Somerville et al., 2002; Taylor et al., 2002). However, as pointed out several times in the literature (Weissmann, 1991), prion samples are not homogeneous in molecular terms and only a portion of PrP molecules in an infectious sample was separated from infectivity, but the infectivity left was never free of PrP molecules. The best explanation would be that the relationship between PrP molecules and infectivity might be heterogeneous within the prion rods, and different parts of the rods are affected differently by detergents or heat. In order to generate one infectious unit, about 100 000 PrP molecules have to be inoculated into the brain and only 4% of it is retained, i.e. not cleared within a very short time (Safar et al., 2005). In our experiments, we cannot differentiate between different portions of PrP molecules within the prion rods.

As infectivity was inactivated much faster than PrP was hydrolysed, we must conclude that the action of acids on scrapie prion rods follows a mechanism consisting of more than one step (Fig. 5). The difference in the time course of prion inactivation and PrP hydrolysis is most obvious from Fig. 2. In the first step, infectious prion rods are transformed into a form of much lower infectivity (Fig. 5). Whilst the peptide backbone was 90% intact at 40 °C, i.e. below the cooperative transition, about 1 log titre of the infectivity of prions (sample SF) was already inactivated at that temperature (15% remaining infectivity at 25 °C and 4% at 45 °C).
The cooperative mode of PrP degradation above 40 °C, connected with a measurable loss of PK resistance. The ‘surface denaturation’ might depend on subtle structural features and was therefore found to be different in samples from San Francisco (SF) and Berlin (BE). These differences were most obvious in the time course of inactivation shown in Fig. 2. Although PK resistance was not studied systematically, the experiments described here show clearly that PK resistance is similar to the resistance against hydrolysis, but not against inactivation of infectivity. After 27 h incubation at 25 °C, >50% of PrP27–30 was left undigested by PK, compared with about 70% of PrP27–30 left unhydrolysed by acid (Fig. 2), whereas infectivity was reduced to 18% (sample BE) or 0·01% (sample SF). Consequently, we conclude that the first step in Fig. 5, i.e. structure denaturation, is not connected with a measurable loss of PK resistance.

The cooperative mode of PrP degradation above 40 °C (Fig. 4) indicates a dissociation of large PrP aggregates, either the prion rods or the rPrP β-aggregates, before cleavage of covalent bonds. If the infectivities remaining after the first step in Fig. 5 are scaled ten-times higher, high cooperativity in exactly the same temperature range was also observed with the infectivity, which decreased by two orders of magnitude. In summary, infectivity was never observed with the dissociated state, but the associated state can exhibit full infectivity (25 °C) or about 1 log-reduced infectivity (45 °C). Conceptually, the dissociated state has to be differentiated from the hydrolysed state, as is obvious from other dissociation studies (Riesner et al., 1996), but we could not resolve the two states unequivocally in our work. Thus, in Fig. 5, two intermediates are postulated between fully infectious prion rods and PrPhydrolysed: these are PrPaggregated and PrPdissociated.

How can the acid treatment of prion rods be explained in molecular mechanistic terms? Treatment of scrapie-infected brain homogenate or scrapie isolates with 0·3 or 1 M NaOH for 1 h at room temperature inactivates infectivity by at least three orders of magnitude (Mould et al., 1965; Prusiner et al., 1981; Brown et al., 1986). This dramatic effect has been attributed to alkaline hydrolysis of the peptide bonds of PrP and a proportional inactivation of prion infectivity. But why is acid inactivation at room temperature so slow compared with the effect of alkali (0·54 h−1 in 1 M HCl vs >105 h−1 in 0·3 M NaOH or >108 h−1 in 1 M NaOH)?

Rates of alkaline or acidic hydrolysis of the peptide bond are approximately equal (Kellner et al., 1999). This is further illustrated by the conditions for quantitative hydrolysis of monomeric proteins for amino acid analysis: either by 4 M NaOH at 110 °C for 18–70 h or by 6 M HCl at 105–110 °C for 24–96 h (Jakubke & Jeschkeit, 1983). Therefore, a similar decrease of infectivity could be expected by either 1 M NaOH or 1 M HCl for 1 h at room temperature if monomeric PrP was the target. This is obviously not the case. Thus, we propose intact prion rods or similar strong amyloid structures as the primary target for the action of acids, which protect against fast hydrolysis.

A closer look into the structure of prion fibrils may provide an explanation for the different action of acids and bases. In the best presently available model of prion rods (Wille et al., 2002), trimers of PrP form the structural subunit; two α-helices and the glycosyl groups are at the outside and the intermolecular interactions are achieved by short, parallel β-sheets. In prion rods, the trimers are stacked on top of each other and assembled into fibrils. This fibrillar assembly is stabilized by a multitude of non-covalent interactions. Hydrogen bonds and hydrophobic interactions probably make the strongest contribution to the interactions stabilizing the PrP fibril assembly.

Hydrogen bonds are more stable against attack by H+ than against attack by OH−, which abstracts protons from proton-donating groups. Proton abstraction destroys hydrogen bonding and adds repulsive negative charges onto the protein β-sheets. Thus, in consequence of the disturbance of fibril-stabilizing hydrogen bonds, prion fibrils are attacked easily by bases (OH−), but are more stable against acids (H+). Acids and bases are both able to hydrolyse the peptide bonds of PrP efficiently, but only bases can penetrate the β-sheet-rich fibril assembly by disrupting fibril-stabilizing hydrogen bonds.

Hydrophobic interactions are promoted by the exclusion of water from the site of hydrophobic interactions, which is an entropic effect. Strong chaotropic agents, such as urea and concentrated HCl, disturb the water structure and produce single, activated water molecules, which are able to penetrate the prion fibrils. This contributes to the explanation of why concentrated HCl, but not dilute HCl, efficiently inactivates and hydrolysates prion rods.

Thus, the fibrillar assembly of PrP in prion rods or other prion structures like plaques, which are stabilized mainly by hydrogen bonds and hydrophobic interactions, provides an explanation for the following results of this study.

(i) Whilst lower concentrations, such as 1 and 3 M HCl (25 °C, 1 h), had only a slow effect on prion infectivity, 8 M

<table>
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<tr>
<th>Denaturation</th>
<th>Dissociation</th>
<th>Hydrolysis</th>
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<td>PrPprion rods</td>
<td>PrPaggregated</td>
<td>PrPdissociated</td>
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**Fig. 5.** Proposed mechanism for acid inactivation and hydrolysis of hamster scrapie prion rods. Prions are inactivated during denaturation and dissociation and only residual infectivity is destroyed by hydrolysis.
HCl completely inactivated prion infectivity and hydrolysed PrP at room temperature within 1 h. At low acid concentrations, the hydrogen bonds in the fibril core are protected against hydrolytic attack. This would be in accordance with a structure denaturation at the surface of the prion rod, which leads to the first step in Fig. 5. Concentrated (8 M) HCl, however, has a strong dehydrating effect. It may remove water molecules from prion fibrils, affecting hydrogen bonds and hydrophobic forces. The fibril assembly is penetrated and massive hydrolysis of peptide bonds ensues, with dramatic further inactivation of infectivity and hydrolysis of PrP.

(ii) A temperature threshold of hydrolysis efficiency at 40–45 °C was observed upon treating scrapie prion rods with 1 M HCl at increasing temperatures. An explanation would be that the fibril assembly, which protects individual PrP monomers or oligomers against attack, disintegrates above that the fibril assembly, which protects individual PrP monomers or oligomers against attack, disintegrates above

(iv) Acid inactivation of prion infectivity has always been found to be faster and more efficient than PrP hydrolysis, which is in agreement with the scheme in Fig. 5.

In summary, HCl is capable of inactivating prion infectivity, but effectively only at elevated temperature or high concentration. Although our studies were carried out more under mechanistic aspects, the results may also be valuable for validating specific chemical processes involving scrapie- or BSE-risk material.

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