2 Materials and methods

2.1 TSE agents

2.1.1 Hamster adapted TSEs

Serial passaging of hamster-adapted scrapie strains 263K, ME7-H, and 22A-H and of a new hamster-adapted BSE isolate, BSE-H, was performed by *i.c.* (intracerebral) infection of outbred Syrian hamsters with 50-µl aliquots of 1% (w/v) hamster-brain homogenates in TBS (10 mM Tris-HCl (Sigma), 133 mM NaCl (Merck), pH 7.4) from terminally ill donors. Hamster scrapie strain 263K (Kimberlin *et al.,* 1977) was originally provided by Dr. R. H. Kimberlin and has been serially passaged for more than 20 years in the Robert Koch-Institute. Strains ME7-H and 22A-H generated by Dr. R.H. Kimberlin after 4 and 7 passages in hamsters, respectively (Kimberlin *et al.,* 1989), were kindly provided by the Institute for Animal Health, Edinburgh, UK. Isolates 'RIV/8' of ME7-H and 'RIV/12' of 22A-H were used for the first passage of these TSE agents at the Robert Koch-Institute. BSE-H was newly derived, after one passage of the BSE agent from cattle in mice and subsequently transmitted to hamsters (Thomzig *et al.,* 2004).

Hamsters with 263K scrapie showed head bobbing, generalized tremor, and ataxia. These animals were frequently and persistently in motion, easily irritated by touch and noise, upon which they often twitched, and had difficulties maintaining balance and rising from a supine position. In contrast, clinically ill animals with the ME7-H or 22A-H scrapie agent exhibited phlegmatic sluggishness with bradykinesia and kyphosis. Unlike 263K scrapie hamsters, ME7-H- and 22A-H-infected animals were frequently and persistently resting, not obviously irritated by touch or noise, and well able to slowly rise from a supine position until very advanced stages of the disease. Head bobbing, generalized tremor, or ataxic gait as in 263K scrapie were not observed, but the animals showed signs of hind limb paralysis. Hamsters infected with BSE-H were also found to rest frequently and continuously in a kyphotic position and then appeared lethargic. In contrast to the phlegmatic sluggishness and apparent unresponsiveness to irritation by touch and noise as observed in ME7-H and 22A-H scrapie, BSE-H hamsters exhibited spontaneous convulsions from a resting position and had an extreme sensibility to touch (especially to the hind limbs) which easily triggered tetanic responses. BSE-H hamsters showed ataxia of gait and hind limb paralysis without bradykinesia. Unlike the 263K scrapie hamsters, they were fairly well able to rise from a supine position and did not show head bobbing and generalized tremor (Thomzig *et al.,* 2004).

For the present study, all examinations were performed in our laboratory on single passages of 263K, 1st and 2nd passages of ME7-H and 22A-H, and 1st and 3rd passages of BSE-H (see Appendix 1), which showed incubation times of 331 ± 16, 287 ± 28, 206 ± 8 and 83 ± 5 days (expressed as mean ± S.D.) until the occurrence of terminal disease, for ME7-H, BSE-H, 22A-H and 263K, respectively. Animals were regularly observed for clinical symptoms and mortified by CO_2 euthanasia at the terminal stage of the disease. The brains were removed, immediately frozen in liquid nitrogen and stored at –70°C until use.

2.1.2 Sporadic CJD samples

Human brain samples with blinded origin, from 22 donors were delivered from Walter Schulz-Schaeffer from the Institute of Neuropathology, Georg-August University, Göttingen, Germany. All samples including cases of sporadic CJD and controls were individually labeled (see Table 3.IV). Each sample was dissected from the frontal cortex, which is a region abundant of PrP^{Sc} (Parchi *et al.,* 1997; Hill *et al.,* 2003). The samples were delivered frozen in dry ice and stored at -70°C until use.

2.2 Extraction and purification of PrP27-30

Originally, scrapie-associated PK resistant prion protein PrP27-30 was purified from 20 *i.c.* infected Syrian hamster brains at terminal stage of the disease, using the so called "10% purification method" developed at Robert-Koch Institute by the group of Prof. Diringer (Diringer *et al.,* 1997). Consequently, the procedure was modified in order to reduce the amount of brain tissue used and to avoid contamination of the purified protein fractions with disintegrated polycarbonate particles from the wall of the used vials (Polycarbonate tube 1x3-1/2 inch; Beckman Coulter GmbH): (i) 5 hamster brains were used instead of 20 as starting material (ii) new polycarbonate vials were used at each step of the purification procedure involving centrifugation (iii) the complete resuspension of the pellets by ultrasound was carried out in glass instead of polycarbonate tubes:

- 5g TSE-infected brains (5 hamster brains) were rinsed once with 4-5 ml H 8.5 (0.2 mM Tris (Sigma) in double distilled H_2O (ddH_2O), pH 8.5) and transferred into 100 ml thick-walled bottle. The bottle was filled up to 50 ml with 10% sarcosyl (sodium dodecyl sarconate, (Serva)) in H 8.5 and 400 ml 1M NaH_2PO_4 (Merck) were added. The brains were homogenized for 30 sec with an Ultra Turrax tissue homogeniser. The homogenate was incubated at room temperature for 30 min, when a few drops of n-octanol (Merck) were added to reduce the persisting foam. The suspension was filtered through a funnel with 4 layers of cheesecloth (lint) and poured in 2 polycarbonate vials (closed with aluminium caps prior to centrifugation).

The human samples were prepared as 10% w/v homogenates, according to their individual weight (see table 3.V).

- The 10% homogenate was centrifuged at 16 000g (12 500 rpm) for 15 min, at 20°C using a 60 Ti-rotor in a Beckman L8-70M ultracentrifuge (Beckman).

- The supernatants (S_{16}) were carefully transferred with a disposal pipette and a vacuum pump in a 50 ml beaker, leaving behind about 1 ml of fluid, a pellet and some lipid material to be discarded. EDTA (4 ml of 0.1M Titriplex III, Sigma) at pH 7.6 were added and mixed properly with the supernatant before being poured in 2 new polycarbonate vials. The supernatant was underlied with 1 ml of 20% sucrose (Sigma) in ddH_2O using a syringe with a needle whose sharp end was covered with a 10 µl tip eppendorf (long size) (Figure 2.1), to avoid scratching of the polycarbonate wall.

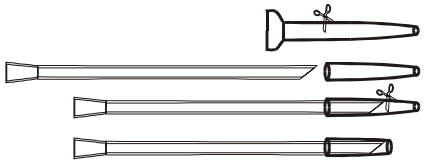

Figure 2.1 Schematic view of the needle protected with a plastic disposable tip.

- S_{16} supernatant was centrifuged at 145 000g (45 000 rpm) for 90 min, at 20°C using a 60 Ti-rotor (Beckman).

- The supernatants were carefully withdrawn with a disposable pipette. Each vial was rinsed once from top to pellet with 1 ml of 0.02M Tris/HCl pH 8.5. Then the pellets (P_{145a}) were roughly resuspended in 1 ml of 0.1% Zwittergent 3,14 (Fluka) (Z3,14; Myristyl sulfobetaine 3-14) in 0.02M Tris/HCl pH 8.5 (Z3,14/Tris) and transferred into a thick-walled glass tube. The vials were rinsed four times with 1 ml of Z3,14/Tris and the suspension was also transferred into the glass tube. The pellet was fully resuspended by sonication applied carefully several times for 3-5 sec using the low power (20 W) of the ultrasonic device (Micro Ultrasonic Cell Disrupter, Kontes). The two pellets were transferred in a single polycarbonate tube, followed by rinsing the glass tube several times with Z3,14/Tris, and filling the centrifuging vial up to 25 ml with Z3,14/Tris. The vial was underlaid with 1.5 ml of 20% sucrose as described above.

- The suspended P_{145a} pellet was centrifuged for 60 min, 145 000 g (45 000 rpm), at 20°C using a 60 Ti-rotor.

- The supernatant was withdrawn with a disposable pipette. The P_{145b} pellet was resuspended roughly, transferred into a glass tube and fully resuspended by ultrasound as described for the P_{145a} pellet, then transferred into a polycarbonate vial and underlaid with 1.5 ml of 20% sucrose.

- The suspended P_{145b} pellet was centrifuged for 60 min, 145 000g (45 000 rpm), at 20°C in a 60 Ti-rotor.

- The supernatant was discarded as described above. The P_{145c} pellet was resuspended in 500 µl of H8.5 and transferred into a glass tube. The vial was rinsed twice with 500 µl

of Tris pH 8.5 and the rinsing volumes were also transferred. 3 µl of 0.5 M $MgCl_2$ (Roth), 2.5 µl RNAse A (2.5µg) (Qiagen), 2.5 µl Benzonase (2.5 E) (Fluka), were added. The mixture was continuously stirred (Teflon-coated magnet) overnight.

- On the next morning 2.5 µl (2.5 µg) of Proteinase K were added and the suspension was stirred for 1 h at room temperature. Then 100 µl of 0.1 M EDTA (pH 7.6) were added and the suspension was stirred for other 15 min. The enzymes-treated suspension was transferred into Ultra-Clear tubes (Beckman) rinsing the glass tube with several volumes of H8.5. The rinsing mixture was added to the main samples as described above. The Ultra-Clear tube was filled with H8.5 and underlaid with 0.5 ml of 20% sucrose.
- The suspension was centrifuged at 200 000 g (40 000 rpm) for 120 min, at 4°C using a TH 641-rotor (Sorvall).
- The supernatant was removed. The pellet was carefully washed once with 200 µl H8.5 then resuspended in 1 ml H8.5 and divided on 2, 1 and 0.5 brain equivalents (BE) i.e. 2, 1, or 0.5 g of the original amount of brain tissue in the sample, in conical Beckman centrifugation tubes, filled up to 1 ml with H8.5. The proteins extracted from the human brains were divided on gram equivalents, according the original weight of brain tissue. For simplicity the human samples were also designated as brain equivalents (BE)
- The fractioned brain equivalents were centrifuged for 60 min at 125 000 g (45 000 rpm) at 4°C in a TL 45-rotor Beckman in a Beckman TL-100 ultracentrifuge.
- The supernatants were completely discarded. The pellets were carefully dried with thin strips of filter paper and stored at -20°C until use.

2.3 Protein analysis

For protein analysis 0.5 BE of the finally purified protein were resuspended in 50 µl sampling buffer (4% (w/v) sodium dodecyl sulfate (SDS) (Serva), 10% (v/v) 2-mercaptoethanol in 120 mM Tris-HCl, pH 6.8, containing 20% (w/v) glycerol (Roth) and 0.05% (w/v) bromphenol blue) and denatured by 5 min incubation of the sample in a boiling water bath. The solution contained 0.1 BE/10 µl and was used as a stock for the preparation of serial dilutions (also in sample buffer) for detection of PrP27-30 and determination of the protein quantity and purity.

2.3.1 Protein quantification

The protein quantity was determined by amido black staining (Neuhoff *et al.*, 1979; Nakamura *et al.*, 1985) of the finally purified PrP27-30 fractions as described (Beekes *et al.*, 1995). The protein samples were blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Watford, UK) using a 96-well vacuum dot blot chamber (Bio-Dot Microfiltration

Apparatus, Bio-Rad). Each well was filled with 400 µl methanol (Roth). 10 µl from the investigated protein samples representing $0.3x10^{-1}$, 10^{-2}, $0.6x10^{-2}$ and $0.3x10^{-2}$ BE were assayed in triplicate. Bovine serum albumin (BSA) and a molecular weight (MW) marker (Pharmacia) were diluted and boiled for 5 min in sample buffer. 1-10 µl corresponding to 20-200 ng protein, respectively, were assayed in duplicate.

The protein samples were blotted on the membrane and consequently stained for 15 min with 5 mg/ml amido black solution (Merck) in methanol. The excess dye was eluated by washing the membrane two times for 5 min in 10% acetic acid (Sigma) in methanol. Then, the membrane was dried at room temperature. When the membrane was completely dried, a black-and-white image was generated by laser scan of the membrane. The intensity of the stained protein spots was evaluated with densitometry software (WinCam, Cybertech, Berlin, Germany). The protein quantities of the analyzed samples were extrapolated to the standard curves produced from the intensities of the BSA and the MW marker samples as described elsewhere (Beekes *et al.,* 1995).

2.3.2 Polyacrylamide gel electrophoresis

The purity of the purified protein fractions was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. PrP27-30 was detected by SDS-PAGE followed by western blotting and immunostaining with 3F4 mAb. The samples were run on 12.5% minigels (Mini-protein II Dual Slab Cell, Bio-Rad) following a SDS-PAGE protocol, based on a discontinuous buffer system (Laemmli 1970). In this system, proteins denatured in the presence of SDS and 2-mercaptoethanol as thiol reducing agent acquired a rod-like shape and a uniform charge-to-mass ratio proportional to their molecular weights. Proteins separate according to their molecular weight, making this system extremely useful for molecular weight determination (Laemmli 1970). The molecular weight of the proteins in the finally purified pellet was determined in respect to standard molecular weight (MW) markers (Pharmacia).

2.3.2.1 Silver staining

The gels were silver stained according to Oakley's procedure (Oakley *et al.,* 1980), briefly described below:

- The gel was incubated for 30 min in 50% methanol and 7.5% acetic acid (Sigma).
- 30 min in 5% methanol and 7.5% acetic acid in ddH_2O.

- 30 min in freshly prepared 5% glutaraldehyde (Fluka).
- The gel was rinsed once in ddH_2O and incubated in water overnight at 4°C.
- 15 min incubation in freshly prepared solution of silver nitrate. 0.4 g $AgNO_3$ (Merck) was solved in 2 ml ddH_2O and added dropwise in solution of 10.5 ml 0.36% NaOH (Merck), 0.7 ml 25% NH_4OH (Merck) in 36.7 ml ddH_2O, under discontinuously stirring.
- The gel was washed two times (each in a new glass plate) for 2 min in ddH_2O.
- Then in another new glass plate the gel was developed for 1-5 min in a fresh solution of 100 µl 5% citric acid (Sigma) and 100 µl 37% formaldehyde (Roth) in 100 ml ddH_2O.
- The reaction was stopped by incubation for 10 min in 5% acetic acid.
- The gel was rinsed two times for 2 min in ddH_2O.
- The gel was incubated 30 min in 10% glycerine and 20 % ethanol and then dried in a gel dryer (Roth).

2.3.2.2 Immunostaining of PrP27-30

For the detection of PrP27-30 the proteins was transferred onto PVDF membrane by Western blotting for 20 min at 200 mA using semidry blotting (Burnette 1981; Tovey *et al.*, 1987) (Fast-Blot B 34, Biometra). PrP27-30 was detected with 3F4 monoclonal antibody (mAb) in combination with CDP-Star reagent (Applied Biosystems). 3F4 mAb detects amino acid residues 109-112 of PrP from human and hamster origin (Kascsak *et al.*, 1987; Rogers *et al.*, 1991). CDP-Star is a 1,2-dioxetane, which produces a luminescent signal when activated by alkaline phosphatase (AP). The AP dephosphorylates the substrates and yields anions that ultimately decompose, resulting in light emission.

The immunostaining was carried out according to the following procedure:

- The blot membrane was incubated 2 h in 3% (w/v) solution of skimmed milk in TBST (TBS (10 mM Tris-HCl pH 7.4, 133 mM NaCl) with 0.05% (w/v) Tween20 (Fluka)) at room temperature.
- The membrane was incubated overnight with 3F4 mAb 1:20 000 (w/v) in 3% skimmed milk in TBST at 4°C
- The membrane was washed shortly three times with TBST and three times for 5 min in 3% skimmed milk in TBST.

- The membrane was incubated for 90 min at room temperature with the second antibody (AP Anti Maus IgG from cattle (Dako, Hamburg) diluted 1:10 000 in 3% skimmed milk in TBST
- The membrane was washed shortly three times with TBST then two times for 5 min and ones for 1 h in 3% skimmed milk in TBST.
- The membrane was incubated 2x5 min in Assay buffer (1M Tris)
- The membrane was partially dried between two sheets of filter paper.
- The membrane, still wet, was placed on a glass plate, soaked with 5 ml CDP-Star 1:50 diluted with Assay buffer and incubated for 5 min.
- The CDP-Star solution was removed by drying the membrane between two sheets of filter paper.
- The membrane was placed in a membrane folder and stored for 1 h into a development cassette
- Kodak XAR-2 Film was exposed to the membrane for 10 min.

Some human samples were analyzed using 6H4 mAb (Prionics, Zurich) and ICSM18 (D-Gen Ltd., London, UK) mAb, which recognizes human PrP residues 144–152 (Korth *et al.*, 1999) and 143-153 (Jackson *et al.*, 1999), respectively. The immunostaining was performed following the procedure described above but (i) in 0.02% casein solution in TBST instead of 3% skimmed milk solution in TBST, when 6H4 mAb was used; (ii) in TBST buffer when ICSM 18 mAb was used, respectively. As a positive control an aliquots of PK digested hamster brain homogenate were co-analyzed. The positive control was prepared as follows:

- 10% brain homogenate was prepared in TBS by ultrasound disruption of scrapie 263K infected hamster brain
- 50 µl of the homogenate were incubated under constant shaking for 1 hour at 37°C in the presence of 5 µl of 13% Sarcosyl and 10 µl of 1 mg/ml PK
- 435 µl of sampling buffer were added to the PK digested brain homogenate and incubated for 5 min in a boiling water bath. The resulting solution presents 10^{-4} BE/10 µl (100 ng PrP27-30) and was stored at -20°C until use.

2.4 FT-IR spectroscopy

OPUS 3.0.2 for OS/2 and OPUS versions 3.1, 4.0, and 5.0 for Windows NT (Bruker) software were used for the row data collection and for all spectral manipulations and evaluations. FT-IR spectra of PrP27-30 were collected with a Bruker IFS28 B or Bruker IFS66 FT-IR spectrometers (Bruker Optics GmbH, Germany). Spectral resolution of 4 cm^{-1} was applied. For apodisation a Blackman-Harris 3-Term function was applied and a zero filling factor of 4 was used, thus encoding the spectra by approximately 1 data point per wavenumber. For each spectrum 128 scans were co-added and averaged. Transmission/absorption FT-IR spectra were collected between 4000 and 400 wavenumbers and were computer stored, while purging the instrument continuously with dried air to reduce water vapour absorption. FT-IR spectra were collected from dried samples and samples suspended in H_2O or D_2O. PrP27-30 samples suspended in D_2O were used to investigate the effect of the temperature gradient and urea on the prion aggregates.

2.4.1 Measurement of dried samples

The protein samples (half brain equivalents) were centrifuged once in ddH_2O to remove the residual sucrose. The supernatants were discarded and the pellets were resuspended in 0.5-1.5 µl ddH_2O to obtain samples with 10 µg/µl protein content. Aliquots of 0.5-1 µl from these suspensions were transferred to a ZnSe window (Korth Kristalle GmbH, Germany) and dried overnight in a desiccator with silica gel, prior FT-IR measurements. The size of the dried protein film spots was in the order of 1-2 mm. A representative number of spectra from each PrP27-30 spot were collected with an IRScope II equipped with a broad-band dMCT detector. Spectra were collected from measuring points in X and Y direction as well as from randomly selected positions (Figure 2.2 A and B).

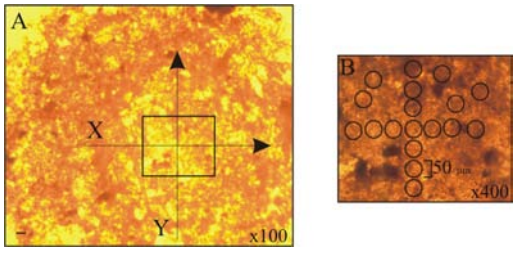

Figure 2.2 Microscopic views of a dried PrP27-30 film spot.

The selected aperture size was 50 µm (designated by circles in Figure 2.2 B). All spectra of a given protein spot with amide I maximum falling within the range of 0.3 and 1.0

absorbance units were averaged. As seen from the microscopic images the PrP27-30 spots were characterized by unequal sample distribution within the dried film. The criteria for choice of the aperture size were the properties of the dried protein film as (i) the heterogeneous sample protein distribution within the dried spot and (ii) the quality changes of the film surface upon drying, which can cause scattering of the IR light; and (iii) the signal intensity which diminished with reduction of the aperture size.

2.4.2 Measurements of hydrated samples

For FT-IR measurements of the samples in D_2O, the proteins from 2 BEs were resuspended and centrifuged twice in Beckman TL-100 ultracentrifuge in a TL 45 rotor at 45,000 rpm for 10 min at 4°C, each time in 1 ml 0.1% Z3,14 in D_2O (Z/D_2O) to obtain a finer protein suspension. H/D exchange was carried out for 1 hour. Aliquots from the supernatants of the second centrifugation were used to collect reference spectra for digital subtraction from the protein/D_2O suspension. The spectra of PrP27-30 were obtained from samples resuspended in 2-6 µl Z/D_2O to obtain final protein concentration of 10 µg/µl. 1.8 µl were transferred to an IR cell constructed from two CaF_2 windows (Korth Kristalle GmbH, Germany), in one of which a cylindrical cavity with approximately 6 µm pathlength was polished (Figure 2.3). The FT-IR spectra were collected in the front measurement channel of an IFS28B FT-IR spectrometer equipped with a DTGS detector.

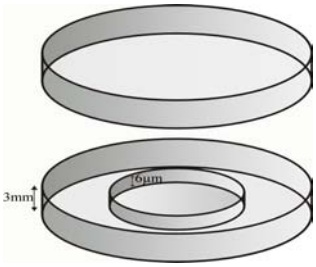

Figure 2.3 IR cell constructed from two CaF_2 windows. The desired pathlength was achieved by a polished cavity in one of the windows.

FT-IR measurements of samples in H_2O were performed as described for the D_2O measurements but 0.1% solution of Zwittergent 3-14 in 0.2M PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) at pH7.2 in H_2O (Z/H_2O) was used instead Z/D_2O for the sample preparation.

2.4.3 Temperature gradient measurements

The effect of different temperatures on the PrP27-30 samples was measured with a Bruker IFS66 FT-IR spectrometer equipped with a DTGS detector. Z/D_2O samples of PrP27-30 were prepared as described above. It is recommended to perform the experiments in D_2O solution since the strong band of the H_2O bending vibration is as well temperature-dependent (Barth *et al.,* 2002). Samples with a final protein concentration of 10 µg/µl were used to investigate the effect of the temperature on the strain specific PrP27-30 aggregates. 3 µl of the suspensions of were measured in a temperature-controlled demountable IR cell with approximately 8 µm pathlength. The samples were hermetically sealed by applying a thin paraffin layer between the two CaF_2 windows. The temperature program was started after 14 hours of incubation of the sample at room temperature. The samples were heated in two subsequent thermo-cycles form 20°C to 90°C and from 90°C back to 20°C by changing the temperature at a constant rate of +/- 1°C/5 min with deviation tolerance of +/-0.05°C, using Haake C25 temperature control equipment (Thermo Electron Corporation).

2.4.4 Spectroscopic and biochemical properties of heated and consequently PK digested PrP27-30 samples

For Western Blot analysis, PrP27-30 samples of 263K (1 BE) were suspended in 100 µl PBS/Z (H_2O) and divided in two parts: 50 µl (0.5 BE) were heated for 30 min at 90°C and then cooled down to 20°C. The heating/cooling cycle was repeated for a second time. For control measurements 50 µl samples were incubated at room temperature for the same time. The heated and control samples were divided in two parts each. One aliquot of these samples was treated with PK (100 µg/ml). The samples containing or not containing PK were incubated for 30 min at 37°C. Then 10 µl of the suspensions were mixed with 10 µl of 2x concentrated sample buffer and boiled for 5 min at 100°C. Aliquots equivalent to $0.5x10^{-4}$ BEs from all samples were analyzed by SDS-PAGE followed by Western blotting and immunostaining with 3F4 mAb as described above. For comparison, an aliquot of 10^{-6} BEs from PK digested, 10% brain homogenate from scrapie 263K animals was analyzed simultaneously.

For FT-IR spectroscopy of samples which had been treated at 90^{o}C and subsequently PK digested, 5 BE from 263K scrapie, were suspended in 500 µl PBS/Z (H_2O). The suspensions were subjected to thermo-cycles and PK digestion as described above. After PK treatment, the suspensions were centrifuged for 5 min at 16 000 g in a Biofuge Pico centrifuge (Heraeus, Germany), and the supernatants were discarded. The protein pellets were then

resuspended in 1 ml Z/D_2O and sampled for FT-IR measurements as described above. For FT-IR microscopy of the dried samples, aliquots of 1 BE suspended in 100 µl PBS/Z in H_2O were used. After treatment at 90°C and subsequent PK digestion, the suspensions were centrifuged and the supernatants discarded. The pellets were resuspended in 1 ml H_2O, and processed for FT-IR microscopy as described above.

2.4.5 Urea induced unfolding of PrP27-30 aggregates

PrP27-30 from 2 BEs were resuspended and centrifuged twice, first in Z/D_2O and than in pure D_2O. The resulting pellet was mixed with 20 µl D_2O and freeze-dried. 20-30 µg of protein were resuspended in 2-3 µl of freshly prepared D_2O solution of 6M deuterated ^{13}C-labeled urea as described by Reinstädler *at al.,* (Reinstädler *et al.,* 1996) to final protein concentration of 10 µg/µl. 1.8 µl were transferred to an IR cell of approximately 6 µm pathlength. The IR cell was hermetically sealed with paraffin as described above. The FT-IR spectra were collected in the front measurement channel of a Bruker IFS28B FT-IR spectrometer equipped with a DTGS detector. The measurements were started half an hour after sample exposition to the denaturant. Spectra were collected at room temperature every 30 min during the first 4 days. Consequently, due to slowing of the intensity changes in time, spectra were collected every 4 hours between the 5-15 days and every 24 hours until the termination of the kinetic study. The use of ^{13}C-labeled urea for the infrared experiments was essential, since it causes the C=O band of urea to shift well below 1600 cm^{-1}, and in this way permits analysis of the amide I band of the protein (Fabian *et al.,* 1995; Reinstädler *et al.,* 1996).

2.4.6 Spectral data processing

2.4.6.1 Spectra subtraction

For the examination of the secondary structure components it is necessary to isolate the protein spectral bands from the spectral bands of the solvent. The spectra subtraction option of the OPUS software allows compensation of the solvent bands without a reference spectrum of precisely the same optical thickness. The spectrum of the sample of interest and the spectrum of the solvent whose spectrum is to be compensated are collected and calculated as absorbance spectra. Compensation was achieved by multiplying the referent spectrum by a factor *k* and subtracting the scaled spectrum from the sample spectrum. The scaled spectrum is calculated such, that

$$(A_1 - kA_2) = 0 \qquad \textbf{Eq. 2.1}$$

for interfering bands, where A_1 is the band absorbance of the sample spectrum and A_2 the absorbance of the interfering band of the reference spectrum.

The protein absorption spectra were obtained after digital correction for Z/D_2O Z/H_2O or 6M ^{13}C urea and water vapour contributions. For the temperature gradient experiments the subtraction for Z/D_2O was done using reference spectra collected at identical temperatures to consider the temperature influence on the IR spectral properties of the solvent (Fabian *et al.*, 2002). Optimal subtraction for Z/D_2O and Z/H_2O was achieved when a flat baseline between 1800-2200 wavenumbers was obtained. The water vapour contributions were subtracted from the protein spectra considering the water vapour contributions in the range 1500-1800 cm^{-1}. The compensation of D_2O and ^{13}C Urea absorptions were performed as described elsewhere (Reinstädler *et al.,* 1996)

2.4.6.2 Averaging

The averaging option of the OPUS software generates an average spectrum from a set of original spectra of the same type. Upon averaging the mean intensity $\bar{y}$ of the n input spectra is calculated:

$$\bar{y} = \frac{\sum_{i=1}^{n} y_i}{n} \qquad \textbf{Eq. 2.2}$$

2.4.6.3 Second derivative

Second derivative spectra of the protein absorption spectra (d^2A/dv^2) were calculated using the Savitzky–Golay algorithm with a 13 smoothing points. This allows a simultaneous smoothing of the spectrum, which reduces the noise generated by the derivatization.

2.4.6.4 Normalization

For visual comparison of the strain specific absorption characteristics, but not the spectra obtained from the temperature gradient or urea induced unfolding studies, the spectra were vector normalized between 1600-1750 wavenumbers or 3240-3330 cm^{-1}, respectively, to obtain similar intensities of the band components. This method calculates the average value of the absorption intensity of each data point y_i in the spectral region of interest, which is then subtracted from the spectrum in a way that a new average value equals zero.

$$x_i = \frac{y_i}{\sqrt{\sum_{i=1}^{n} (y_i)^2}} \qquad \textbf{Eq. 2.3}$$

then the spectra are scaled such, that the sum squared deviation over the indicated wavelengths equals 1:

$$\sum_{i=1}^{n} (x_i)^2 = 1 \qquad \textbf{Eq. 2.4}$$

The intensity and the frequency of each band component of interest were determined using the peak picking options of the OPUS (Bruker) software.