4 Discussion

No differences were found between FT-IR spectra of PrP27-30 samples, purified according to either the modified protocol, or the original one of Diringer *et al* (Diringer *et al.,* 1997), and protein purity and the quality of the materials remained consistent. When using our modified purification protocol protein spectra could be obtained from a minimum amount of brain tissue and the samples were devoid of the IR absorptions of polycarbonate particles. For this reason, only the modified procedure was used to investigate the secondary structure of PrP27-30 from different TSEs in this study.

4.1 Secondary structure characteristics of PrP27-30 from different TSE strains

4.1.1 FT-IR characteristics of PrP27-30 from different laboratories

The TSE strain typing required an easily applicable method able to generate reproducible results. Quite encouraging, common absorption characteristics of the secondary structure components of PrP27-30 from 263K scrapie were found in the spectra presented here with the most pronounced bands in spectra published earlier (Caughey *et al.,* 1991b; Caughey *et al.,* 1998). Namely, the turns plus high frequency β-sheet absorption band at 1671 cm^{-1}, α-helical component at 1656 cm^{-1} and the low frequency β-sheet features at 1637 and 1620 cm^{-1} showed identical (except for the last component) peak positions and band intensities. This implied similarity in the secondary structure of PrP27-30 obtained from the same strain but in different extraction and purification methods. In comparison to previously published data (Caughey *et al.,* 1991a; Caughey *et al.,* 1998), the spectral characteristics of 263K presented in this study differed mainly in the specific features of the intermolecular β-sheet band component. As shown here, it was found at 1625 cm^{-1} and was present at 1626 cm^{-1} in samples suspended in H_2O, as shown by Caughey, at al., (Caughey *et al.,* 1991b) where also the high frequency absorption was centred at 1695 wavenumbers and showed slightly higher intensity than the observed here. More significant differences were observed in the characteristics of the intermolecular hydrogen bond absorption in the spectra obtained from D_2O suspensions. Two components at 1627 and 1619 cm^{-1} were clearly seen in the spectra obtained from samples hydrated in D_2O (Caughey *et al.,* 1991a), whereas the presented results suggested a single main band at 1620-1621 wavenumbers. The possible cause for this dissimilarity might be the use of ultrasound for sample preparation in the above

mentioned literature. Such a treatment might reduce the level of stacked prion rods, favouring the H/D exchange and thus better resolving of the overlapping β-sheet bands. Indications for the existence of different intermolecular β-sheet components were present in the dilution experiments, carried out to examine the effect of the PrP27-30 concentration on the secondary structure characteristic of the prion rods (see section 3.1.5). In the spectra of diluted samples, two β-sheet band components at 1624 and 1619 cm^{-1} were revealed as a consequence of higher H/D exchange as suggested by a characteristic changes in the amide I and II absorption regions. The complex nature of the band component at 1620 cm^{-1} was also suggested by the temperature experiments and urea induced structural changes of PrP27-30 aggregates. These results showed that the component at 1620 cm^{-1} is indeed a complex absorption band and that both PrP27-30 products possess very similar intermolecular hydrogen bonds.

The spectral similarity, observed in spectra from different purification procedures and IR instrumentation, suggests that the application of an optimized and standardized purification procedure, sampling conditions and measuring parameters should be feasible to guarantee a reliable laboratory independent FT-IR based TSE strain typing technique in the future.

4.1.2 Strain-associated spectroscopic differences of PrP27-30 from hamster adapted TSEs

In order to employ the spectral variations between PrP^{Sc} as a tool for TSE strain differentiation the spectral characteristics reflected in the strain specific PrP27-30 absorption spectra were analyzed in details. As already proposed by Bessen and Marsh, 1994 (Bessen *et al.,* 1994), and more detailed discussed recently on the basis of FT-IR data from three scrapie strains (Caughey *et al.,* 1998), the study presented here, with a collection of four different TSE isolates, confirms that PrP^{Sc} from different TSE strains exhibited different and very characteristic protein conformations, expressed as distinct differences in the secondary structure sensitive amide I band components. Although PrP^{Sc} from different isolates is derived from PrP^{C} with the same amino acid sequence, it shows strain dependent conformational diversities, assigned mainly to differences in the β-sheet structure but also to other structural components present in PrP^{Sc} such as turns and α-helices.

Multivariate cluster analysis, on the basis of repetitive measurements of samples purified in independent runs, was used for the fist time in this study to prove objectively that strain specific differences are reflected in the FT-IR second derivative spectra, obtained from dried and hydrated PrP27-30 samples. The absorption characteristics of the amide I region of

PrP27-30 from each sampling technique revealed the strain specific secondary structure characteristics of each particular TSE strain. The FT-IR microscopic measurements of dried prion aggregates allowed to easily obtain spectroscopic information on the gross-composition of the prion rods, such as relative fractions of protein, carbohydrates and lipids. However, it appeared that the PrP27-30 samples dried from H_2O suspensions are not optimally suited for the elucidation of the structural differences between the TSE strains. The band broadening of the spectral patterns as typically observed for dried protein samples suggests that the drying process affects the structure of PrP27-30 aggregates. This may lead to considerable levelling off of the interstrain-specific spectral differences. Safar et al. (Safar *et al.,* 1993b) suggested an intermolecular alignment of external β-sheets, and/or protein secondary structure transitions, when assembling PrP27-30 molecules into the solid state. This may explain the similarity of the PrP27-30 absorption characteristics among the various TSE strains and the lack of two clearly resolved β-sheet band components, which are readily observed in hydrated samples. Purified PrP27-30 hydrated either in H_2O or D_2O showed well resolved spectral features, suggesting the presence of highly ordered secondary structures. This makes such samples more suitable for structural investigations by FT-IR spectroscopy. In addition, D_2O as a solvent creates a region of low absorbance between 1700 and 1600 cm^{-1}, which is an ideal spectral window to investigate the various amide I components of the peptide backbone (Fabian *et al.,* 2000). Some of these amide I band components occurred at lower wavenumbers than in H_2O, due to the downshift effect of the deuterium atoms on the >C=O stretching vibration (Backmann *et al.,* 1996). Obviously H/D exchange-induced small band shifts of nearly all amide I band components of the PrP27-30 depended on the type of the secondary structure involved (Backmann *et al.,* 1996; Barth *et al.,* 2002). The observed differences in the H/D exchange after incubation in D_2O at room temperature are also indicative of structural variations between PrP27-30 from different strains. Most probably strain-specific structural variations give rise to differences in functional groups of the peptide backbone that are exposed to the solvent and/or to different structural flexibilities of sub-structures within the PrP27-30 aggregates. These structural variations are detectable only in partially H/D-exchanged samples and provide additional structural differences between PrP27-30 from different TSE strains.

The FT-IR spectroscopic analyses of hydrated PrP27-30 samples in D_2O gave highly reproducible spectra, which are specific for each examined TSE-strain. Such strain-specific spectra could serve as “fingerprints” for the determination and classification of unknown

TSE-isolates. Nearly all signals in the amide I, II, and A spectral regions exhibited, to a varying degree strain specific features, suggesting that these spectroscopic "traits" can be used for strain typing, best however, by the use of multivariate fingerprinting techniques.

The excellent reproducibility of FT-IR measurements when PrP^{Sc} was passaged in Syrian hamsters suggests stable propagation of the PrP^{Sc} secondary structure during different passages of the TSE strains accompanied by other stable strain characteristics such as incubation time and clinical symptoms (see section 2.1.1). If the self propagation of a particular prion carrying the disease-specific information is defined by the structure of the PrP^{Sc}, the strain properties should be invariable as long as the structure of PrP27-30 is a constant characteristic of the TSE strain. Thus, the secondary structure "fingerprint" of PrP27-30 measured in the infrared spectra could serve as a control for strain identity testing at least for experimental TSEs.

4.1.3 TSE strain differentiation capacity of the FT-IR spectroscopy technique

As shown elsewhere (Thomzig *et al.,* 2004), three of the investigated TSE-isolates (263K, BSE-H and ME7-H or 22A-H) could be distinguished from one another by several biochemical and histochemical methods. However, two of the passaged agents, 22A-H and ME7-H, turned out to cause TSEs with indistinguishable neurological/behavioural clinical symptoms. Both strains showed the same lesion profiles, electrophoretic mobilities or glycosylation patterns of PrP27-30. Even when additional methods for their neuropathological or biochemical differentiation were applied, such as paraffin embedded tissue blot analysis of the distribution of cerebral PrP^{Sc} or Western blot typing of the pathological prion protein after Proteinase K-digestion at different pH values, it was not possible to make a reliable discrimination between ME7-H and 22A-H. The only difference between these TSEs was in their incubation times (331 ± 16 vs. 206 ± 8 days) (Thomzig *et al.,* 2004).

The data obtained from the FT-IR-measurements of PrP27-30 from the examined strains, made possible to achieve reliable discrimination between ME7-H and 22A-H in samples dried or hydrated in H_2O or D_2O. The dried samples showed only subtle variations, which, however, were characteristic for both strains, such as a small difference in the frequency and the width of the β-sheet band components at 1632 and 1631 cm^{-1}, and the α-helices at 1660-1658 cm^{-1} as well as the characteristic weak absorption band at 1644 cm^{-1} in the spectra of 22A-H (Figure 3.13 A). In H_2O or D_2O, both strains differed more clearly since the hydrated samples revealed additional variations in all secondary structures (Figure

3.13 B-D). In D_2O suspension for example, the 22A-H strain showed β-sheet and turn absorption band components at 1630 and 1670 cm^{-1} (see Figure 3.13, Table 3.III), as well as a very characteristic band component at 1642 cm^{-1}, when compared with ME7-H, which exhibits a β-sheet band component at 1634 and a turn or a turn plus a high frequency β-sheet component at 1679 cm^{-1}. Another difference between these two strains can be found in the intensity of the component at 1620 cm^{-1} (more intense and broader for ME7-H) and its proportion to the other secondary structure components. In samples suspended in D_2O significant structural variations between 22A-H and ME7-H strains were observed also in amide A and II absorption regions. Variations in the spectral characteristics used for the discrimination of the four investigated isolates - number of bands, peak positions, intensity, broadness and proportions - are due to the varying extent, orientation and flexibility of the existing secondary structures components.

The strains investigated in this study differed significantly in their incubation time. Thus, the observed structural variations in the spectra could also be due to the different time course of the disease. Therefore, it would be of practical interest to extend the number of the investigated strains to TSE strains that are characterized by similar incubation times, or vice versa, a given strain, at different time points of the disease development. Although the strain-specific conformational diversities may be the molecular basis for TSE-strain variations, it still has to be elucidated, whether different structures in PrP^{Sc}-molecules or other associated factors are responsible for strain-specific pathological phenotypes and whether PrP^{Sc} itself is really the only component of the etiological agent causing TSEs, according to the prion hypothesis (Thomzig *et al.*, 2004). The results from this study, strongly suggest that FT-IR spectroscopy has a significant diagnostic potential for TSE strain differentiation. They show in particular that infrared spectroscopic structural characterization of pathological prion proteins is able to distinguish between PrP27-30 molecules from different TSE agents which is not easily achievable by biochemical PrP^{Sc} typing.

4.1.4 Discrimination of human prion disease and strains of sCJD

4.1.4.1 Biochemical PrP^{Sc} typing

PrP isotypes may account for the phenotypic variations between the different forms of CJD (Parchi *et al.*, 1996). The glycosylation patterns of PrP27-30 from sCJD investigated in the present work showed subtle variations among the samples classified as type 1 or 2 PrP^{Sc}, also in patients with the same PrP genotype and clinical propagation. In general, the

classification of proteinase K resistant PrP isotypes found in the brains of patients suffering from sCJD has proven to be controversial in regard to the possible number and molecular size of the non-glycosylated PrP27-30[3]. Some of the investigated human samples showed two, separate bands of unglycosylated PrP27-30 at 19-21 kDa after PAGE of PK digested PrP^{Sc} samples, suggesting the co-existence of different sCJD strains (Parchi *et al.,* 1996; Hill *et al.,* 2003; Minor *et al.,* 2004). The coexistence of different strains in sCJD has been observed in the different patterns of PrP deposition and severity of the spongiform changes in diseased brain material (Puoti *et al.,* 1999). It was proven also by specific antibody-binding to the N-proximal epitopes of type 1 PrP^{Sc} (cleaved at amino acid 82) but not type 2 PrP^{Sc} (cleaved at amino acids 97 or 86), and by amino acid sequencing of type 1 and type 2 PrP^{Sc} in human prion disease after PK digestion, which revealed the existence of several N-terminal variants in a single human sample (Parchi *et al.,* 2000) Variations in the exact PK digestion site are normally related to conformational variations in PrP^{Sc} (Bessen *et al.,* 1992; Bessen *et al.,* 1994). Thus, the PrP^{Sc} typing results appeared to be an indication for the structural heterogeneity of the PrP27-30 at least in some of the investigated human brain samples.

The controversial lab-internal and lab-external PrP^{Sc} typing of 3 sCJD samples from two patients, samples Bn128, Bn128a and R1181 (see Table 3.IV), introduces additional uncertainty in the stability of the PrP^{Sc} typing based on the electrophoretic mobility of the PrP27-30 glycoforms. A plausible explanation for this confusing result could be the different PAGE systems used to separate the PrP27-30 glycoforms and/or most probably variations in the extraction and purification procedures used in the two different laboratories. In addition, recently published data raise doubts about the biochemical methods available for PrP^{Sc} typing, showing that the 3F4 and several other antibodies binding to type 1 and type 2 PrP^{Sc} do not identify type 1 PrP^{Sc} whenever it is less than 30-40% of the PrP^{Sc} in mixtures of brain homogenates containing both PrP^{Sc} types (Polymenidou *et al.,* 2005).

4.1.4.2 Spectroscopic PrP^{Sc} typing

The results from the strain typing experiments in the hamster model, suggested that the multivariate cluster analysis should be performed on second derivative FT-IR spectra of purified protein samples hydrated in D_2O as an optimal procedure to compare the secondary structure variations of PrP27-30 from different sCJD strains and samples purified from control human brains. The most distinct spectral features observed in the spectra of the sCJD

[3] Ninth Annual Report 2000 Creutzfeldt-Jakob Disease Surveillance in the UK

samples showed a secondary structure composition (see Table 3.V) similar to the one found in a previous investigation of human PrP27-30 preparations i.e. pronounced band components at 1628, 1655-1668 and 1668-1677 cm^{-1} (Aucouturier *et al.*, 1999). Aucouturier et al., have also suggested the presence of three different PrP27-30 structures that is in agreement with the outcomes from the hierarchical cluster analysis performed with the human sCJD samples (Figure 3.32).

However, the hierarchical cluster analysis failed to classify "correctly" spectra generated from samples with the same PrP genotype, PrP^{Sc} type and disease characteristics. This was generally due to the considerable variation in the main secondary structure components of samples with the same CJD characteristics and the secondary structure composition similarities between most of the samples from sCJD patients and control. The dendrograms obtained on the basis of different spectral ranges, showed in general three PrP27-30 "structural groups", which were, however characterized by inconsistencies and misclassifications, including sometimes even misclassified samples obtained from control patients. Indeed, the variations in the outcoming dendrograms strongly resembled the outcomes from the hierarchical cluster analysis in the attempt to classify a PrP27-30 mixture from 263K and ME7-H strains, suggesting that the examined samples are most probably composed from proteins with different structures. As shown (Figure 3.34) the small band component variations observed in the spectra of PrP27-30 mixture (Figure 3.33) result in controversial "objective" classification to 263K or ME7-H strain depending on the frequency range used for the hierarchical cluster analysis. The investigation of PrP27-30 mixture also suggests that a multiple conformations in a single diseased brain would be an obstacle for the objective structural discrimination of such samples. The "correct" strain discrimination should be additionally complicated if the proportions between the structures differ from sample to sample.

Alzheimer's disease (AD), dementia with Lewy bodies (DLB), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease) are chronic neurodegenerative disorders characterized by selective neuronal death and the accumulation of insoluble proteinaceous deposits, such as senile plaques and neurofibrillary tangles in AD and DLB, Lewy bodies in PD, and hyaline- and skein-like inclusion bodies in ALS (Barrachina *et al.*, 2005; Choi *et al.*, 2005). In spite of the abundant spectroscopic data for the presence of amyloid-like aggregates of the protein fractions extracted and purified from brain tissue from patients suffering of different diseases including amyloidosis, it is not

clear whether the spectra do not reflect the absorption characteristics of common protein contaminant/s or not, since there is no direct evidence for the co-relation between the presented spectra with the proteins associated and the diseases described above.

It should be mentioned that several lines of evidence (i) silver stained SDS-PAGEs, (ii) the presence of significant PK-resistant protein fractions in brain samples from individuals suffering from other diseases than sCJD, and (iii) misclassification of samples from sCJD patients and controls, suggested the existence of a common protein "contaminant" in the investigated samples. The silver staining properties of the proteins can differ (Oakley *et al.*, 1980; Rabilloud 1990), therefore it is not clear, whether the human PrP^{Sc} remains invisible after silver-staining or not and whether the reason for this is a low PrP27-30 yield in the finally purified fractions. It could turn out that the observed secondary structure components did not mirror predominantly the secondary structure composition of human prion aggregates, but rather a mixture of residual PK resistant proteins and/or also aggregates, with unknown origin. According to the recent work of Safar and co-workers, the lower amount of PrP27-30 in comparison to the amounts of the protein contaminants in the samples, could be due to the fact that most of the PrP^{Sc} in the frontal cortex of sCJD brains is protease sensitivity (Safar *et al.,* 2005a). The Western blot characteristics of samples before and after PK treatment (see Figure 3.28) of sCJD samples did not show significant variations in the staining intensity between the PrP^{Sc} and PrP27-30. This result indicated that, if present, the protease sensitive PrP^{Sc} fractions should be digested as a result of autoproteolysis prior dissecting a tissue sample for extraction and purification of PrP27-30. The presence of truncated PrP^{Sc} forms in the sCJD samples already before the PK treatment supports this theory (see Figure 3.28 E).

A contaminant common for all protein samples could be the ferritin as suggested by the characteristic silver stained bands at ~20 kDa. The ferritin has been shown to be constantly present in the purified samples from healthy and infected hamster brain tissue (Diringer *et al.*, 1997). However, the possibility should be considered, that the ferritin might be not an "accidental" contaminant in prion rods. As suggested by recently published data, the ferritin facilitates the up take of PrP^{Sc} in the intestine (Mishra *et al.*, 2004). In this line of thoughts the co-localization of PrP^{Sc} with iron (Kühbacher *et al.,* 2005; Wang *et al.,* 2005) and the ferritin deficiency in infected cells (Fernaeus *et al.,* 2005) may open space for speculations about a possible functional and/or structural relation between both proteins.

4.1.4.3 Differences between the experimental and clinical TSEs

The PrP27-30 samples from the 263K strain (see section 3.1.6) investigated here showed that the characteristics of the prion aggregates dominate in the spectra obtained before and after treatment with PK. A comparative FT-IR study of the structure of both PrP^{Sc} and PrP27-30 from different scrapie strains (Caughey *et al.*, 1998), has demonstrated that, in general, the second derivative spectra of the non-PK-treated PrP27-30 preparations of a single strain showed greater heterogeneity in comparison to FT-IR spectra from PK-treated PrP27-30 samples, especially in the regions containing absorption bands from turns and unordered structures. Notwithstanding this variability, the differences in the β-sheet regions of the FT-IR spectra were consistent among the TSE strains in both non-PK treated and PK-treated PrP27-30 samples (Caughey *et al.*, 1998). In contrast, human brain samples not-treated with PK did not show any evidence for the existence of protein aggregates at all. Probably the spectroscopic characteristics of the prion rods are masked from other protein present in these samples.

In contrast to the stable and reproducible data about the secondary structure of the PrP27-30 from hamster-adapted TSEs, the FT-IR spectra from sCJD showed significant and unexpected spectral variations especially of the β-sheet band components. The intra- and intermolecular β-sheet band components were not always clearly resolved in the spectra from the human samples (Figure 3.30), suggesting significant variations between the protein products obtained in the experimental TSE model and the clinical cases. Striking was the lower reproducibility of measurements observed in some of the reproducibility tests performed on samples from one donor purified in independent extraction and purification runs. The lower reproducibility could be due to significant variations in the content of the protein contaminants, different secondary structure of different PrP27-30 aggregates, or from both. Apparently, FT-IR spectra from such samples could not be used for reliable discrimination between strains of sCJD.

The FT-IR approach allowed a successful discrimination of TSEs in the hamster model, where the defined and strictly controlled experimental conditions could be ensured. The present FT-IR analysis of hamster TSEs revealed stable propagation of the structure of PrP27-30 through different passages of the agent. PrP27-30 fractions extracted and purified from hamster-adapted TSE strains show constant glycosylation and FT-IR second derivative spectral patterns. These strain characteristics remain constant between passages of the agent, showing typical incubation times, neuro- and histopathology and therefore can be considered

as “pure” TSE strains. A wealth of data indicates that the propagation of the prions occur by a nucleation-dependent polymerization mechanism (Jones *et al.*, 2005). In the light of the prion hypothesis the co-existence of strains in the human brain samples could be explained by the existence of different PrP^{Sc} conformations which co-propagate in similar proportions. In the hamster model it is obviously possible that only one structure, the most abundant one, is selectively propagated between the passages of the agent. Indeed characteristic changes of the incubation period occurred after separation of co-existing strains in experimental animals, suggesting the possibility that the field isolates often consist of different TSE strains (Kimberlin *et al.*, 1978). Finally in the animal model, the spectrum obtained from a single PrP27-30 preparation represents an average of the PrP27-30 (i) from the whole brain and (ii) of five individual brains. It is entirely possible that the constant secondary structure characteristics of the PrP aggregates observed experimentally in the hamster TSEs are based on this averaging approach, which most possibly statistically levels off existing individual or brain structure-dependent variations in the PrP27-30 conformation.

4.2 The structure of different TSE-strains show a different temperature dependent behaviour

In contrast to previous spectroscopic examinations of dried samples (Safar *et al.*, 1993a) the results obtained here showed that high temperature treatment of PrP27-30 has a significant effect on prion conformation and clearly suggested that dried prions are more resistant to temperature treatment and that the hydration state of prions is critical for their heat stability. The PrP27-30 samples from scrapie strain ME7-H showed significantly higher ratios of H/D exchange at all temperatures than all other strains and, concomitantly, much higher loss of secondary structure. The loss of secondary structure of PrP27-30 as a function of the increasing temperature showed strain-specific transition curves of the secondary structures which are typical of non-cooperative unfolding processes and suggested strain specific mechanisms of temperature induced unfolding and/or denaturation of the prions, independent of the PrP27-30 concentration (data not shown). Interestingly, the intramolecular β-sheets, and the α-helical band components turned out to be the most characteristic spectral differences between the strains.

Differences in temperature-induced structural changes and “residual” H/D-exchange of protons in proteins, resisting deuteration at ambient conditions, can be interpreted in terms of the different stability and flexibility or accessibility of certain secondary structure elements

to the solvent. The additional H/D exchange in the PrP27-30 samples as a function of the increasing temperature is a result of partial unfolding/denaturation of the prion aggregates and/or destabilization of specific secondary structure components. This makes possible the penetration of the solvent (D_2O) to previously inaccessible amide groups of the peptide backbone. The temperature-induced changes in the amide I, II and A absorption regions, observed in this study as a function of temperature, provided clear evidence for the existence of strain-specific structural diversity of PrP27-30 in terms of flexibility, stability, and accessibility of certain sub-structures to H/D-exchange and suggest that the H/D-exchange experiments and temperature dependent measurements of PrP27-30 in D_2O are optimal experimental procedures complementing the strain dependent structural information obtained at room temperature.

4.3 Urea induced unfolding of PrP27-30 aggregates

The urea-induced unfolding of the PrP27-30 aggregates showed characteristic time dependence. Similarly to the temperature induced structural changes, the unfolding of the PrP27-30 at 6 M urea is non-cooperative since each of the secondary structure specific band components, showed individual transition characteristics (Figure 3.25). The second derivative FT-IR spectra obtained at the end of this kinetic study showed that the purified from 263K infected brains aggregates, which persisted a long term exposure to 6 M urea were characterised by residual α-helical and intermolecular β-sheet band components. The transition characteristics and stability of distinct secondary structures are reproducible, suggesting, that they could serve as strain identity markers. Furthermore, it is already known that the PrP^{Sc} from several TSE strains differed in their specific resistance to chemical denaturation (Madec *et al.,* 1997). Thus, FT-IR spectroscopy analysis on the secondary structure changes of PrP27-30 from different strains induced by chemical denaturants could provide additional means and/or alternative approach for discrimination between TSEs.

In agreement with the hierarchical assembly model of amyloids (see section 1.1.4.5), the characteristic band component transitions in the presence of urea suggested an unfolding model in which the intermolecular hydrogen bonds are disrupted prior unfolding/denaturation of single PrP27-30 molecules. The specific transition curves obtained from the urea and the temperature gradient experiments also implicated a different unfolding/denaturation mechanism of the PrP aggregates from 263K exposed to high temperature or urea. The characteristic α-helical band component at 1656 cm^{-1} in the spectra from 263K shows clear

structural rearrangement in the temperature gradient experiments and higher, compared to the other secondary structures, resistance in the presence of 6 M urea. Probably, different secondary structures and/or not yet determined factors that stabilize the protein fold are associated with the unusual resistance of the PrP27-30 aggregates exposed to high temperature or chemical denaturants.

4.4 Protective function of the non protein constituents of prions

The significant decrease in intensity of samples which have been temperature treated and subsequently PK digested (Figure 3.21), suggests, that part of the PrP27-30 samples lack the amino acid residues 109-112 (the epitope recognized by the 3F4 mAb (Kascsak *et al.*, 1987; Rogers *et al.*, 1991), or that part of the PrP27-30 molecules in the aggregate were newly PK digested. Since no changes in the glycosylation pattern of heat-treated and subsequently PK-digested PrP27-30 samples were observed after PAGE analysis, we assume that the relative increase in the IR absorptions between 800 and 1200 cm^{-1} was mainly due to a complex carbohydrate polymer and in the range of 3000-2800 cm^{-1} to lipid constituents of prion rods described elsewhere (Appel *et al.*, 1999; Appel *et al.*, 2001; Riesner 2003). The compositional differences observed between the “control” and the “heat treated and subsequently PK digested” samples indicate that the lipids and carbohydrates in PrP27-30 are integral and heat resistant structural components contributing to the unique properties of prions (at least in the case of 263K strain). However, to what extent and in which way these non-protein components play a protective role in heat resistance or even in protecting the aggregates from PK digestion remains an open question (Appel *et al.*, 2001). Indeed, the presence of lipids and other cellular components was suggested to be important for the resistance of the prion infectivity to autoclaving (Taylor *et al.*, 1998; Appel *et al.*, 2001), and a complex polysaccharide consisting of α-1,4-linked and 1,4,6-branched polyglucose has been discussed to contribute also to the unusual chemical and physical stability of prion rods, acting like a macromolecular “scaffold”. The questions, whether the nature and the relative amount of the non-protein components also exhibit strain specific features and what kind of specific interactions between the prion protein and these components contribute to the stabilization, or even define certain secondary or tertiary structures of PrP27-30, are important issues that have to be addressed in the future.

4.5 Molecular implications of the structural variations among the TSEs

As proposed by Peretz et al. (Peretz *et al.*, 1997), the conversion of PrP^{C} to PrP^{Sc} involves primary structural changes in the N-terminal part of the protein and includes reorganisation of residues 90 to 121 and part of the first α-helix into a β-sheet, while the two C-terminal α-helices remain preserved. When used to explain the structural diversity of PrP27-30 investigated here, this model implicates that depending on the strain, different parts of the first α-helix are unfolded and, due to variations in the length of the unfolded α-helix, the prion protein refolds to different β-sheets formations involved in strain-specific intra- and intermolecular hydrogen bonds. This theory is confirmed by the FT-IR spectra with the individual frequencies of the intramolecular β-sheets and the intensity variations of the intermolecular β-sheet band components.

According to the "protein-only model" of the prion hypothesis, TSE agents replicate through a molecular mechanism in which abnormally folded PrP^{Sc} acts as a catalyst or template nucleus, which recruits cellular prion protein molecules and transforms them into its own "infectious" conformation (Prusiner 1982; Prusiner *et al.*, 1998). If this concept, which has been substantially supported by two recent reports on the *in vitro* generation of infectious scrapie prions by growing amyloid fibrils from recombinant PrP expressed in bacteria (Legname *et al.*, 2004) or by replicating PrP^{Sc} using PMCA (Castilla *et al.*, 2005), is correct, then FT-IR spectral features of the secondary structure of PrP27-30 extracted from brain tissue would represent the replicative strain-specific conformational properties of both infectious "parent" and "offspring" PrP^{Sc} molecules.

This conclusion would not be compromised by the fact, that one infectious unit of the TSE agent has been found associated with more than 10^5 PrP^{Sc} molecules (Prusiner 1982; Beekes *et al.*, 1996). Within the framework of the protein-only-hypothesis, replication of infectivity in acquired TSEs is thought to mechanistically follow a mechanism of seeded aggregation (Glover *et al.*, 1997; Collins *et al.*, 2004; Jones *et al.*, 2005), and under such conditions, the specific biological infectivity of PrP^{Sc} can be expected - according to theoretical considerations (Masel *et al.*, 2001) - to depend on the size distribution of PrP^{Sc} aggregates. A given amount of PrP^{Sc} may form a higher or a lower number of infectious particles (i.e. seeds for PrP misfolding), depending on the size of the aggregates in which it is present (Silveira *et al.*, 2005). On the other hand, smaller particles may be less stable and

more susceptible to clearance. Thus, the ratio of PrP^{Sc} molecules associated with one infectious dose of biological infectivity may vary substantially under the influence of several different factors. At the molecular level, one infectious unit of a given TSE isolate may therefore theoretically correspond to 10^5 or more PrP^{Sc} molecules that all share conformational replicon elements in their strain-specific secondary structure. A potential approach to address this question, would be by performing an up-scaled PMCA (Bieschke *et al.,* 2004; Castilla *et al.,* 2005) with PrP^{Sc} from different TSE strains. Subsequently, the conformation of the PrP^{Sc} used for seeding and the "offspring" PrP^{Sc} should be probed by FT-IR analysis and bioassay for both, their secondary structure and strain-specific infectivity.

4.6 Structural constraints from FT-IR spectroscopy for model building of PrP27-30

X-ray scattering studies showed that the morphology and structural properties of amyloid fibrils from different origin, including TSE, are remarkably similar (Nguyen *et al.,* 1995; Sunde *et al.,* 1997b; Jimenez *et al.,* 2002). These protein aggregates are generally believed to consist of protofilaments, constructed by juxtaposing two or more cross-β units (Sunde *et al.,* 1997a; Khurana *et al.,* 2003; Tycko 2004). An alternative structural model for prion rods, which describes PrP^{Sc} as a parallel β-helical structure has recently been proposed (Wille *et al.,* 2002; Govaerts *et al.,* 2004). Although no unique spectral signature diagnostic for a parallel β-helix structure can be expected in the infrared spectra, recent FT-IR spectroscopic investigations of proteins known to be β-helical (Khurana *et al.,* 2000) leave some space for speculations about a β-helical fold as the characteristic structural feature of PrP^{Sc}

The FT-IR experiments give strong evidence for the presence of two fundamentally different β-sheet structures in PrP27-30. We propose that (i) the FT-IR component band between 1630 and 1637 cm^{-1} corresponds to an intramolecular β-sheet structure and (ii) the component bands between 1620 and 1626 cm^{-1} reflect intermolecular β-sheets. This assignment is based on the fact that the component bands between 1620 and 1626 cm^{-1} clearly respond to the hydration state and H/D-exchange, while the band between 1630 and 1637 cm^{-1}, as well as the α-helical band at 1656 cm^{-1}, is hot affected by H/D-exchange. Dilution experiments in D_2O suggested that this intermolecular β-sheet specific band is a complex band composed of two components evidencing two structurally different intermolecular β-sheet contacts that clearly differ in accessibility to H/D-exchange.

The most intriguing aspect of the temperature stability of PrP27-30 is the reversible narrowing or broadening of the intermolecular β-sheet specific IR band at 1620 cm^{-1} (Figure 3.17), which is accompanied by a shift to higher or lower wavenumbers when elevating or decreasing the temperature in the second temperature cycle. This temperature dependent behaviour most probably indicates the coexistence of two different intermolecular β-sheet structures in PrP27-30, responding differently to high temperatures. In the spectra obtained from PrP27-30 at low concentrations and in the temperature treated and subsequently PK-digested PrP27-30 fractions these two structural components are better resolved (Figure 3.10 and Figure 3.22). The underlying two intermolecular β-sheet structures thus are characterized by distinct strengths of intermolecular hydrogen bonds and different stability/flexibility, with the lower frequency component being reversibly unfolded at 90°C, while the higher frequency component is not. Both components, however, are not accessible to H/D-exchange even at high temperatures. These conclusions clearly indicate that PrP27-30 is a fairly complex, aggregate structure in which significant portions of the secondary structure persist even at 90°C and are not accessible to H/D-exchange while others are reversibly unfolded.

4.7 FT-IR characteristics of isolated PrP27-30 versus prions in infected tissue or recombinant $SHaPrP^{90-232}$

The spectra of dried PrP27-30 isolated from scrapie infected hamster brains exhibit specific low-frequency β-sheet bands centered around 1630 cm^{-1} (Figure 3.13 A), with a main peak component at 1634 cm^{-1} and a shoulder at 1627 cm^{-1}. At 1694 cm^{-1} a high-frequency β-sheet band can be observed, which is usually taken as a diagnostic peak for antiparallel pleated β-sheets. The intense band observed at 1659 cm^{-1} is most probably due to α-helical structures. The increased intensity at 1637 cm^{-1} in the spectra of ganglion cells is typical for intramolecular β-sheet structures in proteins rather than for intermolecular β-sheets, and the appearance of a new β-sheet band at 1631 cm^{-1} indicates β-sheets with similarly strong hydrogen bonds as observed for the purified PrP27-30 isolated from scrapie 263K-infected hamster brains (Kneipp *et al.*, 2004b; Kneipp *et al.*, 2004a).

The method for *in situ* detection of prion aggregates (Kneipp *et al.*, 2000; Kneipp *et al.*, 2002) is potentially promising also for TSE strain typing. However one should consider the higher spectral similarity between the spectra obtained from various TSEs from dried PrP27-30 samples (Figure 3.13 A) and the fact that the FT-IR spectra obtained from tissue sections consist not only of PrP^{Sc} but also of other proteins (Kneipp *et al.*, 2004b; Kneipp *et*

al., 2004a). Therefore, such samples should be analyzed by FT-IR microscopy after hydration in D_2O or at high temperatures. As shown in this study, hydrated samples should help to better discern the spectroscopic features of the prions. Due to H/D exchange the low frequency β-sheets were apparently better resolved, than samples hydrated or dried from H_2O. Significant secondary structure of the PrP27-30 aggregates was present at 90°C in D_2O samples with various concentrations. Dried prions ware even less affected by high temperature as shown elsewhere (Safar *et al.,* 1993a).

The FT-IR spectra published so far and that are typical of the β-forms of recombinant PrP obtained from the predominantly α-helical prion protein for *in vitro* produced prion fibrils or oligomers (Sokolowski *et al.,* 2003; Baskakov *et al.,* 2005) clearly show, that there is still no clear evidence available for identical or even only similar secondary structures between the various β-rich prion isoforms obtained *in vitro* and *ex vivo* (Sokolowski *et al.,* 2005). The spectral features, especially at 1620 and 1691 cm^{-1} (Sokolowski *et al.,* 2003), 1622 and 1693 cm^{-1} (Baskakov *et al.,* 2005) are obviously very different from those of dried PrP27-30 (see Figure 3.13 A) and from PrP27-30 dispersed in H_2O (Figure 3.13 B), suggesting that the *in vitro* and *ex vivo* prion protein structures are substantially different. This is best reflected in the very prominent and specific α-helical infrared band around 1658 cm^{-1} and the intramolecular β-sheet band at 1636 cm^{-1} of PrP27-30, which have no counterparts in the so far *in vitro* produced prion isoforms. The high-frequency band at 1693 cm^{-1} is much less intensive for PrP27-30 and the extremely shifted low-frequency β-band of the recombinant $SHaPrP^{90-232}$ indicates a much stronger, most probably intermolecular hydrogen bonding in the β-form of the recombinant protein (Sokolowski *et al.,* 2003; Kneipp *et al.,* 2004b). The direct comparison of the β-form of recombinant prion proteins and purified PrP27-30 shows that there is still no evidence available for any particular secondary structure similarity between the β-sheet-rich prion proteins obtained in vitro and ex vivo (Kneipp *et al.,* 2004a; Sokolowski *et al.,* 2005).

4.8 Conclusions

The main aim of the present work was to develop a fast, robust and specific method for TSEs strain differentiation. The present results clearly show that the FT-IR technique can be used for a reliable discrimination of the four hamster adapted TSE agents analyzed. As demonstrated here, PrP27-30 can be extracted and purified from minimum amounts of hamster brain tissue according to the slightly modified procedures, originally developed by Prof. Diringer and co-workers (see section 2.2). PrP27-30 samples from four TSE strains adapted to Syrian hamster were differentiated on the basis of their secondary structure variations, reflected in the structure sensitive amide I region. As shown for the first time, these structural variations can be objectively proven by multivariate cluster analysis applied to the second derivative FT-IR spectra from various PrP27-30 samples. The comparative investigation of PrP27-30 as dried samples or samples hydrated in H_2O and D_2O, demonstrate that the structural diversity can be best detected in samples suspended in D_2O. The partly H/D exchange samples revealed novel spectroscopic evidences for differences between the TSE strains present in the amide II and amide A absorption regions, which can be also used to discriminated between the TSE strains.

The structural differences between the strain specific PrP27-30 aggregates could be reproducibly propagated between passages of the agent and proved to be sufficient for strain discrimination. Furthermore, the FT-IR technique turned out to be superior to already existing biochemical methods for PrP^{Sc} typing, which failed to differentiate between the strains ME7-H and 22A-H. The investigation of the biological properties of TSE isolates in experimental animals is currently the only reliable method of TSEs strain typing. Direct comparisons of the animal assay with the FT-IR approach developed here, showed that the latter is also able to discriminate with a high precision between the four strains adapted to Syrian hamster. Furthermore, the FT-IR approach provided the strain typing results with significant time- and cost- efficiency.

The prion rods are complex formations in respect to structure and chemical composition, and apparently multiple factors are responsible for the unique thermal stability of the prions. PrP27-30 samples from 263K ME7-H and 22A-H heated up to 90°C showed strain-specific secondary structure transitions and characteristic thermal stability of the different secondary structures. Thus the temperature gradient measurements of PrP27-30 suspended in D_2O could be applied as an optimal experimental procedure to complement the strain dependent structural information obtained at room temperature.

Discussion

The present results strongly suggest that FT-IR spectroscopy has a significant diagnostic potential for TSE strain differentiation. However, the impurity and/or the PrP27-30 structural heterogeneity in the PK resistant pellets obtained from the human brain samples hampered the objective strain discrimination in the case of sCJD. Thus, a "final" evaluation of the ability of the FT-IR technique to discriminate human TSEs can most probably be performed by investigating highly purified PrP27-30 fractions. Obviously the purification procedure used in this study requires an optimization in respect to the reduction of residual protein contaminants. The main contaminants observed in purified PrP27-30 samples are ferritin and an unknown protein with a molecular mass of ~17 kDa. The latter appeared to be partially digested by PK in samples subjected to PK treatment. The partial degradation of the 17 kDa protein could be explained with insufficient enzyme activity. Therefore, it seems that this contaminant could be completely removed by the use of higher PK concentrations (or by applying an additional PK digestion step) in the purification procedure. Getting rid of the ferritin, which showed only some PK resistance, would require more sophisticated methods for protein separation. This protein can form aggregates of approximately 500 000 kDa (Arosio *et al.,* 1978) and a simple step of size dependent separation could result in the loss of small-sized amyloid aggregates as well. More suitable for the removal of ferritin would thus be a separation step based on the magnetic beads technology[4] or immunoprecipitation with a ferritin specific antibody. The efficacy and the applicability of the proposed changes remain to be established in the future.

The results presented in this work lead to the necessary of further additional series of experiments in order to establish the applicability of the FT-IR spectroscopy technique for TSEs strain differentiation as well as the exploration of the molecular base of the structural variations and stability of the PrP27-30 aggregates and their relation to the strain properties of the TSE agent. These still open issues could be addressed by the following experiments:

- Extending the number of hamster-adapted TSE strains investigated by FT-IR spectroscopy. Examination of the influence of the disease duration on the secondary structure of the PrP27-30 aggregates. Comparison of the secondary structure characteristics of PrP27-30 samples extracted and purified from different brain regions;
- Examination of the strain characteristics of temperature treated and consequently PK digested PrP27-30 samples;

[4] http://biophoton.snu.ac.kr/paper43.pdf

Discussion

- Investigation of the urea induced unfolding/denaturation of PrP27-30 from different TSE strains. Examination of the secondary structure and chemical composition of prion aggregates persisting prolonged exposure to 6M urea;
- Adaptation of the PrP27-30 extraction and purification procedure to human brain material;
- Investigation of PrP27-30 from hamster with different PrP genotype.