Strain-dependent Differences in β-Sheet Conformations of Abnormal Prion Protein*

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Strain diversity in the transmissible spongiform encephalopathies (TSEs) has been proposed to be determined by variations in the conformation of the abnormal, protease-resistant form of prion protein (PrP-res). We have investigated whether infection of hamsters with three TSE strains resulted in the formation of PrP-res with different conformations using limited protease K (PK) digestion and infrared spectroscopy. PrP-res isolated from the brains of hamsters infected with the hyper (HY), drowsy (DY), and 263K TSE strains yielded similar SDS-polyacrylamide gel electrophoresis profiles prior to PK treatment. However, after limited digestion with PK, the PrP-res from the DY strain exhibited a fragmentation pattern that was distinct from that of the other two strains. Infrared spectra of HY and 263K PrP-res each had major absorption bands in the amide I region at 1626 and 1636 cm⁻¹ both prior to and after digestion with PK. These bands were not evident in the DY PrP-res spectra, which had a unique band at 1629–1630 cm⁻¹ and stronger band intensity at both 1616 and 1694–1695 cm⁻¹. Because absorbances from 1616 to 1636 cm⁻¹ of protein infrared spectra are attributed primarily to β-sheet structures, these findings indicate that the conformations of HY and 263K PrP-res differ from DY PrP-res at least in structural regions with β-sheet secondary structure. These results support the hypothesis that strain-specific PrP-res conformers can self-propagate by converting the normal prion protein to the abnormal conformers that induce phenotypically distinct TSE diseases.

The transmissible spongiform encephalopathies (TSEs) or prion diseases are characterized in part by the accumulation of the abnormal protease-resistant isoform of prion protein (PrP-res), primarily in the brain (for review see Ref. 1). PrP-res is an amyloidogenic protein that is post-translationally derived from the normal protease-sensitive prion protein (PrP-sen) (3, 4). The properties of PrP-res that distinguish it from PrP-sen include the formation of fibrils both in vivo and in vitro (5–8), insolubility in nondenaturing detergents (9), and a high β-sheet secondary structure composition (10, 11). The TSEs, like classical amyloid diseases, can have a sporadic or genetic (linked to mutations and insertions in the PrP gene) etiology, but the TSEs can also be infectious (for review see Ref. 1). Strong circumstantial evidence supports the hypothesis that PrP-res is the major component of the infectious agent, but direct evidence for this is lacking.

The existence of TSE strains presents a major challenge to the protein-only hypothesis for the TSE agent. Although differences in the host PrP genotype can influence the phenotype of TSE disease for a single TSE strain, TSE strains also contain host-independent information and can be discriminated within a single host species or PrP genotype by reproducible differences in incubation period, clinical signs, vacuolar pathology, and PrP-res deposition (for review see Ref. 12). The basis of strain diversity among conventional pathogens is determined by variations in their nucleic acid genome, but no TSE agent-specific nucleic acid has been identified despite extensive searches. There is evidence suggesting that TSE strain propagation is determined by the self-propagation of PrP-res molecules that differ in conformation, polymeric states, and/or ligand associations (13–15). However, the molecular nature of the strain-dependent differences in PrP-res is poorly understood.

Strain-specific properties of PrP-res were first seen among murine scrapie strains that differed in the relative proportions of PrP-res glycoforms (i.e. PrP molecules with zero, one, or two N-linked glycans) (16, 17). Evidence for the existence of multiple conformations of PrP-res has come from observations that PrP-res associated with different strains of TSEs are cleaved at different N-terminal sites by protease K (PK). This was first documented with the hyper (HY) and drowsy (DY) strains of transmissible mink encephalopathy (TME) passaged in Syrian golden hamsters (13, 18). The HY and DY strains of TME can be serially propagated in hamsters, giving rise to distinct incubation periods, clinical symptoms and brain patterns of PrP-res deposition (19). After treatment with PK, the PrP-res from HY-infected hamsters had a ~2-kDa larger molecular mass than PrP-res from DY-infected hamsters in SDS-PAGE analysis. This size shift was not due to additional glycosylation because it was observed in both unglycosylated and glycosylated PrP-res molecules. N-terminal sequencing revealed that PK cleaved additional residues from the N terminus of DY PrP-res compared with HY PrP-res. Because the DY and HY PrP-res molecules are derived from the same Syrian hamster PrP-sen precursor, it was concluded that the observed difference in cleavage by PK was due to strain-dependent differences in conformation and/or ligand binding.

Evidence that the two strain-associated conformations of hamster PrP-res could self-propagate from the same hamster PrP-sen was obtained using a cell-free conversion reaction (14). In these studies, HY and DY PrP-res were each incubated with

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3. The abbreviations used are: TSE, transmissible spongiform encephalopathy; PrP-res, protease-resistant prion protein; PrP-sen, protease-sensitive prion protein; PK, protease K; TME, transmissible mink encephalopathy; HY, hyper; DY, drowsy; FTIR, Fourier transform infrared; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
The cell was then assembled and adjusted to a 0.1% sulfobetaine in PBS. SDS-PAGE, silver staining, and immunoblotting analysis of the PrP-res fractions were performed as described previously (13, 29, 30).

Perkin-Elmer system 2000 FTIR instrument equipped with a TGS detector and water vapor subtraction was factored to minimize water vapor bands in the infrared spectra. The buffer spectrum subtraction was factored previously by comparing the integrated areas of the negative spectra (10, 27) from the brains of hamsters in the clinical phase of disease after intracerebral inoculation with the designated TSE strain. At least three separate isolates of each type of PrP-res were analyzed by PTIR. To obtain PK-treated PrP-res, 100 µl of p4 fraction of PrP-res (1 mg/ml in 0.1% sulfobetaine in PBS (130 mM NaCl, 10 mM sodium phosphate, pH 7.4)) was diluted to 500 µl in 50 mM Tris-HCl, pH 8, 150 mM NaCl and digested with 5–10 µg/ml PK for 1 h at 37 °C. PefablocSM (Boehringer Mannheim) was then added to 1 mM to inhibit PK. To remove the PK and PefablocSM, the PrP-res aggregates were washed three times by centrifugation at 12,000 × g for 20 min at 4 °C and resuspension in 0.1% sulfobetaine in PBS. SDS-PAGE, silver-staining, and immunoblotting analysis of the PrP-res fractions were performed as described previously (10, 28).

**FTIR Spectroscopy**—PrP-res pellets containing 100–200 µg of protein were suspended in 8 µl of 0.1% sulfobetaine in PBS and spotted onto a CaF2 window of a variable pathlength infrared cell (International Crystal Labs). The cell was then assembled and adjusted to a ~6-µm pathlength. After purging of the sample compartment with dried air to reduce water vapor contributions, spectra were collected at 23 °C in a Perkin-Elmer system 2000 FTIR instrument equipped with a TGS detector. Spectral parameters: 54 scans; 4 cm⁻¹ resolution; 0.2 cm/s optical path difference velocity; Kaiser-Bessel apodization. Protein spectra were obtained by sequential digital subtraction of the buffer and water vapor spectra. The buffer spectrum subtraction was factored to flatten the baseline between 1800 and 2300 cm⁻¹. The water vapor subtraction was factored to minimize water vapor bands in the infrared spectra. The spectra were subjected to a nine-point Savitsky-Golay smoothing. Estimation of secondary structure compositions was done as described previously (10, 28).

**RESULTS**

**SDS-PAGE Analysis of PrP-res Preparations**—PrP-res was purified from the brains of hamsters infected with the HY, DY, and 263K TSE strains by detergent extraction and differential ultracentrifugation. Analysis by SDS-PAGE followed by silver staining or immunoblotting indicated that the three major bands were of similar size among the TSE strains prior to PK treatment. However, PK treatment of the DY PrP-res generated three resistant PrP core fragments in the 19–29-kDa range that migrated slightly faster (~2 kDa lower in apparent molecular mass) than the corresponding PK-treated HY and 263K PrP-res bands (Fig. 1). The observed difference between DY and HY PrP-res was most obvious when the PK-treated DY and HY PrP-res was most obvious when the PK-treated HY and DY PrP-res was most obvious when the PK-treated HY and DY PrP-res samples were deglycosylated with N-deglycosylase (PNGase) (25). Molecular mass markers are designated in kDa on the left of the panels.

**Conformational Variations in PrP-res**

These studies have examined the absorbance in the protein amide I region, an area sensitive to differences in protein secondary structure, and demonstrated that PrP-sen has a low β-sheet content compared with PrP-res from 263K scrapie-infected hamsters. In the present study, we have compared the conformations of PrP-res in the HY, DY, and 263K hamster TSE strains and found striking differences in secondary structures. These results provide evidence that differences in PrP-res conformation characterize TSE strain diversity at the molecular level.
4–6), indicating that it is derived primarily from the C-terminal portion of PrP. This low molecular mass band was not detected on immunoblots developed with the C-terminal antibody when the PrP-res was deglycosylated, indicating that these C-terminal fragments contained N-linked glycans and that without them, the polypeptide fragments were too small (<~6 kDa) to resolve on the gel. The observation that the PK-treated HY and 263K PrP-res fragments migrated similarly to one another in SDS-PAGE but differently from the DY PrP-res fragments suggested that the DY PrP-res conformation differed from the other two.

**FTIR Analyses**—To further compare the conformations of these different types of hamster PrP-res, we analyzed multiple PK-treated and non-PK-treated PrP-res preparations from each strain by FTIR. Transmission FTIR spectra of the PrP-res preparations were obtained after sequential subtraction of buffer and water vapor spectra. Representative primary spectra are shown in Fig. 2. To better resolve the absorbance bands comprising the primary spectra, deconvolution (Fig. 3) and second derivative calculations (Fig. 4) were performed. In second derivative spectra, negative bands were obtained that correspond in wave number to positive absorbance maxima in the primary or deconvoluted spectra. The reproducibilities of the spectra are shown by overlaying second derivative spectra from independent preparations. Using this approach, the secondary structure of PrP-res molecules derived from the same PrP-sen primary sequence but having distinct conformations were distinguished. The region of protein FTIR spectra that is most informative regarding conformation is the amide I region from ~1600–1700 cm\(^{-1}\), which is dominated by the absorption of the carbonyls in amino acyl linkages of the polypeptide backbone. The frequency of the absorption of a given carbonyl is modulated by the secondary structure in which it resides (for reviews see Refs. 30 and 31). The FTIR spectra of the PK-treated 263K and HY PrP-res were similar; major bands were found at 1626, 1636, and 1657 cm\(^{-1}\) that were visible as absorbance maxima or shoulders in the primary spectra but were emphasized as positive absorbance maxima in the deconvoluted spectra (Fig. 3) and as negative bands in the second derivative spectra (Fig. 4). The bands at 1626 and 1636 cm\(^{-1}\) are indicative of \(\beta\)-sheet, and the 1657 cm\(^{-1}\) band is generally assigned to \(\alpha\)-helix. Another \(\beta\)-sheet band, at 1694–1695 cm\(^{-1}\), was more intense in the HY spectra than in the 263K spectra.

Comparison of the PK-treated HY and 263K PrP-res spectra to those of PK-treated DY PrP-res indicated a striking and consistent difference in \(\beta\)-sheet absorbances (Figs. 2–4); the peak bands at 1626 and 1636 cm\(^{-1}\) characteristic of HY and 263K PrP-res were not present in the DY spectra, but prominent bands at 1616, 1629–1630, and 1694–1695 cm\(^{-1}\) were observed. The 1629-cm\(^{-1}\) band was restricted to the DY spectrum, but the 1616- and 1695-cm\(^{-1}\) bands were also present at lower intensity in the HY spectra. Because absorbances in the 1616–1636 cm\(^{-1}\) region of the protein infrared spectrum are usually attributed to \(\beta\)-sheets (30, 31), these results suggest that the strain-dependent conformational differences are associated with \(\beta\)-sheet secondary structures. This difference in conformation likely involves most, if not all, of the \(\beta\)-sheet secondary structures, because the bands at 1626 or 1636 cm\(^{-1}\) of HY and 263K PrP-res were not evident in the deconvoluted or second derivative DY PrP-res spectra, and conversely, there was no evidence of the 1629 cm\(^{-1}\) band in the HY or 263K PrP-res spectra. These spectral differences provide physical evidence that DY PrP-res has a distinct conformation from HY and 263K PrP-res even though they are derived from PrP-sen molecules with identical primary structures.

The second derivative spectra of the non-PK-treated PrP-
secondary conformational difference was intrinsic to the PrP-res samples both before and after PK treatment.

Secondary Structure Compositions—Secondary structure content of PrP-res was estimated by comparing the relative integrated intensities of bands in the second derivative spectra (10, 30). The secondary structure compositions of HY, DY, and 263K PrP-res preparations both before and after PK treatment indicated that each had a high \( \beta \)-sheet content (~50% before PK treatment and ~61% after) relative to \( \alpha \)-helix (~12% before PK treatment and ~19% after) (Table I). PK treatment decreased the amount of turn and undefined secondary structure and increased the relative proportions of both \( \beta \)-sheet and \( \alpha \)-helix. No significant differences in secondary structure composition were observed among the PrP-res preparations from the different TSE strains. These results provide physical evidence that DY PrP-res differs from HY and 263K PrP-res in the character rather than the proportion of \( \beta \)-sheet structures.

DISCUSSION

TSE Strain-dependent Conformational Diversity of PrP-res—In this study we used infrared spectroscopy to demonstrate that types of PrP-res associated with distinct hamster TSE strains can have different conformations even though they are derived from PrP-sen with the same amino acid sequence. The HY and 263K PrP-res spectra were nearly indistinguishable but differed from DY PrP-res, principally in absorbances related to \( \beta \)-sheet structures. As reported previously, DY PrP-res had a distinct pattern of PK cleavage among the strain-associated PrP-res molecules in the 19–30-kDa size range (13), but we now report that an additional glycosylated ~7-kDa fragment from near the C terminus of PrP-res was also found at higher levels in DY PrP-res compared with HY or 263K PrP-res after PK treatment. These distinguishing physical and biochemical properties of DY PrP-res could encode the distinct phenotypic properties of DY TME, which is characterized by incubation periods of 6 months, a progressive lethargy, and prominent PrP-res deposits along white matter tracts in the brain (18). Conversely, the similarity of the FTIR spectra and PK cleavage patterns of PrP-res between the HY and 263K strains were consistent with this alternative strain-associated conformation of PrP-res having a role in determining the biological properties of these shorter incubation TSE pathogens that cause hyperexcitability, ataxia, and a widespread distribution of PrP-res in the brain gray matter (18). Based on our findings, we propose that HY TME is an independent reisolation of the well studied 263K scrapie strain and, more importantly, that the clinicopathological phenotypes of these hamster TSE strains are causally linked to conformational differences in PrP-res.

Nature of Conformational Difference—The FTIR spectrum of DY PrP-res differed most prominently from those of HY and 263K PrP-res in the region that is typically dominated by \( \beta \)-sheet absorbances of proteins. The difference was in the type rather than the proportion of \( \beta \)-sheet because HY and 263K PrP-res were characterized by major bands at 1626 and 1636 cm\(^{-1} \), and DY PrP-res was characterized by an absorbance band at ~1629 cm\(^{-1} \), whereas the percentage of \( \beta \)-sheet was similar for each strain-associated PrP-res (Table I). The frequencies of absorption of \( \beta \)-sheet carbonyls can be modulated by several factors, including strength/length of hydrogen-bonding, distortions of sheet structure, solvent exposure, and the dielectric constant of the immediate environment (32). Thus, DY PrP-res may be unique in the pattern of intramolecular or intermolecular hydrogen bonds linking \( \beta \)-strands or in the exposure of its \( \beta \)-sheet structures to the aqueous medium or a strain-specific ligand(s).

Other differences among the TSE strains in the FTIR spectra
assigned to β-sheet were found at 1616 and 1695 cm⁻¹; these bands were more intense in the DY spectra compared with HY and 263K PrP-res, and in each case, the intensity of these bands was proportional within a given spectrum. Increases in the intensities of bands with similar wave numbers has been observed in proteins that have been induced to aggregate by thermal denaturation and have been ascribed to intermolecular antiparallel β-sheets in protein aggregates (for review see Ref. 30). The presence of these bands is consistent with the aggregated nature of PrP-res; however, the fact that these bands were usually lacking in 263K PrP-res spectra indicates that these structural features associated with the 1616 and 1695 cm⁻¹ bands are not present in all types of PrP-res. Furthermore, we have observed prominent bands at 1616 and 1690 cm⁻¹ (but not within the region from 1626–1636 cm⁻¹) in aggregates of recombinant PrP expressed in Escherichia coli that is PK-sensitive and noninfectious,² indicating that the associated conformational features are not restricted to PK-resistant and TSE-associated forms of PrP.

The fact that PK treatment reduced the intensities of FTIR absorbance bands associated with disordered and turn structures is consistent with previous demonstrations that the N-terminal portion of PrP-res molecules removed by PK consists of undefined and extended/turn secondary structures (33, 34). The observation that PK digestion did not eliminate the β-sheet bands or alter their wave numbers (1616–1636 and 1695 cm⁻¹) indicates that the structures responsible for these bands are in the PK-resistant C-terminal part of the molecule. **Mechanistic Considerations and TSE Strain Typing by FTIR**—Although the high resolution structure of PrP-res is not known, it is clear that it has more β-sheet content than PrP-sen (10, 11, 13) and is usually, if not always, found in ordered polymeric structures such as amyloid fibrils or plaques (5–8). Furthermore, in vitro studies have demonstrated that PrP-res aggregates can induce the conversion of PrP-sen to PrP-res by a mechanism that results in the inclusion of the conversion product in the PrP-res aggregate, as is the case with amyloid formation by other proteins (35–38). This has been hypothesized to be a nucleated polymerization mechanism whereby pre-existing PrP-res polymers or oligomers acts as seeds for the recruitment and conformational conversion of PrP-sen into additional PrP-res (35). Our previous demonstration that HY and DY PrP-res induce the conversion of hamster PrP-sen to strain-specific conversion products in vitro led us to propose that different self-propagating conformers and/or packing arrangements of PrP-res seeds could act as different TSE agent strains in the same host species (14). The present study provides additional support for this concept by showing the first direct spectroscopic evidence that PrP-res can adopt different strain-associated secondary structures from the same PrP-sen molecule. The ability of FTIR to detect these conformational differences in PrP-res suggests that it may be a rapid method for TSE strain typing.

**Potential Influences of Different PrP-res Conformations on TSE Pathogenesis**—The existence of distinct strain-dependent conformations of PrP-res of a given amino acid sequence is consistent with the hypothesis that conformational diversity of PrP-res could account at least in part for TSE strain diversity (13, 14). These different conformations may affect interactions of PrP-res with PrP-sen and/or other factors in host cells in a way that differentially affects pathogenesis. For instance, differences in the stability, targeting, self-propagating activities, or toxicities of HY and DY PrP-res could affect their rates and sites of accumulation in the brain, their neuropathic effects, and ultimately, the incubation periods and clinical manifestations of their respective infections. This view must be tempered by the uncertainty as to whether PrP-res is, in and of itself, the infectious agent and whether conformational variation of PrP-res is a cause or an effect of TSE strain diversity in vivo. Nonetheless the present results, by demonstrating that different strain-associated conformers of PrP-res exist, is consistent with the view that the self-propagation of conformational variants of PrP-res plays a role in TSE strain diversity.

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