Infrared Microscopy Detects Protein Misfolding Cyclic Amplification (PMCA)-induced Conformational Alterations in Hamster Scrapie Progeny Seeds*

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**Background:** It is currently under discussion whether protein misfolding cyclic amplification (PMCA) alters strain properties of prions.

**Results:** An improved infrared microspectroscopic approach combined with biochemical and bioassay data revealed altered strain properties of hamster 263K scrapie prions due to PMCA.

**Conclusion:** PMCA can alter strain properties of 263K prions.

**Significance:** Our analytical approach may help to improve the understanding of prion strain conversion.

The self-replicative conformation of misfolded prion proteins (PrP) is considered a major determinant for the seeding activity, infectiousness, and strain characteristics of prions in different host species. Prion-associated seeding activity, which converts cellular prion protein (PrPC) into Proteinase K-resistant, infectious PrP particles (PrP<sup>TSE</sup>), can be monitored in vitro by protein misfolding cyclic amplification (PMCA). Thus, PMCA has been established as a valuable analytical tool in prion research. Currently, however, it is under discussion whether prion strain characteristics are preserved during PMCA when parent seeds are amplified in PrP<sup>C</sup> substrate from the identical host species. Here, we report on the comparative structural analysis of parent and progeny (PMCA-derived) PrP seeds by an improved approach of sensitive infrared microspectroscopy. Infrared microspectroscopy revealed that PMCA of native hamster 263K scrapie seeds in hamster PrP<sup>C</sup> substrate caused conformational alterations in progeny seeds that were accompanied by an altered resistance to Proteinase K, higher sedimentation velocities in gradient ultracentrifugations, and a longer incubation time in animal bioassays. When these progeny seeds were propagated in hamsters, misfolded PrP from brain extracts of these animals showed mixed spectroscopic and biochemical properties from both parental and progeny seeds. Thus, strain modifications of 263K prions induced by PMCA seem to have been partially reversed when PMCA products were re inoculated into the original host species.

Prions represent a class of infectious agents that are thought to transfer strain characteristics based on their different protein conformations and not by nucleic acids (as do bacteria or viruses) (1–3). Once cellular prion protein (PrP<sup>C</sup>) is converted into a β-sheet-rich misfolded and aggregated isoform (PrP<sup>TSE</sup>), further propagation of PrP<sup>TSE</sup> by seeded polymerization multiplies PrP<sup>TSE</sup> seeds and finally can cause fatal neurodegenerative diseases in mammals (transmissible spongiform encephalopathies (TSEs) (4)). Several different co-factors possibly modulate the seeding event, but bacterially expressed and in vitro misfolded PrP has been shown to induce disease in reporter animals also in the absence of any seed-associated co-factors (5–8). A classification of different prion strains can be achieved by bioassay typing in vivo, by in vitro approaches using protein misfolding cyclic amplification (PMCA), and by biochemical strain typing (9–11). Spectroscopic approaches, in particular when just minute amounts of protein are available, have been shown to be powerful tools for the direct structural typing of PrP<sup>TSE</sup> (12–15). Caughey et al. (16) demonstrated in a pioneering study the possibility to structurally discriminate different prion strains by Fourier transform-infrared (FTIR) spectroscopy. Such strain typing is important because different conformational arrangements of prions (although they share identical amino acid sequence) can correlate with different specific infectivities and clinical features.

Here we present a technical advance that enabled us to obtain spectra by infrared microspectroscopy (IR-MSP) from prion protein extracts of as little as about 2.8 ng. This unprecedented sensitivity allowed not only the analysis of brain-derived PrP<sub>27-30</sub> (the Proteinase K (PK)-resistant core of PrP<sup>TSE</sup>)
Infrared Microspectroscopy on PMCA-derived Prion Protein

extracted from small amounts of starting tissue but also the investigation of minute amounts of PMCA-derived PK-resistant prion protein (PrPres).

For secondary structure analysis of proteins by FTIR spectroscopy, the amide I band is the most useful infrared absorption band and originates essentially from C=O stretching vibrations of amide groups of the protein backbone (17–19).

PMCA has been established as a valuable analytical tool in prion research that mimics the conversion of PrPC into PrP\(^\text{TSE}\) in vitro (20). It is currently unclear whether strain characteristics are preserved during PMCA when parent seeds are amplified in PrPC substrate from the identical host species (21). Along with others, Gonzalez-Montalban et al. (22, 23) reported on the change of PrP\(^\text{TSE}\) properties during PMCA. To further test these findings of altered functional properties of prions caused by PMCA, we characterized the following samples at a structural level by IR-MSP as well as in biochemical assays and hamster bioassays: (i) 263K-brain, prion isoform in the brain of 263K-infected Syrian golden hamsters; (ii) 263K-PMCA, prion isoform produced by 263K-seeded PMCA; and (iii) 263K-PMCA-brain, prion isoform in the brain of Syrian golden hamsters that had been intracerebrally inoculated with 263K-PMCA.

Our spectroscopic, biochemical, and bioassay data support the notion that structural alterations of prions can occur during PMCA and additionally show that such alterations can be partially propagated in bioassay animals. However, our findings suggest that such conformational alterations may be also reversed, at least partially, upon repassage into the original host species.

EXPERIMENTAL PROCEDURES

Animals, TSE Agents, and PrP\(^\text{C}\) Substrates—Hamster scrapie strain 263K was originally provided by R. H. Kimberlin (24) and has been serially passaged for many years in our laboratory. All surgery was performed under Ketavet/Rompun anesthesia, and all efforts were made to minimize suffering. Animal experiments were conducted under the European directive regarding the protection of animals used for experimental and other scientific purposes in strict accordance with the German Animal Welfare Act (Tierschutzgesetz) and adhering to the guidelines for the practical implementation of the German Animal Welfare Act published by the Charité-University Medicine Berlin (a joint institution of the Free University of Berlin and the Humboldt-University of Berlin, Germany). Protocols for the intracerebral inoculation of hamsters with scrapie prions were reviewed and approved by the responsible Committee on the Ethics of Animal Experiments (“Tiererversuchskommission-Berlin”) affiliated with the Authority for Animal Protection in Berlin (“Landesamt für Gesundheit und Soziales Berlin”; permit number G0073/07).

The sacrifice of normal Syrian golden hamsters for the preparation of PrP\(^\text{C}\) substrate from hamster brains did not require approval by animal protection institutions or ethic committees, according to German regulations. However, the sacrifice of these hamsters by exposure to CO\(_2\) for our study was reported to and registered by the responsible authority in Germany (Landesamt für Gesundheit und Soziales Berlin, Berlin, Germany; registration number T0220/07).

Intracerebral Inoculation of Hamsters—Hamsters were intracerebrally inoculated with PrP\(^\text{TSE}\), seeding active PrPres produced by PMCA or control samples in a volume of 50 \(\mu\)l (diluted in PBS). The following samples were used for inoculation. (i) \(1 \times 10^{-6}\) g of homogenized brain tissue from 263K scrapie hamsters was used as positive control. (ii) \(5 \times 10^{-9}\) g of 263K-brain tissue was used as a negative control. This represented the residual amount of 263K seeding material after seven rounds of PMCA when the reaction had been started with \(1 \times 10^{-12}\) g of brain tissue of 263K-infected hamsters (see below). (iii) Seeding active PrPres produced by PMCA (using normal hamster brain as substrate) in an amount equivalent to that present in \(1 \times 10^{-6}\) g of 263K-infected brain tissue (see sample (i)). To obtain this inoculum, seven rounds of PMCA were performed with a starting seed of \(1 \times 10^{-12}\) g of brain tissue of a 263K-infected hamster. (iv) The PMCA product obtained after seven rounds of PMCA with an unseeded reaction was used as a negative PMCA control.

Hamsters were regularly observed for clinical symptoms and euthanized by inhalation of CO\(_2\) when they showed pronounced health problems (due to TSE or not related to prion infection) or after asymptomatic survival for an observation period of 513 days post-infection (dpi).

Paraffin-embedded Tissue (PET) Blotting—PET blotting was performed as described previously (25) with midsagittal brain sections.

Polycrylamide Gel Electrophoresis (PAGE) and Western Blot Typing of Misfolded Prion Protein—Samples were subjected to PAGE and Western blotted as described elsewhere (26).

Silver Staining of Polycrylamide Gels—Silver staining was performed as described elsewhere (27).

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed by Proteome Factory (Proteome Factory AG, Berlin, Germany). For isoelectric focusing (IEF), 4 times the amount of PrP\(^\text{S}\) to 263K-brain shown in Fig. 1B, either alone or mixed with 10 \(\mu\)g of IEF protein marker (IEF Marker 3-10 (Serva)), was loaded onto IEF gels. Test samples containing PrP\(^\text{S}\) had been purified according to the protocol for FTIR analysis. The protein solution was applied to vertical rod gels (9 \(\mu\)murea, 4% acrylamide, 0.3% PPA, 5% glycerol, 0.06% TEMED, 2% carrier ampholytes (pH 2–11), 0.02% ammonium persulfate) for isoelectric focusing at 8820 V-h in the first dimension. After focusing, the IEF gels were incubated in equilibration buffer, containing 125 mM trisphosphate (pH 6.8, 40% glycerol, 65 mM DTT, and 3% SDS for 10 min and subsequently frozen at –80°C. The second dimension SDS-polyacrylamide gels (7 \(\times\) 8 \(\times\) 0.1 cm) contained 375 mM Tris-HCl buffer (pH 8.8), 12% acrylamide, 0.2% bisacrylamide, 0.1% SDS, and 0.03% TEMED. After thawing, the equilibrated IEF gels were immediately applied to SDS-polyacrylamide gels. Electrophoresis was performed using 150 V for 75 min until the front reached the end of the gel. After two-dimensional gel electrophoresis separation, the gels were stained with silver (Proteome Factory).

Protease K Stability Assay—For analyzing the stability of brain- and PMCA-derived 263K-associated PrP to protease digestion, different concentrations of PK were used. 10\(^{-4}\) g of
Irradiated Microspectroscopy on PMCA-derived Prion Protein

brain tissue or equal amounts of PMCA products, in terms of PrPres levels, were adjusted to a final volume of 50 μl with PBS. 1.0% sarkosyl and 0.06% SDS were present as well as PK (stock 1 mg/ml; Roche Applied Science) in concentrations of 75, 400, 600, and 700 μg/ml, respectively. Samples were incubated for 1 h at 55 °C. Reactions were stopped with 2 mM PMSF, and samples were centrifuged at 13,000 × g for 1 min. 37.5 μl of supernatant was mixed with 12.5 μl of sample buffer (4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol in 120 mM Tris-HCl, pH 6.8, containing 20% (w/v) glycerol and 0.05% (v/v) bromphenol blue). Samples were boiled for 10 min, and 10 μl were loaded on SDS gels. Monoclonal antibody 3F4 (28) was used for prion detection by Western blot. When brain tissue of infected animals was tested for the presence of PrP 

Densitometry was performed using the open source software ImageJ (National Institutes of Health) as described previously (26). For each Western blot, the strongest triplet PrP signal was set to 100%.

Sedimentation Velocity Assay Using Iodixanol Gradients—Sedimentation velocity analysis of PrP particles was performed as described by Tixador et al. (29) with the modifications described below. This centrifugation method, basically a zonal or gradient centrifugation, separated proteins based on their sedimentation coefficient. Accordingly, the sedimentation velocity of PrP assemblies primarily depended on their mass and shape (i.e. particles with a higher mass sedimented faster than particles with a lower mass (similar shape), and more compact particles sedimented faster than particles with a larger shape (similar mass)).

Ultracentrifugations were performed with a swinging bucket TLS-55 rotor (Beckman Coulter) and a 2-ml 12–33% (w/v) iodixanol gradient in 2.2-ml thin wall polyallomer tubes (Beckman Coulter). Ultracentrifugation in the absence of protein sample was performed for 47 min at 55,000 rpm at 4 °C to build up iodixanol gradients. Subsequently, 10 μl of 10% brain (w/v) homogenates or PMCA products containing equal amounts of PrPres were carefully loaded on the top of the gradient, and centrifugation was repeated under identical conditions. 80-μl fractions were collected under constant flow from the bottom of ultracentrifugation tubes after puncture with a syringe needle using a peristaltic pump. The density of gradients was recorded in each run by refractometric analysis of a similarly processed and collected iodixanol gradient that had not been loaded with PrP. For Western blot analysis, collected gradient fractions were digested with 100 μg/ml PK for 1 h at 37 °C. For sedimentation velocity analysis of PrP27-30 extracts that had been prepared according to the protocol for FTIR analysis with or without additional sonication, the same ultracentrifugation protocol was used. However, here a PK digestion of collected gradient fractions was omitted because these samples had already been treated with PK during the purification procedure.

PMCA—PMCA was performed as previously described (26). PMCA experiments using hamster-adapted 263K as seed were started with 10⁻⁶ g of homogenized brain tissue in 140 μl of normal 10% hamster brain homogenate. Samples were sonicated for 40 s every 60 min and passed in a 1:5 ratio in fresh normal 10% hamster brain homogenate. Aliquots were digested with 150 μg/ml PK for 1 h at 55 °C. All PMCA products were detected by mAb 3F4.

Extraction of PrP27-30 from Brain Tissue and of PrPres from PMCA Reactions for IR-MSP Analysis—Extraction of PrP27-30 from brain tissue (263K-brain, 263K-PMCA-brain) was based on a protocol by Diringer et al. (30) modified by Thomzig et al. (12). We further customized this protocol for the extraction of 263K-brain and 263K-PMCA-brain from only 100 mg of homogenized brain tissue and for the extraction of PrPres from 263K-PMCA.

In brief, Buffer H (200 μl of 1 M Tris-HCl in 1 liter of double-distilled H₂O (ddH₂O)) and buffer T (20 mM Tris-HCl) were adjusted to pH 7.5 for all preparations. 100 μg of brain tissue were incubated as starting material were adjusted to a final volume of 2 ml with homogenization buffer (10% (v/v) sarkosyl in buffer H containing 8 mM Na₂HPO₄) and sonicated. For the purification of PrPres after PMCA, 1 ml of PMCA product (from the third round of PMCA, as described above) was adjusted to a final volume of 2 ml with homogenization buffer and centrifuged at 3000 × g for 1 min at room temperature. Supernatant was transferred to a new vial and again centrifuged as before. Subsequently, supernatant or samples derived from brain tissue were incubated for 30 min at room temperature in homogenization buffer. 1 ml of each was transferred to an ultracentrifugation vial (1.5-ml Microfuge Tube Polyallomer, Beckman) and centrifuged at 16,000 rpm (TLA-55 rotor) for 8 min at 20 °C. Supernatants were removed from pellets and pooled before adding 160 μl of 0.1 M EDTA (pH 7.6). After mixing, solutions were filtered in 1-ml portions into new vials. Each 1-ml sample was underlaid with 40 μl of 20% (w/v) sucrose and centrifuged at 55,000 rpm (TLA-55 rotor) for 52 min at 20 °C. Supernatants were removed from pellets and pooled before adding 160 μl of 0.1 M EDTA (pH 7.6). After mixing, solutions were filtered in 1-ml portions into new vials. Each 1-ml sample was underlaid with 40 μl of 20% (w/v) sucrose and centrifuged at 55,000 rpm (TLA-55 rotor) for 52 min at 20 °C. Supernatants were decanted, and each pellet was resuspended in 30 μl of buffer T containing 0.1% (v/v) Z3.14 (Zwittergent) and pooled. The pooled sample was adjusted to a final volume of 2 ml with buffer T containing 0.1% (v/v) Z3.14 and sonicated. Subsequently, the sample was dispersed in two 1-ml portions into new vials. After mixing, samples were underlaid with 60 μl of 20% (w/v) sucrose and centrifuged at 55,000 rpm (TLA-55 rotor) for 35 min at 20 °C. Supernatants were decanted, and each pellet was resuspended in 30 μl of buffer H and pooled. The pooled sample was adjusted to a final volume of 2 ml with buffer H and sonicated. Subsequently, the sample was dispersed in two 1-ml portions into new vials. After mixing, samples were underlaid with 60 μl of 20% (w/v) sucrose and centrifuged at 55,000 rpm (TLA-55 rotor) for 35 min at 20 °C. Supernatants were decanted, and pellets were resuspended in 50 μl of buffer H each and pooled. 200 μl of buffer T were added, and the sample was sonicated. 0.6 μl of 0.5 M MgCl₂, 0.5 μl of RNase A solution (100 mg/ml; Qiagen), and 0.5 μl of Benzonase (Fluka) containing 2.5 units were added, and the sample was incubated overnight at room temperature on a shaking device.

The following day, 2 μl of PK of a stock solution (1 mg/ml in ddH₂O) were added, and the sample was incubated for 1 h at room temperature on a shaking device. 80 μl of 0.1 M EDTA in ddH₂O (pH 7.6) were added, and the sample was incubated for 15 min at room temperature. The sample was dispersed in equal parts into two new vials, and buffer H was added to a final
volume of 1 ml/vial. After mixing, samples were underlaid with 40 μl of 20% (w/v) sucrose and centrifuged at 55,000 rpm (TLA-55 rotor) for 66 min at 4 °C. Supernatants were decanted, and pellets were resuspended in 100 μl of buffer H each, pooled, and subsequently sonicated for further dispersion. The resulting suspensions (total volume 200 μl) were transferred, in 25-μl aliquots, into new vials, and 975 μl of buffer H were added. After mixing, suspensions were centrifuged at 55,000 rpm (TLA 55 rotor) for 2 h at 4 °C. Supernatants were decanted, and pellets were stored at −80 °C until analysis. For IR-MSP spectroscopy, PrP27-30 pellets were thawed, resuspended in 1 ml of ddH2O, and centrifuged at 55,000 rpm (TLA-55 rotor) for 2 h at 4 °C. Supernatants were decanted, and pellets were again resuspended in 1 ml of ddH2O and centrifuged at 55,000 rpm (TLA-55 rotor) for 2 h at 4 °C. Supernatants were decanted, and pellets were resuspended in 5 μl of ddH2O and sonicated in a water bath prior to analysis.

**IR-MSP**—For each prion isoform, three independently prepared protein samples were used for spectroscopy. 1 μl of PrP27-30/PrPres suspensions (corresponding to ~2.5 mg of the starting brain tissue) were transferred for drying onto a CaF2 window of a thickness of 1 mm (Korth Kristalle GmbH, Altenförde, Germany). Infrared microspectroscopic measurements of dried PrP27-30/PrPres extracts were carried out in transmission mode using an IFS 28/B FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany) linked to an IR microscope IRscope II (Bruker). The microscope was equipped with a small sized 100 × 100-μm2 MCT (HgCdTe) detector element and connected to a motor-driven microscope stage. A specially designed Plexiglas chamber allowed purging of the sample compartment by dry air. IR absorbance spectra were recorded using an aperture diameter of 900 μm and a 15× Cassegrain objective with a numerical aperture of 0.4 giving a spatial resolution of ~60 μm. Nominal spectral resolution was 4 cm⁻¹, and a zero filling factor of 4 was applied, giving a point spacing of ~1 cm⁻¹ (31, 32).

For each background and sample spectrum, 512 individual scans were averaged. The co-adding of 512 individual measurement scans was done in order to increase the signal/noise ratio. The signal-averaged, zero-filled interferograms were apodized using a Blackman-Harris three-term apodization function before Fourier transformation. Background spectra were repeatedly acquired before each sample measurement at window positions outside of the PrP27-30/PrPres sample. To further increase the signal/noise ratio and in order to address the aspect of within-sample heterogeneity, ~15 single point absorbance spectra were acquired for each sample. Spectra were corrected for spectral contributions of residual water vapor using an OPUS macro developed in house (OPUS is the data acquisition software package supplied by Bruker). Using OPUS, second derivative spectra were calculated using a nine-smoothing point Savitzki-Golay derivative filter function. This specific number was chosen to increase the signal/noise ratio in order to optimize the balance between the reduction of noise and the loss of subtle spectral information. If not otherwise stated, the derivative spectra were vector-normalized in the wave number region between 1610 and 1700 cm⁻¹.

**Cluster Analysis**—Agglomerative hierarchical cluster analysis was used to objectively assess clustering of the IR microspectra obtained from the different prion isoforms. For details of cluster analysis application, see Helm et al. (30). Hierarchical clustering was performed using the cluster analysis module of OPUS version 5.5 software (Bruker). For cluster analysis, average derivative spectra from three independent preparations were used for each prion isoform. The spectral range was focused on the structure-sensitive amide I region between 1610 and 1700 cm⁻¹. Using this region, interspectral distances were calculated as so-called D values (redefined Pearson’s correlation coefficients), whereas Ward’s algorithm was used for hierarchical clustering.

**Atomic Force Microscopy (AFM)**—Extracted protein samples (see “Extraction of PrP27-30 from Brain Tissue and of PrPres from PMCA Reactions for IR-MSP Analysis”) were diluted with ddH2O to a final protein amount corresponding to ~25 μg of brain tissue. Measurements were performed on a Nano-Wizard II atomic force microscopy system equipped with a TAO stage (IPK Instruments AG). AFM head and stage were mounted on an inverse optical microscope frame (Olympus IX 71). All scans were performed in non-contact mode with commercially available silicon-based AFM probes (resonance frequency, 320 kHz; force constant, 42 newtons/m). Measurements were performed at different positions within a sample. Similar results were obtained for independently prepared samples.

**RESULTS**

**Purification and Preparation of 263K-Brain, 263K-PMCA, and 263K-PMCA-Brain Samples for IR-MSP**—In comparison with previously published approaches for the IR spectroscopic characterization of prion protein isolates, the IR-MSP method offers the advantage of significantly reduced amounts of required sample material. This allowed us to also reduce the amount of brain tissue needed for PrP27-30/PrPres extraction (100 mg versus 5 g) but also had the consequence that our previously published extraction protocol (12) had to be adapted (for details, see “Experimental Procedures”). Based on protein concentration determination by Amido Black staining, the amount of PrP27-30 extracted from a 263K-infected hamster brain (1 g of tissue) by our devised protocol was in the range of 10–30 μg (data not shown).

The modified PrP27-30/PrPres extraction protocol could also be applied to PMCA-derived samples. Because the amount and purity of IR sample material is of utmost importance for correct spectral interpretation, the following control measures were taken. (i) The presence of PrP27-30/PrPres was tested and confirmed by Western blot (Fig. 1A). (ii) The protein samples were subjected to SDS-PAGE using gels with different concentrations of acrylamide (10–20%). Gels were silver-stained afterward, and no detectable contamination by other proteins of higher or lower molecular weight could be found (Fig. 1B). A co-purification of ferritin as described for other purification procedures (mainly for mouse-derived but not for hamster-derived PrP*SE* (33)) was not detectable. (iii) Samples were also analyzed by two-dimensional gel electrophoresis, as shown in Fig. 2. For the detection of proteins other than PrP, we used a standard two-dimensional gel electrophoresis protocol and sil-
ver staining of gels. By this approach, reference proteins (10 μg of IEF Marker 3–10, Serva) that had been added to our PrP27-30 extracts, but no protein contaminations, could be detected in the second dimension gels (Fig. 2). (iv) To eliminate any components putatively present in buffers used for protein extraction, the sample solutions were extensively washed, diluted, and placed on microscope slides for drying at room temperature. Dried samples were then scanned by using AFM for the detection of potential impurities (Fig. 1C). An overview is shown with two enlarged sections demonstrating the appearance of the preparation, which consisted essentially of fibrillar material made up of ultrapure PrP27-30 (A and B and Fig. 2). Identical results were obtained for independently prepared samples. Ultrapure, essentially fibrillar PrPres or PrP27-30 was also identified in extracts from 263K-PMCA and 263K-PMCA-brain, respectively.

The overall picture of these analyses shows that our extracts for IR-MSP consisted of ultrapure (in terms of protein content) and essentially fibrillar PrP27-30/PrPres. For IR-MSP, small amounts of such PrP extracts were transferred onto calcium fluoride (CaF₂) windows. After drying, prion protein particles of different sizes could be visualized by microscopy (Fig. 3).
Infrared Microspectroscopy on PMCA-derived Prion Protein

IR-MSP of 263K-Brain, 263K-PMCA, and 263K-PMCA-Brain Samples—Although all samples of the tested prion isolates had been extensively sonicated before they were loaded on CaF₂ slides (see “Experimental Procedures”), particles of different size and shape were apparent within a dried protein spot (see Fig. 3). To illustrate that the aggregates differ only in size and not in other structural features or in their biochemical composition, and to demonstrate the accuracy and reproducibility of the IR-MSP approach, we exemplarily show spectra obtained from fine grained and dense particles of 263K-brain extracts (Fig. 3). In this figure, intensity-normalized mean spectra from ~20 individual sample spectra per class are indicated with the respective S.D. spectra. Visual inspection of the individual mean IR-MSP data demonstrated significant intensity differences but no major qualitative changes in the amide I region. These conclusions could be corroborated by hierarchical cluster analysis (HCA), a multivariate unsupervised classification technique. HCA was carried out on individual vector-normalized second derivative spectra using exclusively the information content between 1610 and 1700 cm⁻¹.

Subsequent HCA of the amide I patterns of the mean spectra (Fig. 4, blue curves) demonstrates the presence of four band components in the structure-sensitive amide I region at 1626, 1635, 1659, and 1694 cm⁻¹, respectively. Note that maxima in IR absorbance spectra appear as minima in second derivative spectra. These spectra suggest low levels of dissimilarity, possibly a result of insufficient compensation of water vapor absorption, spectral noise, and the between-sample variance.

Spectra obtained from three isolates of the 263K-PMCA isoform (Fig. 4, red traces) demonstrate the presence of amide I band components at 1627, 1658, 1681, and 1694 cm⁻¹, respectively. Compared with spectra of the 263K-brain isoform, the peak at 1635 cm⁻¹ is clearly absent, whereas the between-sample variance is at a similar level. Second derivative IR microspectra obtained from 263K-PMCA-brain preparations (green curves) exhibit a certain level of similarity with spectra of the 263K-brain isoform. Absorption features in the amide I region were found at 1627, 1658, 1681, and 1695 cm⁻¹. The within-sample variance is comparably large. At least in two mean spectra of 263K-PMCA-brain, there are slight indications of the presence of a band component at 1635 cm⁻¹ that was present in spectra of 263K-brain but not in 263K-PMCA (see arrow in Fig. 4/4).

Subsequent HCA of the amide I patterns of the mean spectra was carried out using the information content between 1610 and 1700 cm⁻¹, D values as interspectral distance measures, and Ward’s algorithm as the clustering method. The dendrogram of Fig. 4B illustrates that the spectral between-sample distances of the individual prion isoforms were always smaller compared with the distances between the different PrP isoforms. This demonstrates the high reproducibility of the extraction protocol and the IR-MSP-based detection technique.

As shown by the dendrogram of Fig. 4B, HCA indicates the similarity of amide I band contours in IR microspectra of both 263K-PMCA and 263K-PMCA-brain preparations.
Infrared Microspectroscopy on PMCA-derived Prion Protein

There are indications for the presence of a band component at 1635 cm\(^{-1}\) in the following 263K isoforms: 263K-brain (time-sensitive amide I region) obtained from three independent samples of PMCA-brain (the spectral region between 1485 and 1715 cm\(^{-1}\)). The frequency positions specific for tyrosine residues are indicated at 1515 cm\(^{-1}\) in the spectra of 263K-brain. Peak positions specific for tyrosine residues are "Discussion." See "Discussion." Weak absorption peak indicated.

**TABLE 1**

| Prion protein isoform | β-Sheet (low frequency) | α-Helix* | Turns/loops | β-Sheet (high frequency) |
|-----------------------|-------------------------|---------|-------------|-------------------------|
| 263K-brain            | 1626/1635               | 1659    |             | 1694                    |
| 263K-PMCA             | 1627                    | 1658    | 1681        | 1694                    |
| 263K-PMCA-brain       | 1627/(1635)*            | 1658    | 1681        | 1694                    |

* See "Discussion."  

In parallel, we analyzed the sedimentation velocity of PrP27-30 in the mildly (without glass beads) and extensively (with glass beads) sonicated PrP extracts from 263K-PMCA-brain by ultracentrifugation. These samples were derived from identically extracted ultrapure fibrillar PrP of identical origin and differed only with respect to an additional ultrasonication in the presence of glass beads (i.e. similar to PMCA conditions). Sonication with beads has been shown to efficiently fragment amyloid fibrils of recombinant PrP into smaller particles (34). We found that the FTIR spectra from "mildly" or "extensively" sonicated samples showed no detectable differences (Fig. 5).

**FIGURE 4.** Infrared microspectroscopy and cluster analysis of spectra from PrP27-30/PrPres samples. A, vector-normalized second derivative IR microspectra in the spectral region between 1485 and 1715 cm\(^{-1}\) (includes the conformation-sensitive amide I region) obtained from three independent samples of the following 263K isoforms: 263K-brain (blue), 263K-PMCA (red), and 263K-PMCA-brain (green). The black trace denotes a second derivative spectrum of a control sample (spectrum not normalized). All spectra represent averages of at least 15 individual IR-MSP measurements (see "Experimental Procedures" and "Results" for details). Note the additional band component at 1635 cm\(^{-1}\) in the spectra of 263K-brain. Peak positions specific for tyrosine residues are indicated at 1515 cm\(^{-1}\). At least in two mean spectra of 263K-PMCA-brain, there are indications for the presence of a band component at 1635 cm\(^{-1}\) (see arrow). AU, absorbance units. B, dendograms obtained by hierarchical cluster analysis of the mean microspectra using the information content in the amide I region (1610–1700 cm\(^{-1}\)), D value as interspectral distance measure, and Ward’s algorithm as the clustering method. The distances between different sample preparations for each prion isoform were always smaller compared with the distances between the different prion isoforms. Furthermore, spectra from 263K-brain and 263K-PMCA-brain cluster in the same branch, whereas spectra of 263K-PMCA form a separate second cluster.

**IR-MSP Was Not Influenced by the Size of PrP Assemblies**—In order to address whether FTIR spectral differences observed between our different sample materials may have been influenced by the size of PrP assemblies, we carried out a control experiment. For this purpose, IR-MSP was performed on highly purified PrP27-30 from 263K-PMCA-brain that had been subjected to mild or harsh treatment for differential aggregate fragmentation. The samples were either treated by "mild" sonication, as was usually done during the PrP27-30 extraction procedure and for sample resuspension before IR spectroscopic measurements, or by additional extensive sonication in the presence of glass beads (i.e. similar to PMCA conditions). Sonication with beads has been shown to efficiently fragment amyloid fibrils of recombinant PrP into smaller particles (34). We found that the FTIR spectra from "mildly" or “extensively” sonicated samples showed no detectable differences (Fig. 5).

In parallel, we analyzed the sedimentation velocity of PrP27-30 in the mildly (without glass beads) and extensively (with glass beads) sonicated PrP extracts from 263K-PMCA-brain by ultracentrifugation. These samples were derived from identically extracted ultrapure fibrillar PrP of identical origin and differed only with respect to an additional ultrasonication in the presence of glass beads. Under these experimental conditions, PrP sedimentation profiles could be expected to reflect the size of PrP assemblies in the analyzed samples. In accordance with previous observations by Tixador et al. (29), we found that the purification of PrP27-30 produced faster sedimenting PrP aggregates than observed in non-purified 263K scrapie brain homogenates (not shown). However, irrespective of this purification effect, the PrP sedimentation profiles significantly differed between the mildly and extensively sonicated preparations in that there was a substantially higher proportion of slower sedimenting (i.e. smaller) PrP aggregates in the “extensively” sonicated sample (not shown). When combined, the findings from these IR-MSP and ultracentrifugation control experiments show that differences observed in our spectra between 263K-brain, 263K-PMCA, and 263K-PMCA-brain samples were not due to the size of the examined objects.
Digestion Patterns of 263K-Brain, 263K-PMCA, and 263K-PMCA-Brain after Treatment with Proteinase K—PMCA with glass beads was performed as described previously for three rounds to obtain 263K-PMCA products (26). When 263K-brain, 263K-PMCA, and 263K-PMCA-brain samples were treated with increasing concentrations of PK, different digestion profiles were obtained (see Fig. 6). 263K-brain was resistant to the protease even at PK concentrations of 700 μg/ml while the 263K-PMCA product was partially degraded already at PK concentrations of 400 μg/ml. For 263K-PMCA-brain products, the digestion profile looked intermediate. These experiments were repeated three times and resulted in similar digestion profiles as shown in Fig. 6 after densitometric analysis. This again is indicative for conformational differences between the tested PrP27-30/PrPres samples before and after PMCA.

Characterization of PrP Assemblies from 263K-Brain, 263K-PMCA, and 263K-PMCA-Brain by Sedimentation Velocity Assay—263K-brain, 263K-PMCA, and 263K-PMCA-brain aliquots in assay buffer were loaded on the top of iodixanol gradients and subjected to sedimentation velocity analysis (Fig. 7) that differentiated PrP assemblies based on mass and shape. PrP\textsuperscript{TSE} from 263K hamster brains (263K-brain) and brains of animals that had been infected with 263K-PMCA-products (263K-PMCA-brain) were found in gradient fractions showing a density of 22–26% or 22–25%, respectively. In contrast, PMCA products (263K-PMCA) were focused in two fractions of the gradient with a density between 12 and 14%. This indicated a more heterogeneous composition of 263K-brain- and 263K-PMCA-brain-associated PrP\textsuperscript{TSE} assemblies as compared with PrPres from 263K-PMCA. Furthermore, the centrifugation findings showed that PrP\textsuperscript{TSE} assemblies from 263K-brain and 263K-PMCA-brain had a higher sedimentation velocity (and thus a higher mass or a more compact shape) than PrPres assemblies from 263K-PMCA. Although the centrifugation results for 263K-brain and 263K-PMCA-brain samples looked rather similar, subtle differences indicate the presence of a proportion of faster sedimenting PrP\textsuperscript{TSE} assemblies in 263K-brain. Sedimentation velocity experiments were repeated three times and produced consistent results with only small variations.

Intracerebral Challenge of Hamsters with 263K-Brain or 263K-PMCA—It has been previously reported that prolonged incubation times can be detected when hamsters are inoculated with PMCA-derived products compared with animals inoculated with parental seeds (22, 35). We now inoculated hamsters with the PMCA product 263K-PMCA. As shown in Fig. 8, A and B, and Table 2, animals infected with 263K-PMCA succumbed to disease between 152 and 208 dpi (179 ± 23 dpi, mean ± S.D.), whereas control animals challenged with authentic 263K-brain seeds succumbed to disease between 85 and 106 dpi (95 ± 8 dpi). All of these animals were inoculated with similar amounts of PrP\textsuperscript{TSE} and PrPres as judged by Western blotting (Fig. 8C). For quantification of PK-resistant PrP, samples were digested with 150 μg/ml Proteinase K. This is a concentration for which similar stability for brain- and PMCA-derived PrP\textsuperscript{TSE}/PrPres is expected according to Fig. 6 and further control experiments (not shown). All bioassay animals that had been tested positive for the presence of PrP\textsuperscript{TSE} by Western blotting (not shown) were also found to be positive when analyzed by PET blotting (Fig. 8, A and B, and Table 2). Brains of hamsters inoculated with an unseeded PMCA control did not reveal any PrP\textsuperscript{TSE} deposits (Fig. 8B). To exclude the possibility that PrP\textsuperscript{TSE} accumulation in animals inoculated with PMCA products resulted from residual 263K seed, we inoculated hamsters with 5 × 10^{-19} g of 263K-brain tissue because this represents the theoretical amount of residual 263K seeding material (see “Experimental Procedures” for details). As demonstrated in Fig. 8A, these hamsters, which remained clinically inconspic-
uous, did not show any PrP\textsuperscript{TSE} in the brain. The clinical symptoms of diseased animals were similar and were typical for 263K scrapie in recipients of 263K-brain and 263K-PMCA inoculum. The same held true for the spatial distribution pattern of PrP\textsubscript{27-30} in PET blots from midsagittal brain sections of diseased animals (Fig. 8, A and B).

**DISCUSSION**

PMCA has become a frequently used in vitro technique to test the seeding activity of prion seeds in different substrates (20). PMCA-derived PrPres seeds have been shown to be autocatalytically active and infectious (21, 35, 36); however, it has not been conclusively elucidated whether PMCA alters PrP strain properties. There is now accumulating evidence that changes in prion replication environment can cause prion strain modifications in PMCA reactions (23). If PMCA altered the conformation and biological properties of prions, this would highlight the need for a careful interpretation of PMCA-derived data, especially when PMCA is used to assess the intra- and interspecies transmissibility of prions. To address the question of whether changes of prion strain properties occur during PMCA, we devised a protocol for the purification of PMCA samples on the basis of which ultrapure PrPres fractions can be prepared for and structurally analyzed by specifically adapted infrared microspectroscopy.

**Infrared Microspectroscopy on PMCA-derived Prion Protein**

![Figure 7. Sedimentation velocity analysis of PrP assemblies.](image)

263K-brain, 263K-PMCA, and 263K-PMCA-brain samples in assay buffer were loaded on the top of iodixanol gradients, and PrP assemblies were separated by their sedimentation velocity in gradient ultracentrifugation. Fractions of 80 μl were collected from the bottom of punctured centrifugation tubes, and the density of the gradient fractions was determined by refractometry of a control gradient. Gradient fractions were digested with PK, and the antibody 3F4 was used for the detection of PrP by Western blotting. Experiments were repeated three times with consistent results.

![Figure 8. PET blot images of hamster brains inoculated with PrP\textsuperscript{TSE}/PrPres and Western blot for the quantification of inocula.](image)

A, microphotographs of PET blots obtained from midsagittal hamster brain sections. Hamsters were intracerebrally inoculated with brain homogenate obtained from 263K scrapie hamsters. Left, PET blot of a brain section (clinical stage of scrapie). Inoculum was 1 × 10\textsuperscript{-6} g brain homogenate from 263K scrapie hamsters. Right, section of a control brain inoculated with a control agent (5 × 10\textsuperscript{-10} g of 263K-brain tissue). B, microphotographs of PET blots obtained from midsagittal hamster brain sections. Animals were inoculated with seeding active PrPres or a control product, both produced by PMCA. Left, section of a brain (clinical stage of scrapie). Inoculum was seeding active PrPres produced by PMCA. Right, negative PMCA control. Shown is a PET blot of a hamster brain inoculated with a product obtained after seven rounds of an unseeded PMCA reaction. C, quantification of the inocula used in A and B by Western blotting for PrPres. A 1:30 dilution of a PMCA product passaged for seven rounds when the reaction was started with 10\textsuperscript{-12} g of 263K-infected brain tissue in normal hamster brain homogenate as substrate showed staining intensities in Western blots similar to 263K-brain material corresponding to 1 × 10\textsuperscript{-6} g of 263K-infected brain tissue. Quantification was confirmed by three independent repeats with similar results. Samples had been loaded on the same gel, and the blot was scanned and digitally rearranged afterward.
Infrared Microspectroscopy on PMCA-derived Prion Protein

TABLE 2
Bioassay results after intracerebral challenge of hamsters with brain homogenate from 263K scrapie hamsters or seeding active PrPres produced by PMCA

| Inoculum                        | Attack rates | Western blot and/or PET blot | Duration of clinical observation (dpi) |
|---------------------------------|--------------|-----------------------------|---------------------------------------|
| Brain homogenate of 263K scrapie | 5/5          | 5/5                         | 85, 92, 92, 99, 106                   |
| Brain homogenate of normal hamsters (control) | 0/5          | 0/5                         | 370, 475, 513, 513, 513               |
| Seeding active PrPres produced by PMCA | 5/5          | 5/5                         | 152, 159, 187, 187, 208               |
| PMCA control                     | 0/5          | 0/5                         | 405, 475, 513, 513, 513               |

Hamsters were challenged by intracerebral injection with materials as indicated by the first column (see “Experimental Procedures” for details). Detection of cerebral PrP deposition was performed by Western blotting and/or PET blotting. Some animals of the control groups had to be humanely sacrificed prior to termination of the experiment due to intermittent health problems not related to TSE. Attack rates are specified as the ratio of animals that developed TSE symptoms and the number of challenged animals. Duration of clinical observation is provided in dpi.

Infrared Microspectroscopy on PMCA-derived Prion Protein

IR-MSP Revealed Secondary Structure Differences between 263K-Brain, 263K-PMCA, and 263K-PMCA-Brain—The frequencies, intensities, and widths of absorption bands in the amide I region of infrared spectra provide biophysical information for a structural characterization of protein conformations. It has long been established that specific absorption bands in the IR spectrum of proteins closely correlate with secondary structure elements (i.e. the pattern of intra- and intermolecular hydrogen bonds between backbone amide and carbonyl groups) in local protein segments. This correlation is used in FTIR spectroscopy to assign characteristic frequencies of structure-sensitive amide bands to different types of secondary protein structure, such as α-helices or β-sheets. There are nine amide bands in the IR spectrum (37). The amide I band used in our study, in which secondary structure elements, such as α-helix, β-sheet, or turns and loops, absorb IR light at different wavelengths, has been established as the most useful band for the analysis of secondary protein structure (for details, see Refs. 37–39).

The amide I absorption maxima of the tested prion isoforms, and their assignment according to current standards to β-sheets, α-helices, and turns/loops are summarized in Table 1. In this context, it has to be noted that the association between specific IR absorption bands and α-helical structure elements in PrP is currently under discussion (38, 40). For our study, however, this is not critical because we focused on IR bands that are assigned to β-sheets according to the conventional practice described in the literature (37, 39). We could detect conformational differences for β-sheet structures in the structure-sensitive amide I region (1610–1700 cm⁻¹) between the tested prion isoforms. Our spectroscopic results obtained for dried 263K-brain samples were in good agreement with previously published data (13). With our modified purification protocol and refinements of the IR data acquisition technique, we are now able to obtain an analytical sensitivity for dried protein samples that was previously only achieved when measurements were performed in H₂O or D₂O (as indicated by the clear double peak at 1626/1635 cm⁻¹ in the low frequency position of β-sheets in the spectrum of 263K-brain (13)).

Of the different secondary structure elements contributing to our amide I absorption spectra, all but intermolecular β-sheets (previously detected in PrP27-30 by Spassov et al. (13)) refer to the internal structure of the protomers in the examined oligo- or polymeric PrP assemblies. Accordingly, FTIR absorption maxima relating to intramolecular β-sheets, turns and loops, or possibly α-helices can be due only to the secondary structure of the protomers (i.e. the individual PrP molecules from which the PrPres aggregates are built).

Our extracts for IR-MSP consisted of ultrapure and essentially fibrillar PrP27-30/PrPres with (possibly) different size distributions of PrP aggregates. In these samples, larger fibrillar
Infrared Microspectroscopy on PMCA-derived Prion Protein

PrP assemblies should plausibly contain protomer-linking intermolecular \(\beta\)-sheets of greater size than smaller PrP aggregates. Thus, differences in FTIR spectra that we assigned to \(\beta\)-sheet structure (without further intra- and intermolecular differentiation) needed to be further clarified in terms of whether they had resulted from differences in the size of PrP assemblies or from differences in the secondary structure of the PrP protomers and/or their linking intermolecular \(\beta\)-sheets.

The influence of the size of amyloid aggregates on FTIR spectral absorption patterns was examined by Xue et al. (41) using the example of \(\beta\_2\)-microglobulin amyloid fibrils (amyloid fibrils of \(\beta\_2\)-microglobulin had been previously shown by Fabian et al. (42), and were later confirmed by Debelouchina et al. (43), to contain intermolecular \(\beta\)-sheets). Xue et al. (41) found that mechanical fragmentation of \(\beta\_2\)-microglobulin amyloid fibrils into shorter fibrillar units (which must have been accompanied by a fragmentation of larger intermolecular \(\beta\)-sheets into smaller ones) did not significantly alter the FTIR spectrum of the samples in the amide I region. Thus, the findings by Xue et al. (41) indicated that the position of FTIR spectroscopic absorption peaks caused by intermolecular \(\beta\)-sheets and other secondary structure elements is not sensitive to the mere size of amyloid aggregates. This is in good accordance with the control experiment that we carried out in this study to address the question of whether FTIR spectral features may have been influenced by the size of the examined PrP assemblies (Fig. 5).

Accordingly, we conclude that the FTIR spectral differences found between our PrP27-30/PrPres extracts from 263K-brain, 263K-PMCA, and 263K-PMCA-brain can only have resulted from secondary structure differences between the PrP protomers and/or their linking intermolecular \(\beta\)-sheets. Because the PrP extracts that were used for IR-MSP had been identically prepared, their secondary structure differences must have plausibly originated from secondary structure differences between the PrP protomers and/or protomer-linking intermolecular \(\beta\)-sheets in native 263K-brain, 263K-PMCA, and 263K-PMCA-brain prions. The relatively large spectral variance of 263K-PMCA-brain samples would be consistent with (although not definitely proving) different stages of reemerging native 263K PrP\textsuperscript{TSE} in bioassay animals that had been inoculated with 263K-PMCA. In any case, the spectral distances shown in the dendrogram of Fig. 4B provided an indication that secondary structure differences of 263K-brain and 263K-PMCA-brain were less pronounced between each other than compared with 263K-PMCA.

Different Resistance of 263K-Brain, 263K-PMCA, and 263K-PMCA-Brain Homogenates to Degradation by Proteinase K—
The resistance of the prion protein to PK was highest in 263K-brain, lowest in 263K-PMCA, and intermediate in 263K-PMCA-brain (Fig. 6). Because the efficacy of the degradation of PrP27-30/PrPres by PK essentially depends on the conformation of PrP aggregates (44), this finding also indicated structural differences between 263K-brain, 263K-PMCA, and 263K-PMCA-brain.

Sedimentation Velocity Analysis of PrP from 263K-Brain, 263K-PMCA, and 263K-PMCA-Brain in Iodixanol Gradients—
In our ultracentrifugation experiments using iodixanol gradients (29), PrP\textsuperscript{TSE} from 263K-brain and 263K-PMCA-brain exhibited a higher sedimentation velocity (indicating a higher mass or a more compact shape) than PrPres from 263K-PMCA. The sedimentation properties of 263K-brain and 263K-PMCA-brain looked rather similar when compared with each other, whereas they substantially differed from those observed for 263K-PMCA. This, again, provides an indication for a closer structural relationship of PrP27-30 from 263K-brain and 263K-PMCA-brain as compared with the PrPres from 263K-PMCA.

Bioassay Analysis of 263K-Brain and 263K-PMCA Prions—
Upon intracerebral inoculation of hamsters with similar amounts of PrP\textsuperscript{TSE} and PrPres from 263K-brain and 263K-PMCA, respectively, we observed a significantly prolonged incubation period for the latter (Table 2) in accordance with data previously published by others (21, 22, 35). However, clinical and PET blot findings were indistinguishable for both groups of recipients.

The longer incubation periods observed for 263K-PMCA versus 263K-brain in our bioassay may be due to differences in secondary structure elements or to a smaller size of PMCA-derived PrPres assemblies as compared with authentic PrP27-30 aggregates. The latter explanation would be consistent with both our findings in the sedimentation velocity assay (Fig. 7) and results previously by Weber et al. (35). Data from the study of these authors suggest that the infectivity of smaller PrPres particles generated by PMCA is relatively lower than that of larger native PrP\textsuperscript{TSE} aggregates from 263K hamster brains because smaller PMCA-derived PrPres particles are more susceptible to biological clearance. Alternatively, as discussed by Klingeborn et al. (45), only a fraction of PMCA-generated PrPres may correlate with infectivity. In this scenario, a proportion of PrPres generated by PMCA is thought to lack conformational properties or co-factors required for biological infectivity.

Why and at Which Molecular Level Did PMCA Induce Conformational Alterations in Progeny Seeds?—
Taken together, we could demonstrate conformational changes in PMCA-derived 263K-associated PrP progeny seeds by (i) infrared microspectroscopy and objective cluster analysis of IR spectra, (ii) biochemical characterization of PrP27-30/PrPres samples with respect to PK resistance, and (iii) sedimentation velocity assays using iodixanol gradient ultracentrifugation. We conclude from the described analyses that our PMCA procedure induced molecular differences between 263K parent and progeny seeds at two structural levels: (i) the secondary structure of PrP protomers and/or their linking intermolecular \(\beta\)-sheets and (ii) the quaternary structure (size and/or shape) of PrP assemblies. Notably, the secondary structure differences detected in our IR-MSP analyses were found not to be due to the size of the examined objects. Although extensive ultrasonication in the presence of glass beads may plausibly explain a fragmentation of larger into smaller PrP aggregates during PMCA, the exact mechanisms underlying the observed modifications of secondary and quaternary structure characteristics in their entirety are not completely clear.

The efficiency and course of PrP\textsuperscript{Sc} into PrPres conversion by PMCA is known to depend on both the PrP seeds and reaction conditions (such as incubation time between sonications, duration and intensity of sonication, and the presence or absence of beads or (bio)chemical co-factors (e.g. nucleic acids or other polyanions) (46)). If prion strains were coded by distinct PrP conformers that are, apart from their size, structurally homog-
Infrared Microspectroscopy on PMCA-derived Prion Protein

De novo, procedural features of our PMCA, such as repeated ultrasonication and/or the presence of beads in reaction mixtures, may have caused a conformational switching within PrP amyloid fibers (47, 48). This could have produced and propagated homogenous or heterogeneous PrPres conformers that structurally differ (in addition to mere size variations) from their parent seeds. If, in contrast, prion strains existed in their biological hosts as stable ensembles of different self-replicative PrP conformers, as suggested by the “quasispecies” model (9, 49), specific PMCA conditions, such as those used in our study, may have favored a non-natural selection of certain PrPPr con-

In any case, our observation of 263K-strain modifications by PMCA is supported by data recently published by Gonzalez-Montalban et al. (22, 23). These authors detected an increase of incubation periods in reporter animals for prion agents that had been subjected to PMCA and additionally showed an increase of the percentage of the diglycosylated PrP glycoforms.

However, Castilla et al. (21) could not find biochemical, biophysical, or immunohistological evidence for changed properties of 263K after PMCA. This discrepancy could be explained by the fact that we used beads for PMCA, which significantly increases the efficiency and robustness of PMCA, as described elsewhere (26, 34). In addition, IR-MSP of highly purified protein samples substantially improved the collection and interpretation of IR spectroscopic data as well as the sensitivity of IR analyses. As outlined above, the efficiency of the seeded conversion of PrPSc into specific PrPres conformations crucially depends on the reaction conditions of PMCA. Whereas Gonzalez-Montalban et al. (22, 23) and we performed PMCA in the presence of beads, Castilla et al. (21) carried out PMCA without beads.

Findings from Gonzalez-Montalban et al. (22, 23) showed that the presence of beads led to the production of rather uniform and small PrP aggregates by PMCA. This is consistent with our gradient centrifugation analysis in which we found that 263K scrapie brain homogenate (that had been only mildly sonicated without beads) contained PK-resistant PrP with a sedimentation velocity faster and more heterogeneous than that detected in PMCA products. Thus, the PMCA products generated by Castilla et al. (21) without using beads were, in terms of aggregate size and/or shape, possibly more similar to native 263K scrapie seeds than our or Gonzalez-Montalban’s PMCA products (22, 23). Without a direct comparison of PMCA products from the different research groups, however, such a notion remains speculative.

Conceptual Implications and Outlook—Our spectroscopic and biochemical analyses suggested a closer relatedness, in terms of secondary and quaternary structure, of 263K-PMCA-brain to 263K-brain than to 263K-PMCA. In addition, the clinical symptoms and PET blot findings in bioassay hamsters were indistinguishable after inoculation of 263K-brain and 263K-PMCA samples. Thus, the conformational modifications of 263K prions induced by our PMCA seem to have been at least partially reversed when PMCA products were repassaged into the original host species. It remains to be tested in future studies whether further passages in hamsters will eventually lead to a complete reestablishment of the authentic 263K scrapie agent.

A (partial) reversion of PMCA-induced conformational alterations in scrapie 263K progeny seeds by biological passage of such seeds in hamsters is basically compatible with both scenarios: that PMCA had (i) modified the relative proportion of PrP conformers in the 263K quasispecies or (ii) caused a conformational switching within PrP fibers and subsequent propagation of previously non-existent PrP conformers. Model (i) would explain the phenomena observed in PMCA and bioassay with a quantitative shift and reshift in the frequency distribution of different PrP conformers mediated by preferential selection under different conditions. Model (ii), in contrast, would implicate a qualitative deterministic structural switching and reswitching of specific PrP conformers. Although model (i) seems to be less demanding (and thus more probable) in mechanistic terms, we cannot rule out the occurrence of conformational switching and reswitching. However, our experimental approach provides the possibility for further in depth IR-MSP studies on this subject. Such studies should also include the issue of putative co-factors of PrP conversion and their possible influence on PrP structure.

Perspectively, biophysical techniques that allow a higher spatial resolution of structural features (e.g., tip-enhanced Raman spectroscopy or near field infrared spectroscopy) may be applied to separated and ultrapurified PrP conformers. When combined with functional analyses, in vitro and in vivo, of the seeding activity of such PrP conformers, this can substantially facilitate the painstaking exploration of the molecular basis of prion strain phenomena.

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