Formation of Critical Oligomers is a Key Event During Conformational Transition of Recombinant Syrian Hamster Prion Protein

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Running title: Conformational Transition of Recombinant Prion Protein
Summary

We have investigated the conformational transition and aggregation process of recombinant Syrian hamster prion protein (SHaPrP^{90–232}) by Fourier transform infrared spectroscopy, circular dichroism spectroscopy, light scattering, and electron microscopy under equilibrium and kinetic conditions. SHaPrP^{90–232} showed an infrared absorbance spectrum typical of proteins with a predominant α-helical structure both at pH 7.0 and at pH 4.2 in the absence of guanidine hydrochloride. At pH 4.2 and destabilizing conditions (0.3–2 M guanidine hydrochloride), the secondary structure of SHaPrP^{90–232} was transformed to a strongly H-bonded, most probably intermolecularly arranged antiparallel β-sheet structure as indicated by dominant amide I band components at 1620 and 1691 cm\(^{-1}\). Kinetic analysis of the transition process showed that the decrease in α-helical and the increase in β-sheet structures occurred concomitantly according to a bimolecular reaction. However, the concentration dependence of the corresponding rate constant pointed to an apparent third order reaction. No β-sheet structure was formed within the dead time (190 ms) of the infrared experiments. Light scattering measurements revealed that the structural transition of SHaPrP^{90–232} was accompanied by formation of oligomers, whose size was linearly dependent on protein concentration. Extrapolation to zero protein concentration yielded octamers as smallest oligomers, which are considered as “critical oligomers”. The small oligomers showed spherical and annular shapes in electron micrographs. Critical oligomers seem to play a key role during the transition and aggregation process of SHaPrP^{90–232}. A new model for the structural transition and aggregation process of the prion protein is described.
Introduction

The prion protein (PrP) is following the protein-only hypothesis – the sole agent causing a group of neurodegenerative disorders (1,2), the so-called prion diseases or prionoses (3). The most important ones among them are bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and Creutzfeldt-Jakob disease (CJD) in humans.

The crucial step in transmission and manifestation of prion diseases is the conversion of benign monomeric cellular prion protein (PrPC), which has a mainly α-helical secondary structure, to pathogenic multimeric scrapie prion protein (PrPSc), which is predominantly folded into β-sheets (4,5). Noteworthy, PrPC and PrPSc do not differ in their amino acid sequence.

Similar mechanisms play an essential role in a number of other neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's diseases. Therefore, the coupled processes of protein misfolding and aggregation, the kinetics of these processes, and the molecular species involved are of fundamental interest.

Late products of the conversion are amyloid fibrils and amyloid plaques, which are widely considered to be direct effectors of the above-mentioned disorders. However, evidence is accumulating that intermediates or by-products of the assembly process could be the pathogenic form of PrP (6,7) and other disease-related proteins (8).

In vitro transition of unglycosylated recombinant fragments of PrPC into aggregated, β-rich, yet not infectious isoforms has been reported for a number of sequences from different species (9-11). A particularly interesting β-rich oligomer has recently been reported (10,11). This oligomer was described to consist of at least 8 monomers and to appear as a transient species under conditions favoring the formation of mature fibrillar structures (acidic pH, 2–4 M urea, incubation at 37 °C, constant agitation) (10) or as a relatively stable end product under conditions optimized for the formation of the oligomer (pH 4 and 2 M GuHCl or pH 2 and 5 M urea, incubation at
23 °C) (11). The structural formation of the β-rich oligomer takes place with PrP exhibiting the intact disulfide bridge and comprises the important initial reorganization of the secondary structure, namely a decrease of α-helical and an increase of β-sheet structure. Since the β-rich oligomer could be both an important precursor of later stages of the assembly process and a toxic species in itself, we have studied its formation, structural properties, and subsequent structural transitions in more detail using Fourier transform infrared (FTIR) spectroscopy, static (SLS) and dynamic light scattering (DLS), circular dichroism (CD) spectroscopy, and electron microscopy (EM). The combination of all these techniques provided solid information on secondary, tertiary, and quaternary structural changes and morphological properties of PrP.

**Experimental Procedures**

**Protein production and purification**

Since our expression and purification system differed from those published earlier (12-16), the procedure is described here in detail. It is worth mentioning that it led to a product with > 99% high performance liquid chromatography (HPLC) purity.

SHaPrP$^{90-232}$ was expressed in *Escherichia coli* strain BL21(DE3) (Novagen, USA) transformed by a pET-15b vector (Novagen) in which the sequence corresponding to amino acids 90–232 of the prion protein of the Syrian hamster was cloned. huPrP$^{91-231}$ was expressed in an equivalent system. The pET-15b vector contains the sequences for a His$_6$ tag and a thrombin cleavage site directly C-terminal to this tag. For cultivation 100 µl bacteria suspension were grown for 7–8 h at 37 °C in 10 ml Luria-Bertoni medium containing 100 µg/ml ampicillin (LB + Amp). This culture was used to inoculate 1 l LB + Amp. After growth for 16 h the culture was centrifuged for 10 min at 2350 × g. The pellet was resuspended in 5 ml LB medium and used to inoculate 1 l of LB + Amp. After growth for 30 min at 37 °C the over-expression of PrP was induced by 1 mM
isopropyl-β-D-thiogalactoside. After additional 5 h at 37 °C the cells were harvested by centrifugation at 2350 × g. The cell pellet was resuspended in 50 ml lysis buffer (100 mM Tris, 200 mM NaCl, 10 mM Na-EDTA, 0.2 % Triton-X-100, pH 7.2) and stirred for 30 min. 1 mmol MgCl₂ (final concentration: 19 mM) and 500 U Benzonase (Roche, Germany) were added. After additional 5 min the lysate was centrifuged at 10240 × g. The pellet was washed with 50 mM Tris, pH 7.4.

For purification PrP was extracted from cell lysate equivalent to 2 l of cell culture and reduced by resuspending the pellet in 40 ml reduction buffer (50 mM Tris, 6 M GuHCl, 50 mM DTT, pH 7.4). After centrifugation (30 min at 25280 × g) and vacuum filtration through a 0.45 µm filter the protein solution was applied to 10 ml nickel-nitrilotriacetic acid Agarose (Qiagen, Germany), which was pre-washed in guanidine buffer (50 mM Tris, 6 M GuHCl, pH 7.4), and incubated for 30 min under shaking. Non-specifically bound proteins were removed by washing 3 times with guanidine buffer and once with guanidine buffer plus 1 mM Imidazol. The Agarose with bound PrP was filled into an “Econo-Pac” column (Biorad, USA). PrP was eluted by 20 ml guanidine buffer plus 250 mM Imidazol. To prevent formation of oligomers, eluted PrP was collected in 50 ml guanidine buffer. The concentration of PrP was determined by HPLC analysis (ET 250/4 Nucleosil 300-7 C8 column, Macherey-Nagel, Germany; HP 1050 device, Hewlett-Packard, Germany). Elution was performed by a linear gradient of 30–60 % eluent B (0.1 % trifluoroacetic acid (TFA) in acetonitrile) in eluent A (0.1 % TFA in water) over 12 min. The solution was diluted to 0.25 mg/ml with guanidine buffer and oxidized by adding 2 µM CuSO₄ and stirring overnight at room temperature. Completion of oxidation was monitored by HPLC analysis. After concentration to 1–2 mg/ml, the PrP solution was diluted 1:10 into Tris buffer (20 mM Tris, pH 8.0) to allow refolding of the protein. After 1–2 h precipitated PrP was removed by filtration. The solution was concentrated to a final volume of 100 ml and dialyzed against Tris buffer.
During dialysis 10 U thrombin per mg PrP were added. Cleavage of the His<sub>6</sub> tag was allowed to take place overnight at room temperature. Completion of cleavage was checked by SDS PAGE. Due to the vector construction, the expressed and cleaved PrP contained N-terminally four additional amino acids (Gly-Ser-His-Met). The PrP solution was filtrated, 10 µl of protease inhibitor (‘Complete, Mini, EDTA-free’, Roche, Germany) were added, and the solution was concentrated to a final volume of 25 ml. After dialysis overnight against phosphate buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0), PrP was analyzed by HPLC, sterile filtrated, and stored at –20 °C until final purification.

For final purification GuHCl was added to the pre-purified PrP samples to obtain a concentration of 6 M. 10–15 mg PrP were loaded on an EP 250/16 Nucleosil 300-7 C8 column (Macherey-Nagel). A solvent port had to be used to load the column with the sample. To prevent precipitation of the protein in the pump, the HPLC system was pre-washed thoroughly with 6 M GuHCl at a flow rate of 8 ml/min. Elution was performed by a linear gradient of 30–40 % eluent B in eluent A over 20 min at a flow rate of 10 ml/min. PrP containing fractions were pooled, lyophilized, resuspended in guanidine/phosphate buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 6 M GuHCl, pH 7.0) and dialyzed against the same buffer to remove residual TFA, which has a very intense C=O stretching band at 1680 cm<sup>−1</sup> in the amide I region. Afterwards PrP was refolded by diluting 1:10 with phosphate buffer. After 1–2 h precipitated protein was removed by filtrating. The solution was dialyzed against phosphate buffer. For examination at pH 4.2, PrP was furthermore dialyzed against acetate buffer (20 mM sodium acetate, pH 4.2). Purity of the protein was determined by HPLC and SDS PAGE; identity of the protein was verified by mass spectroscopic analysis.
**FTIR measurements**

**Stopped flow device**

All measurements were performed using a novel stopped flow device, linked to an IFS 28/B FTIR spectrometer (Bruker Optics, Germany) equipped with a rapid scan option. The stopped flow device was designed specifically for high-precision FTIR kinetic and difference spectroscopy of biological macromolecules in $^1$H$_2$O, and is described in detail elsewhere (17). Briefly, the principle elements of the system are a two-channel high pressure syringe pump, a microstructured diffusional mixer, and a specially designed thin-layer infrared flow cell. HPLC tubing and fittings are used throughout. Samples are injected into a continuous flow of distilled water via HPLC sample injection valves under computer control, and the measurement is triggered after the flow is stopped when the samples have filled the flow cell. For the measurements described in the present work, a flow rate of 3 ml/min and a sample loop volume of 15 µl were used. The dead time of the experiment, *i.e.* the time delay between mixing the samples and obtaining the first spectrum, was an average value of 190 ms. Kinetic measurements of the transition of PrP were performed by mixing GuHCl and PrP solutions in the stopped flow device, and the appropriate control experiments were performed with GuHCl or PrP alone. The particular advantages of this stopped flow device for the present work were the extremely precise and reproducible cell pathlength, the short time between sample and reference data acquisitions, and the very gentle mixing achieved with the diffusion micromixer. These benefits resulted in the unprecedentedly high quality of the FTIR spectra presented below.

**FTIR parameters**

The interferograms were recorded double sided (forward-backward) at a mirror frequency of 200 kHz. The upper and lower frequency folding limits were 7899 and 0 cm$^{-1}$, respectively. A Blackman-Harris 3 term function and a zero filling factor of 4 were used for Fourier
transformation resulting in spectra encoding approximately 1 data point per 1 cm⁻¹. The pathlength of the flow cell was approximately 8 µm. The non-linearity of the MCT detector was corrected prior to Fourier transformation.

**Acquisition of protein spectra**

The initial PrP concentration was 8 mg/ml. Since all PrP solutions were diluted 1:2 in the stopped flow device, measurements were performed at a concentration of 4 mg/ml (≈ 0.24 mM). The GuHCl-containing solutions were buffered by either 20 mM NaH₂PO₄ or 20 mM sodium acetate to ensure proper pH values of 7.0 or 4.2, respectively. Steady state spectra of either buffer or PrP + buffer were measured independently 5 times with 256 scans in each case and finally averaged. Buffer absorbance was subtracted from PrP + buffer spectra.

Time-resolved spectra of buffered GuHCl solutions and buffered PrP + GuHCl mixtures were measured in the rapid scan mode. All spectra were recorded continuously; the time lag between two spectra was determined by the number of scans per spectrum (see below). The acquisition processor of the FTIR spectrometer had a capacity to store 60 spectra. The early ones were recorded with a low number of scans to get spectra with high time resolution but low signal to noise ratio, the later ones were recorded with more scans to get spectra with a higher signal to noise ratio. Since the reaction kinetics of the α-to-β-transition was a function of GuHCl concentration, the distribution pattern of scans per spectrum was adjusted as needed (Table 1).

**Table 1, near here**

The pure PrP spectra were obtained by subtracting GuHCl and buffer spectra. To obtain the time-dependent changes, the difference spectra between each of the 60 single spectra and the last spectrum (i.e. the 60th spectrum) were calculated. Due to small instabilities of the FTIR spectrometer, baseline shifts in the range of ± 0.0002 AU were observed between subsequent spectra and were offset corrected using the region from 1900 to 1750 cm⁻¹, which is free of
spectral features, as a reference. Second derivatives were calculated by applying the Savitzky and Golay algorithm with 9 smoothing points (18).

For evaluation of structural changes of PrP taking place within the experimental dead time, the difference between the first of the 60 spectra obtained after mixing PrP with GuHCl and a steady state spectrum of PrP in the absence of GuHCl was calculated.

**Light scattering**

SLS and DLS were measured simultaneously with one and the same instrument at a scattering angle of 90°. The laboratory-built apparatus, presently equipped with a diode-pumped, cw laser Millennia IIs (Spectra-Physics, USA) and a high quantum yield avalanche photodiode, has been described in detail (19). Apparent molar masses were estimated from the relative scattering intensities using toluene as a reference sample and applying a refractive index increment \((\partial n/\partial c) = 0.19 \text{ ml/g}\). The translational diffusion coefficients \(D\) were obtained from the measured autocorrelation functions using either the program CONTIN (20) or applying the method of cumulants (21). The diffusion coefficients were converted into Stokes radii via the Stokes-Einstein equation \(R_S = k_B T/(6\pi\eta_0 D)\), where \(k_B\) is Boltzmann's constant, \(T\) is the temperature in K, and \(\eta_0\) is the solvent viscosity. For kinetic light scattering experiments two experimental schemes were used. At high protein concentrations, prefiltered (100 nm pore-size) solutions and solvents were rapidly mixed in 100 µl fluorescence cells. At low concentrations, mixing was done before filtration into 30 µl flow-through cells.

**CD measurements**

CD measurements were carried out at protein concentrations between 0.2 and 1.2 mg/ml on a J-720 (JASCO, Japan) CD spectrometer, which was calibrated with (+)-camphorsulfonic acid at 290.5 and 192.5 nm (22). 1 mm and 0.1 mm pathlength cells were used at low and high protein
concentrations, respectively. Mean residue molar ellipticities $[\theta]$ were calculated using the mean residue weight of 113.9. Kinetic measurements were done by manually mixing the protein (buffered by 20 mM sodium acetate, 50 mM NaCl, pH 4.2) with a certain amount of GuHCl-containing buffer to obtain a final concentration of 1 M GuHCl. The dead time of mixing was about 20 s; the first complete spectrum was obtained 1 min after initiation of the experiment.

**Electron microscopy**

Before preparation for electron microscopy, samples were diluted to a protein concentration of about 30 µg/ml with the corresponding solvent. Specimens were visualized by negative staining with 1 % uranyl formate using a double-carbon film technique. 400 mesh copper grids covered with a carbon-coated Triafol microgrid were used as support. Micrographs were taken with an EM910 electron microscope (LEO, Germany) at 80 kV and a magnification of 63,000.

**Analysis of kinetic data**

The time series of FTIR spectra between 1200 and 1900 cm$^{-1}$ was analyzed by singular value decomposition (SVD) using the implemented SVD routine of the program package “Mathematica” (version 4.2, Wolfram Research, USA). A fundamental question concerning the evaluation of the kinetic data, particularly in the present case, is the reaction order of the process under study. Since aggregation is involved in the transition process, an apparent higher order reaction must be taken into consideration. First order reactions follow exponential functions 

$$A(t) = A(0) \cdot e^{-(k \cdot t)}$$

while higher order reactions are characterized by a power law of the form

$$A(t)^{(n-1)} - A(0)^{(n-1)} = (n-1) \cdot k \cdot t$$

Eq. 1

where $A$ stands for any physical quantity, $n$ is the reaction order and $k$ is the intrinsic reaction rate. A good test of the reaction order without explicitly specifying the type of the functional
dependence is to plot \((A(t) - A(0))/t \) versus \(A(t)\) (23). This plot is strictly linear for \(n = 2\), allowing to estimate \(k\) from the slope of the data, and shows characteristic curvatures for \(n = 1\) and higher order reactions. Both FTIR and CD kinetics were treated in this way. In special cases \((n > 1)\) the kinetics were fitted directly by a power law function with arbitrary \(n\). If \(A(t)\) is directly the monomer concentration \(c(t)\) or is proportional to \(c(t)\), from Eq. 1 follows

\[
\text{Eq. 2} \quad c(t) = c(0) \left( \frac{1}{1 + (n-1)c(0)^{n-1} \cdot k \cdot t} \right)^{-\frac{1}{n-1}}
\]

This enables one to calculate the reaction order \(n\) from the concentration dependence of the apparent reaction rate \(\tau^{-1} = (n-1)c(0)^{n-1} \cdot k\) by plotting \(-\ln(\tau)\) versus \(\ln(c(0))\).

**Results**

Large quantities of recombinant PrP (10–20 mg per liter of *E. coli* culture) were obtained in > 99 % purity as proven by SDS PAGE, HPLC, and MS (data not shown) due to the two-step purification procedure. The purified PrP was quantitatively oxidized as proven by HPLC (data not shown).

**Steady-state FTIR spectroscopy**

*Fig. 1, near here*

The stopped flow device with its sealed flow cell enabled the recording of highly reproducible FTIR spectra of the prion protein in H\(_2\)O with a high signal to noise ratio of 250 at the amide I peak near 1653 cm\(^{-1}\) as is exemplarily illustrated in Fig. 1A. This figure shows superimposed 7 independently measured PrP spectra (note the small absorbance values due to the relatively low PrP concentration and the 8 \(\mu\)m pathlength of the measurement cell). For band assignment and detailed analysis of spectral features, second derivatives were calculated in order to enhance the apparent spectral resolution (Fig. 1B). Band assignments were made according to the relevant
literature (see e.g. (24-26)). Briefly, the prominent band centered at 1653 cm\(^{-1}\) (see Fig. 1A) is due to the amide I mode (essentially comprising the C=O stretching vibration of all backbone amide groups), while the band at 1550 cm\(^{-1}\) is due to the amide II mode (essentially due to the N–H in-plane bending and C–N stretching motions of the secondary amide functional groups) (24). Both bands are conformation sensitive and – particularly the amide I band – are frequently used to describe the secondary structure of proteins (for reviews see (25-27)). Calculation of second derivatives (Fig. 1B, curves 1 and 2) revealed a fine structure of several component bands that can be assigned to different secondary structure elements and specific amino acid side chains. The prominent bands at 1652 and 1550 cm\(^{-1}\) are both due to \(\alpha\)-helical and the amide I component at 1678 cm\(^{-1}\) to turn and/or loop structures (25,26). Only a very weak band is visible at 1628 cm\(^{-1}\) due to \(\beta\)-sheet structure. The band at 1517 cm\(^{-1}\) is attributed to the amino acid side chain absorbance of tyrosine, the bands at 1575, 1597, and 1615 cm\(^{-1}\) are tentatively assigned to vibrational modes of aspartate, glutamine, and predominantly tyrosine residues, respectively (28). Between pH 7.0 and pH 4.2 practically no structural changes of PrP could be detected as judged from the second derivative spectra of Fig. 1B (curves 1 and 2) and the difference spectrum between both (Fig. 1B, curve 3). The small difference feature around 1646 cm\(^{-1}\) is possibly due to a small frequency shift and intensity change of the \(\alpha\)-helix band between PrP at pH 7.0 and at pH 4.2 indicating some minor differences in helical structure. This finding is in good conformity with our CD and DLS data, which indicated a small decrease in helical content and compactness of PrP on the transition from pH 7.0 to pH 4.2 (see below). The band at 1415 cm\(^{-1}\) remains unassigned.

Only very slight differences were observed between the FTIR spectra of hamster and human PrP at pH 7 (data not shown). This is not surprising, because both proteins have a high sequence homology (87 % of the amino acids are identical) and exhibit almost identical three dimensional
structures as was proven by NMR spectroscopy (29,30). All experiments presented in the following were carried out using hamster PrP.

We have reexamined unfolding of PrP at room temperature under the influence of chaotropic agents using FTIR spectroscopy. Previous investigations by CD spectroscopy (see (31) for review) have indicated a completely reversible two-state unfolding/refolding transition at pH 7. However, an intermediate state could be detected at pH 4.0 and 1–1.5 M GuHCl (13), pH 3.6 and 0.75–1.75 M GuHCl (32), pH 5.0 and 2–2.5 M GuHCl (33), or pH 4.0 and 3.5–4.5 M urea (34), respectively.

The differences in unfolding at pH 7 and pH 4.2 could be easily seen when PrP was subjected to 1 M GuHCl. Fig. 1C, curve 1 indicates that the secondary structure of PrP underwent almost no changes at pH 7.0, but was dramatically transformed at pH 4.2 (Fig. 1C, curve 2). The latter spectrum as well as the difference spectrum between curve 2 and curve 1 (Fig. 1C, curve 3) prove that the structural transition was mainly characterized by a loss in α-helical structure (positive bands at 1652 and 1550 cm⁻¹) and an increase in β-sheet structure (negative bands at 1691, 1620, and 1537 cm⁻¹).

To investigate the influence of pH and denaturant concentration on the secondary structure of PrP over a broader denaturant concentration range, kinetic and equilibrium data were collected for GuHCl at concentrations of 0.1 to 2.0 M both at pH 7.0 and pH 4.2.

**Fig. 2, near here**

As monitored by the amide II α-helix band at 1550 cm⁻¹, PrP was cooperatively unfolded by GuHCl at pH 7 (Fig. 2A). The α-helical band at 1550 cm⁻¹ remained almost constant at GuHCl concentrations up to 1.0 M. Above this concentration the band decreased steeply in intensity while a broad band centered also at 1550 cm⁻¹, which stands for the unfolded protein species, appeared. No increase in β-sheet content was detected at 1620–1640 cm⁻¹ in the amide I region.
In contrast, dramatic changes of the secondary structure of PrP could be observed at pH 4.2 already in the GuHCl concentration range of 0.3–1.0 M (Fig. 2B): The decrease in intensity at 1550 cm\(^{-1}\) (\(\alpha\)-helices) was observed concomitantly with an increase in intensity of the broad band centered around 1537 cm\(^{-1}\) (\(\beta\)-sheets). At the same time, the appearance of the diagnostic \(\beta\)-sheet band at 1620 cm\(^{-1}\) in the amide I region (see Fig. 2B) together with a band at 1691 cm\(^{-1}\) (see Fig. 1C and Fig. 3A) is indicative of newly formed extended antiparallel pleated \(\beta\)-sheet structures which can often be found in aggregated proteins (35) (see also Discussion). The almost isosbestic point at approximately 1544 cm\(^{-1}\) underlines the finding of a simultaneous disappearance of the \(\alpha\)-helical rich native structure and the appearance of a new PrP species rich in \(\beta\)-sheet structure. This species turned out to be a multimeric state of PrP, which could be unfolded by high concentrations of denaturant, but which was stable in the absence and at low concentrations of denaturant. The kinetics of formation and the structural properties of this \(\beta\)-rich, multimeric state were the subject of the investigations described below.

**Kinetic FTIR spectroscopy**

Since 1.0 M GuHCl at pH 4.2 appeared to be optimal to study the transition process, we investigated the structural changes of PrP under these conditions in greater detail. To kinetically detect these changes, difference spectroscopy was applied as described in the Experimental Procedures section.

**Fig. 3, near here**

The reaction-induced difference spectra obtained at different times after a GuHCl concentration jump from 0 to 1 M (Fig. 3A) clearly show that the main events were a loss in \(\alpha\)-helical structure (decrease of the band at 1653 cm\(^{-1}\)) and formation of extended antiparallel \(\beta\)-sheet structure (increase of the bands at 1621 and 1691 cm\(^{-1}\)). The kinetic curve obtained from the time
evolution of the difference peak at 1621 cm\(^{-1}\) indicating the increase in \(\beta\)-sheet structure (Fig. 3B) could be fitted very well by a function of the form

\[
A_{1621}(t) - A_{1621}(0) = a \left(1 - \frac{1}{1 + \frac{t}{\tau}}\right), \text{ with } a = 0.0026 \text{ and } \tau = 2.42 \text{ s.}
\]

This time dependence is consistent with a second order reaction. A further helpful visual test concerning the order of the apparent reaction rate is to plot \((A_{1621}(t) - A_{1621}(0))/t\) versus \(A_{1621}(t)\) (inset of Fig. 3B). Such a plot must be linear for a second order reaction, while remarkable and characteristic deviations from linearity are expected for both first and higher order reactions (see Experimental Procedures). No systematic deviation from linear behavior could be detected. A kinetic analysis of the difference intensities at 1653 cm\(^{-1}\) indicating the loss of \(\alpha\)-helix led to the same results. Particularly, the apparent rate constants obtained of the changes of the bands at 1621 and 1653 cm\(^{-1}\) agreed within the experimental error. SVD analysis of the entire series of FTIR spectra yielded only two significant components according to the criterion developed by Henry and Hofrichter (36). No further significant components could be constructed by using the so-called rotation procedure. Accordingly, the spectral changes observed point to an apparent two-state transition.

At the experimental conditions chosen (1 M GuHCl, pH 4.2) no \(\beta\)-sheet was formed within the experimental dead time. This was inferred from the absence of a difference band at 1621 cm\(^{-1}\) in the difference spectrum (Fig. 3C, curve 1) between the first spectrum obtained after mixing PrP with GuHCl and the steady state spectrum of PrP in the absence of GuHCl (see also Experimental Procedures). For comparison the total spectral changes observed for the transition process in 1 M GuHCl are also shown (Fig. 3C, curve 2). The large difference band at 1621 cm\(^{-1}\) represents the newly formed \(\beta\)-sheet.
**CD spectroscopy**

**Fig. 4, near here**

CD spectra measured in the absence of GuHCl at pH 7.0 and pH 4.2 are shown in Fig. 4, curves 1 and 2, respectively. The nearly identical spectra confirmed the FTIR results (Fig. 1B) that the secondary structure was essentially unchanged on the transition from pH 7 to pH 4.2. The spectra had a pattern that is typical of a protein that has predominantly helical secondary structure.

After a GuHCl concentration jump from 0 to 1 M at pH 4.2, the CD spectrum changed with a strongly concentration-dependent rate. The spectrum after complete transition (Fig. 4, curve 4) had the characteristic features of proteins with β-rich secondary structure. It is an important question, whether this α-to-β transition proceeded already in the monomeric state or was entirely coupled with the aggregation process detected, e.g., by light scattering (see below). Therefore the first CD spectrum measured 1 min after mixing with GuHCl at low protein concentration (0.27 mg/ml) is also shown (Fig. 4, curve 3). The influence of aggregation should be negligible at this time, because SHaPrP was expected to be still in the monomeric state. This was confirmed by size exclusion chromatography (SEC, data not shown). This spectrum (Fig. 4, curve 3) was clearly distinguishable from both the spectrum in the absence of GuHCl (curve 2) and that after transition has been completed (curve 4). The pattern of the spectrum was, however, more similar to that of the initial helical state.

**Fig. 5, near here**

The specific ellipticity at 215 nm, [θ]_{215}, was suitable to monitor the kinetic changes of the CD signal at different SHaPrP concentrations. The kinetic curves are shown in an appropriate form (Fig. 5A, see also Experimental Procedures) allowing to get an idea of the approximate reaction order. Apart from some deviations at small values for the time t, the kinetic curves were linear in this representation, suggesting the existence of an apparent second order reaction. To estimate the
concentration dependence of the apparent reaction rate, we have plotted the values of 
\[ |m| = k_{\text{app}} = \tau^{-1} \] obtained from the slopes \( m \) in Fig. 5A versus \( c \) in a double logarithmic plot (Fig. 5B). The slope of a linear fit was 1.6. This corresponded to an apparent reaction order of 2.6. The inconsistency of the apparent reaction order estimated either from the time dependence of the CD signal or from the concentration dependence of \( \tau \) hinted at a more complicated transition process consisting of at least two reaction steps.

**The aggregation process studied by light scattering and electron microscopy**

Fig. 6, near here

We have measured SLS and DLS at different concentrations for the stable states in the absence of GuHCl at pH 7 and pH 4.2. The size distributions obtained from the DLS data (Fig. 6) consisted of only one peak, which could be attributed to monomers, at pH 7, but of two peaks at pH 4.2, where the second smaller peak originated from the presence of small amounts of aggregates. The ratio of the peak areas could be used to separate the contributions of monomers and aggregates to the static light scattering intensity. Extrapolating light scattering from monomers to zero protein concentration yielded molar masses \( M \) of (16,000 ± 2,000) g/mol and (18,000 ± 2,000) g/mol at pH 7 and pH 4.2, respectively. Diffusion coefficients obtained from DLS were extrapolated to zero protein concentration yielding Stokes radii \( R_S \) of 2.36 ± 0.04 nm and 2.66 ± 0.05 nm for pH 7 and pH 4.2, respectively. This means that the PrP molecules were slightly less compact at pH 4.2 as compared to pH 7. \( M \) and \( R_S \) obtained at pH 4.2 in the absence of GuHCl were used as reference values for the relative increases in both quantities during aggregation.
Fig. 7, near here

The time course of aggregation after addition of GuHCl by manual mixing was monitored by SLS and DLS at 20 °C. The increase in the relative molar mass is shown for 3 selected protein concentrations in Fig. 7A. The strong concentration dependence of the rates is clearly visible. The highest concentration was close to that used for the FTIR experiments. However, the aggregation kinetics at concentrations above 2 mg/ml was too fast to be measured using manual mixing techniques. The transient maximum in the average mass of the oligomers formed during the initial stage of aggregation depended on protein concentration. An “overshoot” in size was significant at high protein concentrations. Extrapolation of this transient maximum to zero protein concentration yielded a relative mass corresponding to 7.9 ± 1.0 (Fig. 7B). The corresponding average Stokes radii of the oligomeric species ranged from 6.6 to 12.3 nm. Plotting the Stokes radii versus the corresponding maxima in the relative mass (Fig. 7C) enables one to estimate the dimensionality of the oligomers. The apparent rate constants of the size transition were obtained from the half-times of the transitions. The concentration dependence of the half-times is shown in Fig. 7D. The slope of this plot (s = 2) is consistent with a reaction order of 3. During long-time storage of samples a second slow increase in both the relative mass and the relative Stokes radius was observed. The entire growth process is shown for a protein concentration of 0.5 mg/ml in Fig. 7E.

Fig. 8, near here

In order to determine the morphology of the growing particles, small amounts of this sample were withdrawn 25 days (3.6·10^4 min) and 55 days (7.9·10^4 min) after starting the transition and subjected to electron microscopy (Fig. 8). Fig. 8A demonstrates that the particles formed during the first kinetic phase had mostly spherical or annular structures with diameters of 10–15 nm (arrows and inset in Fig. 8A). Moreover, some larger spherical particles with diameters of about...
20 nm and a few fibrillar structures can be seen. Comparison of Fig. 8A and Fig. 8B shows that the increases in mass and Stokes radius at later times can be attributed to the length growth of curved fibrillar structures.

Discussion

The transition of different recombinant prion protein sequences into β-rich, aggregated structures has been studied by several groups (9-11,31,37,38). The consistent finding of the latest studies is the appearance of a particular β-rich oligomer during the assembly process. At present it is not fully established, whether the oligomer itself is a toxic species, an on-pathway precursor of later stages of aggregated PrP, or an off-pathway by-product of the assembly process. Since the oligomer was formed by the full-length-protein (11) as well as by truncated sequences (37) under a variety of conditions, mostly at acidic pH, a more general role of the oligomer for the transition process can be anticipated. The formation of the oligomer involves dramatic changes of secondary structure during the transition of the prion protein. The aim of this study was to obtain more detailed information about these secondary structure changes, the size distribution and morphology of the relatively stable oligomers, the kinetics of the transition reaction, particularly its concentration dependence, and the sequence of steps involved in this process.

FTIR spectroscopy

To elucidate the transition process of recombinant PrP on the level of secondary structure in more detail, the use of FTIR spectroscopy was a very appropriate technical approach. In this study FTIR spectra of high quality as shown in Figs. 1 and 2 were obtained. Note that the FTIR spectra were measured in H₂O at concentrations that are low for FTIR. Spectra published so far were measured in D₂O or as either dried films or liquid spots (H₂O) by attenuated total reflection.
The use of the specially designed micro stopped-flow device in combination with FTIR rapid scan techniques enabled us to monitor the α-β-transition (which is relatively fast at protein concentrations used in this study) in real time with sufficiently high time-resolution and excellent reproducibility. The small structural changes which occurred within the dead time of the experiments will be discussed later.

Up to now the α-β-transition of recombinant PrP was studied mainly by CD spectroscopy (13,34,37,38,42). The advantage of FTIR spectroscopy is that bands specific for α-helices, β-sheets, turns, and a number of amino acid side-chains are well separated. Thus, changes in these types of structures can be detected independently. Our results demonstrate that under the conditions chosen formation of β-structure and loss of α-helical structure occurred simultaneously. This finding does not exclude a transition through a more or less unfolded state on the pathway from the α-rich to the β-rich structure. However, the population of such intermediate states, if existing, must be very low. Furthermore, FTIR spectroscopy can distinguish between different β-structures: Antiparallel β-sheets show an additional high frequency band around 1690 cm\(^{-1}\) that could not be observed in parallel β-sheets as has been shown for model peptides (43,44). Additionally, FTIR can distinguish between intermolecular and intramolecular β-strands, which generally differ in length and/or hydrogen bonding strength (24,26,35). No infrared spectrum of globular proteins has been reported in the literature showing intramolecular β-sheet bands at frequencies as low as 1621 cm\(^{-1}\) in H\(_2\)O or even in D\(_2\)O. The occurrence of difference bands at 1621 cm\(^{-1}\) as well as at 1691 cm\(^{-1}\) (see Fig. 3A) suggests that the β-structures formed during the transition process of PrP must essentially involve extended antiparallel β-sheets (35). Additionally, the frequency position of the β-sheet band at 1621 cm\(^{-1}\) specifies rather strong hydrogen bonding. Taken together with our DLS data (Fig. 7) we assume...
that PrP has most probably formed extended intermolecular β-sheet aggregates upon incubation in appropriate concentrations of GuHCl at pH 4.2.

In comparison to the FTIR spectra of aggregates from recombinant PrP obtained in this study, FTIR spectra of PrP-res (i.e. the scrapie-associated, proteinase K resistant core of PrP\textsuperscript{Sc}) isolated from infected hamster brains show remarkable differences. When measured as aqueous suspensions, the main bands of PrP-res indicative for β-sheet were found at 1694, 1636, and 1626 cm\(^{-1}\) (45), representing most likely both intra- and intermolecular β-structure with different hydrogen bonding properties. The exact frequency positions of the β-bands differ between the different PrP-res strains measured\(^2\). Hence, the β-sheet hydrogen bonds of the aggregated recombinant PrP seem to be significantly stronger than those found in isolated PrP-res. Moreover, all PrP-res samples so far documented exhibited a characteristic and prominent amide I band component at 1658 cm\(^{-1}\). These differences have to be studied by future FTIR experiments in more detail.

The strong tyrosine band at 1517 cm\(^{-1}\) did not shift during the partial unfolding or transition process of PrP. However, when PrP was unfolded completely by 6 m GuHCl at pH 7, the band shifted to 1516 cm\(^{-1}\) (data not shown). A shift in this range is significant and typical for un- and refolding processes of proteins (46,47). This indicates that the alterations of the secondary structure during the transition process did not change the micro-environments of the tyrosine residues as strongly as a complete unfolding procedure did. This observation is especially interesting, since the primary structure of SHaPrP\textsuperscript{90–232} contains 5 tyrosine residues in α-helices, 2 tyrosines in β-sheets, and 3 tyrosines in loops and turns. It is expected that an α-β-transition of the secondary structure of PrP should lead to a dramatic change in the micro-environment of all tyrosines involved in the reassembly process and thereby to distinct spectral changes of these
groups. One possible explanation for our experimental finding of no tyrosine peak shift might be mutual cancellation of the individual tyrosine peak shifts to a net difference of zero.

**Folding Kinetics**

Important information about the transition process could be obtained from the kinetic traces and the concentration dependence of the kinetics. For kinetic FTIR experiments under the given solvent conditions (H₂O, 1 M GuHCl), protein concentrations of at least 2–3 mg/ml were needed. Thus, FTIR and CD spectroscopy had to be combined in order to monitor changes in secondary structure down to concentrations as low as 0.1 mg/ml. Kinetic far UV CD spectra were measured from 260 nm down to 207 nm. The restricted lower wavelength limit was due to presence of 1 M GuHCl.

Elucidating the average starting conformation of the monomeric prion protein after transferring it into conditions optimal for the α-β-transition is important for studying the whole transition process. Both FTIR and CD spectroscopy have consistently demonstrated that changes in pH alone, namely from strictly native conditions at pH 7 to pH 4.2, do not significantly influence the secondary structure. This finding is in agreement with previously published results (13,32). From the evaluation of the dead time events (Fig. 3C, see also Experimental Procedures) we conclude that no β-sheet structures were formed within the dead time of the experiment. However, other structural variations must have occurred, since spectral changes at 1720–1500 cm⁻¹ and 1320–1200 cm⁻¹ were observed which have to be interpreted in future approaches. The evaluation of a possible loss in α-helical structure was unfortunately prevented by the strong noise level around 1653 cm⁻¹ caused by the strong absorbance of water and GuHCl within the amide I region.

A detectable loss in helical structure was indicated by the pattern of the first CD spectrum after mixing. Although the duration of dead time was much longer in the case of CD (~ 1 min), aggregation taking place within the dead time was negligible because of the low PrP
concentration. Quantitative estimations of the secondary structure from CD data could not be performed because of the limited spectral range (207–260 nm).

Within the time range available in this study, both FTIR and CD spectra point to an apparent two-state transition of the secondary structure. This was proven by the singular value decomposition technique and by comparing the time evolution of several bands of the FTIR difference spectra. Accordingly, by our spectroscopic methods we could not detect any intermediates during the monomer-oligomer-transition. This means that intermediates were either only weakly populated (see below) or their spectral features were close to those of the initial or final states. The kinetic traces of the FTIR band at 1621 cm⁻¹ and the CD amplitude at 215 nm were consistent with a second order reaction, because the data could be fitted by a function according to Eq. 1 with \( n = 2 \). However, the concentration dependence of the rate constant \( r^{-1} \) of the CD changes yielded a reaction order of \( 2.6 \pm 0.3 \). Notwithstanding the experimental errors of these estimations, the variations should be taken as an indication for a more complicated transition of at least two reaction steps.

**Aggregation process**

The aggregation process accompanying the secondary structure transition was studied by SLS and DLS at concentrations between 0.2 and 2.9 mg/ml. At the highest concentration, which is comparable to that used for the FTIR experiments (4 mg/ml), the initial aggregation process was practically completed within the dead time of the SLS/DLS experiment. A more direct comparison between changes in secondary structure and aggregation was possible on the basis of CD and light scattering data between 0.2 and 0.5 mg/ml. Comparable half-times of 4.2 min and 7.7 min were measured at 0.5 mg/ml by SLS and CD, respectively. A better understanding of the entire aggregation process was achieved by determining the morphology of the growing species.
by electron microscopy at particular times after starting the transition. The aggregation process revealed several interesting features.

1. The average mass of the oligomers at the transient maximum was found to depend linearly on protein concentration (Fig. 7B). This result possibly explains why different sizes of the oligomer have been reported in the literature (10,11).

2. Linear extrapolation of the transient maximum value to zero protein concentration (Fig. 7B) yielded a relative mass of 7.9 ± 1.0. This leads to a stoichiometry of 8 monomers forming a stable misfolded oligomer. Accordingly, mostly octamers were formed at low protein concentrations. Additionally, the larger oligomers formed at higher concentration showed a tendency to disaggregate at later times (“overshoot”, Fig. 7A). Thus, the octamers appear to be the most stable species and will be termed “critical oligomers” according to similar observations during the assembly process of other proteins towards fibrillar structures (48). The oligomers appeared to be stabilized by intermolecular β-sheets with strong hydrogen bonds according to our FTIR data. Particularly, the detection of these octamers stabilized by strongly hydrogen bonded β-sheets is fully consistent with the observations of Baskakov et al. (10).

3. It is important to measure the hydrodynamic Stokes radii and the mass of individual molecules or aggregates independently, e.g. by DLS and SLS, respectively. Estimations of the mass from the measured Stokes radii, as it is frequently done, requires the knowledge of the correct scaling law of the form $R = a \cdot M^{1/d}$, where $a$ is constant and $d$ is the dimensionality of the particles. Compactly folded, spherical structures ($d = 3$) are often implicitly used in estimations of $M$ from $R_S$. The other way round, measurements of pairs of $M_{rel}$ and $R_S$ at different concentrations (see Fig. 7C) make it possible to determine the dimensionality of the oligomers. From the slope $s = 1/d = 0.345$ in Fig. 7C we obtained $d = 2.98$, indicating that the oligomers had a compact, spherical structure.
4. The kinetic traces of the changes of the relative mass were used to obtain apparent rate constants of aggregation. For this purpose we have determined the half-times \( t_{1/2} \) for reaching half of the transient maximum as approximate values of \( \tau \), because direct fits of the kinetic curves were not possible due to the overshoot phenomenon at high concentrations (Fig. 7A). The slope \( s = 2.0 \pm 0.2 \) in Fig. 7D is consistent with an apparent reaction order of 3. Combining this result with those obtained from the kinetic spectroscopic data, our estimations of the reaction order fall into the range between 2 and 3. In any case, we find a reaction order smaller than the apparent fifth order obtained by Baskakov et al. (10), when the \( \beta \)-oligomer was formed at 5 M urea starting from the unfolded state at 10 M urea.

5. The spherical shape of the rapidly formed aggregates was also confirmed by electron microscopy (Fig. 8). Closer inspection of the morphology reveals annular shapes, particularly among the smaller particles. It is conceivable that the most stable octamer consists of a ring structure of 8 monomers.

6. The rapidly formed oligomers were able to assemble further during a second, much slower process (Fig. 7E) in a linear fashion leading to curved protofibrillar structures, as can be judged from electron micrographs (Fig. 8). Protofibril formation seems to be a side reaction of a possible subpopulation of the oligomers, since the amount of oligomers was only partly reduced even after observation times of about 2 months. Furthermore, the growth rate of a sample with fourfold higher concentration was only slightly higher as compared to that shown in Fig. 7E. Straight mature fibrils were not observed under the conditions used in this study. Therefore we suppose that other structures larger than the protofibrils observed cannot be obtained without further rearrangement of the basic internal structure of the oligomers. However, the oligomers may provide an ideal pool of pre-aggregated material, enabling further cooperative structural transitions under modified external conditions, e.g. at elevated temperatures.
7. Up to now we failed to detect any smaller oligomers than the critical octamers, particularly by measuring SEC chromatograms at different times using protein concentration of 0.08 mg/ml (data not shown). Similar conclusions can be drawn from the SEC data reported by others (10,38). On the other hand, the measured apparent reaction order $\leq 3$ and the apparent bimolecular step involved in the changes of the spectroscopic properties demand the transient population of smaller oligomers such as dimers or tetramers.

**A possible model for the aggregation process**

*Fig. 9, near here*

In the following, we will summarize the most important data of our study and previous findings in a model shown in Fig. 9. Upon the change from native environmental conditions to those favoring the transition (1 M GuHCl, pH 4.2) PrP$^C$ is transferred into the helical state $\alpha'$. In the $\alpha'$-state, PrP$^C$ is destabilized and has a somewhat less helical structure and an enhanced tendency to aggregate. The subsequent steps are presently still not resolved very well and are buried within the “gray box” on the pathway from $\alpha'$ to the oligomer. However, they must fulfill some kinetic requirements. Furthermore, we have indications from spectroscopic data and SEC that the intermediate states involved in these steps are only extremely weakly populated. Presumably, all reactions steps on the left side of B in Fig. 9 are reversible. However, once a critical oligomer size with its specific stabilizing interactions is reached, the backward reaction constant becomes very small, making the transition on the right hand side of B in Fig. 9 to an irreversible one. The critical oligomer size is 8 monomers in our case. The linear concentration dependence of the oligomer size is indicative of additional bimolecular steps leading to multimers of octamers. The slow disintegration of larger oligomers (“overshoot” phenomenon) cannot be explained adequately at present.
Though we cannot resolve the details within the “gray box” in Fig. 9, our kinetic data and the observed stoichiometry pose some constraints on the possible mechanisms. The apparent bimolecular reaction \((n = 2)\) revealed by the kinetic traces of our optical data together with the apparent third order reaction consistent with the concentration dependence of the reaction rates \((2.6 < n < 3)\) can be explained consistently by assuming two consecutive bimolecular steps with a weakly populated intermediate. At least one additional reaction step is required to fulfil the stoichiometric constraints to build up the critical octamer. A plausible scheme is shown in Fig. 9, however until now no clear signature of this particular scheme was found in our kinetic data.

The observed kinetic features of the reaction leading to misfolded \(\beta\)-octamers are in marked contrast to proposed mechanisms of misfolding and amyloid formation (49-51). The involvement of oligomers larger than dimers in conformational transition rules out the heterodimer mechanism (49). The secondary structure transition according to a bimolecular reaction contrasts with the postulated unimolecular reaction of conformational transition of unstructured oligomers in the so-called nucleated conformational conversion mechanism (50). The involvement of the \(\beta\)-sheet rich octamer in a sequence of bimolecular steps leading to larger multimers cannot be explained assuming a nucleated polymerization mechanism (51). The exclusion of a nucleated polymerization mechanism is further supported by the absence of any lag phase. These considerations lead to the conclusion that a distinctive, in its details so far unknown mechanism must be responsible for the observed transition process.

**Toxicity of Oligomers**

Whether the oligomers and particularly the critical octamers are themselves a toxic species, is still an open question. Such a role could be inferred from observations that small oligomers of the A\(\beta\) peptide are toxic at early stages of Alzheimer’s disease (52). A possible toxic role was also postulated for small aggregates of \(\alpha\)-synuclein. Volles and Lansbury (53) have shown that small
annular aggregates of α-synuclein enhance the permeability of model membranes by a pore-like mechanism. Note that annular structures have been observed for the critical oligomers of PrP (Fig. 8). Chiesa and Harris (6) have demonstrated that toxicity and infectivity are not necessarily correlated in prion diseases. Up to now, infectivity could not be shown for β-rich forms of recombinant SHaPrP, neither for the oligomeric nor for the amyloid forms (10). Even as a transient species the critical oligomers could play an important role among the β-rich isoforms described in the literature. The oligomer represents a high local protein concentration behind the energy barrier between the α- and β-isoforms and is therefore a good candidate for further structural transitions. Baskakov et al. (10) have shown that the oligomer is not a substructure of the amyloid form. However, amyloid formation without the appearance of oligomers was not found.

Further investigations are needed to resolve the transition mechanism, particularly the “gray box” reaction steps. Possible ways could be the use of appropriate changes in environmental conditions or the use of selected mutant proteins. Vanik and Surewicz (54) found an increased propensity for conformational transition of a disease-associated mutation of recombinant human PrP\textsuperscript{C}. These and further strategies should be applied to enhance the population of the transient states on the pathway to the oligomer in order to study their structures and kinetic roles. An interesting question is, whether there are stable oligomers smaller than the octamers observed. Wille et al. (55) have deduced a hexameric arrangement of monomers in two-dimensional crystals of purified infectious fractions of prion protein using electron crystallography. This finding supports the idea that hexamers could also exist under presently unknown conditions. Whether octameric or smaller oligomers obtained \textit{in vitro} are toxic and/or infectious, is an exiting question.
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Footnotes

1 The abbreviations used are: AU, absorbance unit; BSE, bovine spongiform encephalopathy; CD, circular dichroism; CJD, Creutzfeldt-Jakob disease; cw, continuous wave; DLS, dynamic light scattering; EM, electron microscopy; FTIR, Fourier transform infrared; GuHCl, guanidine hydrochloride; HPLC, high performance liquid chromatography; huPrP^{91–231}, fragment comprising amino acids 91–231 of the human prion protein; LB + Amp, Luria-Bertoni medium containing 100 µg/ml ampicillin; PrP, prion protein, PrP^C, cellular isoform of prion protein; SEC, size exclusion chromatography; SVD, singular value decomposition; PrP-res, proteinase K resistant core of PrP^Sc; PrP^Sc, scrapie isoform of prion protein; SHaPrP^{90–232}, fragment comprising amino acids 90–232 of the Syrian hamster prion protein; SLS, static light scattering; TFA, trifluoroacetic acid.

2 Sashko Spassov, personal communication.

Figure Legends

Fig. 1: FTIR spectra of SHaPrP^{90–232}. A, 7 superimposed and independently measured absorption spectra of SHaPrP^{90–232} at pH 7.0. Spectral contributions from water and buffer are subtracted. B, second derivative spectra of SHaPrP^{90–232} at pH 7.0 (curve 1) and at pH 4.2 (curve 2); the difference spectrum between both (pH 4.2 – pH 7.0) is shown by curve 3. Only very small differences between both spectra are observed. The spectra 1 and 2 are vector normalized with reference to the strong tyrosine band at 1517 cm⁻¹. Marked bands are discussed in the text. C, second derivative spectra of SHaPrP^{90–232} at 1.0 M GuHCl and pH 7.0 (curve 1) and at 1 M GuHCl and pH 4.2 (curve 2). Curve 3 shows the difference spectrum between curves 1 and 2 (pH 4.2 – pH 7.0). Dramatic differences between both spectra are visible, particularly in the structure.
sensitive amide I and amide II region. Spectra 1 and 2 are vector normalized with reference to the tyrosine band at 1517 cm\(^{-1}\).

Fig. 2: Second derivative spectra of SHaPrP\(^{90-232}\) in the amide I and amide II region at different concentrations of GuHCl and at different pH values. \(A\), pH 7.0; \(B\), pH 4.2. The GuHCl concentrations are 0 M (curves 1), 0.3 M (curves 2), 0.7 M (curves 3), 1.0 M (curves 4), 1.5 M (curves 5), and 2.0 M (curves 6). The spectra are corrected for buffer, GuHCl, and – if necessary – water vapor contributions. The spectral range between 1640 and 1688 cm\(^{-1}\) is dominated by an intensive absorption band of GuHCl which resulted in a spectral noise too high to evaluate the \(\alpha\)-helix band at 1653 cm\(^{-1}\) quantitatively at GuHCl concentrations \(\geq 0.7\) M. Clearly visible is the evolution of \(\beta\)-sheet structure at pH 4.2 (see bands at 1620 and 1537 cm\(^{-1}\)). At pH 7.0 no \(\beta\)-sheet is formed.

Fig. 3: \(\alpha\)-\(\beta\)-transition of PrP monitored kinetically by FTIR spectroscopy. \(A\), difference spectra of SHaPrP\(^{90-232}\) after mixing with GuHCl to a final concentration of 1 M, buffered by 20 mM sodium acetate at pH 4.2. Spectra from bottom to top at 1621 cm\(^{-1}\): average of difference spectra 1–5, 6–10, 11–15, 16–20, 21–25, 26–30, 31–35, 36–40, 41–45, 46–50, 51–55, and 56–60. The 60 difference spectra are obtained by calculating the difference between the last (60\(^{th}\)) spectrum and each single spectrum obtained at a particular time, \textit{i.e.} spectrum 1 – spectrum 60, spectrum 2 – spectrum 60 etc. Band assignments are made in the text. \(B\), kinetic curve for the appearance of \(\beta\)-sheet structure by monitoring the difference peak at 1621 cm\(^{-1}\). The fitting curve is represented by a solid line. To check the reaction order, the same kinetic data is displayed in the plot of the inset. A second order reaction is expected, because this plot does not deviate systematically from linearity (see Experimental Procedures). \(C\), comparison between the structural differences of
SHaPrP^{90–232} occurring within the dead time of the experiment (curve 1) and the differences observed during the whole observation time (curve 2). Within the dead time no β-structure is formed, as can be seen by the absence of a difference band at 1621 cm\(^{-1}\).

Fig. 4: CD spectra of SHaPrP^{90–232} in the natively folded states at 20 mM NaH\(_2\)PO\(_4\), pH 7.0, c = 1.13 mg/ml (1), 20 mM sodium acetate, pH 4.2, 50 mM NaCl, c = 1.19 mg/ml (2) as well as 1 min (3) and 116 h (4) after addition of GuHCl (1 M final concentration) to 20 mM sodium acetate, pH 4.2, 50 mM NaCl, c = 0.27 mg/ml. All measurements were done at 23 °C using 0.1 mm (curves 1 and 2) and 1 mm cells (curves 3 and 4), respectively. Because of the strongly absorbing GuHCl, spectra 3 and 4 are available only for wavelengths above 207 nm.

Fig. 5: A, kinetics of secondary structure transition monitored by the ellipticities \([\theta]\) at 215 nm for SHaPrP^{90–232} concentrations of 0.125 (▲), 0.2 (▼), 0.32 (□), 0.35 (■), 0.47 (○), and 0.50 mg/ml (●) in 20 mM sodium acetate, pH 4.2, 50 mM NaCl, 1 M GuHCl at 23°C. B, concentration dependence of the apparent rate constants \(k_1^{-1}\) calculated from the slopes in Fig. 7A.

Fig. 6: Size distributions in terms of light scattering intensity versus populated Stokes radii obtained from DLS data for SHaPrP^{90–232} in the natively folded state in 20 mM NaH\(_2\)PO\(_4\), pH 7.0, c = 1.3 mg/ml (gray bars) and in 20 mM sodium acetate, pH 4.2, 50 mM NaCl, c = 1.1 mg/ml (black bars). The width of the size classes (bars) results from the grid applied in the calculations of the size distribution function using the program CONTIN. Thinner gray bars are used for a clearer presentation of data only.
Fig. 7: A, initial step of aggregation of SHaPrP<sup>90–232</sup> monitored by the increase in the relative masses after mixing with GuHCl (final concentration 1 M) at protein concentrations of 1.25 mg/ml (1), 0.50 mg/ml (2) and 0.20 mg/ml (3) in 20 mM sodium acetate, pH 4.2, 50 mM NaCl, T = 20°C. B, concentration dependence of the transient maximum of the relative mass. C, double logarithmic plot of the relation between the transient maximum of the Stokes radius and the transient maximum of the relative mass. The linear fit yields 1/d = 0.345. D, concentration dependence of the half-time t<sub>1/2</sub> of the changes in the relative mass. E, increase in both the relative mass (●) and the Stokes radius (□) of SHaPrP<sup>90–232</sup> at 0.50 mg/ml, conditions as in A. M and R<sub>S</sub> were normalized to the values in native-like state at pH 4.2 in the absence of GuHCl.

Fig. 8: Electron micrographs of aggregated SHaPrP<sup>90–232</sup>, c = 0.5 mg/ml incubated in 20 mM sodium acetate, pH 4.2, 50 mM NaCl, 1 M GuHCl for 25 days (3.6·10<sup>4</sup> min) (A) and 55 days (7.9·10<sup>4</sup> min) (B). Samples were diluted 25-fold before staining. Annular shapes are frequently observed among the small oligomeric structures. Three of them are marked by arrows; the lower one is shown at higher magnification on the top right in panel A. The late stage is characterized by an increased length of protofibrillar structures.

Fig. 9: Model for the assembly of critical oligomers. The hypothetical sequence of steps between A and B is only one plausible explanation for the observed kinetic features satisfying the stoichiometric requirements.
Tables

Table 1: Distribution pattern of scans per spectrum for all measured GuHCl concentrations. Since no more than 60 spectra could be stored for each rapid scan measurement, the number of scans and thus the time of measurement (larger numbers of averaged scans result in longer times of measurement) for each of the 60 spectra had to be chosen in order to observe the whole reaction with an appropriate time resolution.

| measurement at 0.3 M GuHCl | measurement at 0.5, 0.7, and 1.0 M GuHCl | measurement at 1.5 and 2.0 M GuHCl |
|---------------------------|------------------------------------------|----------------------------------|
| no. of spectra | no. of scans averaged per spectrum | no. of spectra | no. of scans averaged per spectrum | no. of spectra | no. of scans averaged per spectrum |
| 10 | 2 | 10 | 2 | 15 | 2 |
| 10 | 4 | 10 | 4 | 10 | 4 |
| 10 | 8 | 10 | 8 | 10 | 16 |
| 10 | 32 | 10 | 32 | 10 | 32 |
| 10 | 128 | 15 | 128 | 10 | 128 |
| 10 | 512 | 512 | 512 | 512 | 512 |
| total time of measurement: 380 s | total time of measurement: 273 s | total time of measurement: 243 s |
Fig. 1

A

amide I
amide II

B

C

\frac{\partial A}{\partial \nu} (\text{arbitrary units})

\frac{\partial^2 A}{\partial \nu^2} (\text{arbitrary units})
Fig. 2

A

B

\[ \frac{d\alpha}{d\nu^2} \text{ (arbitrary units)} \]

wavenumber (cm\(^{-1}\))

1640 1620 1600 1580 1560 1540 1520 1500
Fig. 3

A

B

C
Fig. 4
Fig. 7

A

B

C

D

E
Fig. 9

\[ \alpha \rightarrow \alpha' + \alpha' \]

monomers

weakly populated intermediates

sequence of bimolecular reactions

stable k-mer (k ≥ 8)
Formation of critical oligomers is a key event during conformational transition of recombinant syrian hamster prion protein.

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