Physical Studies of Conformational Plasticity in a Recombinant Prion Protein[†]

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ABSTRACT: PrP^{Sc} is known to be the major, if not the only, component of the infectious prion. Limited proteolysis of PrP^{Sc} produces an N-terminally truncated polypeptide of about 142 residues, designated PrP 27−30. Recently, a recombinant protein (rPrP) of 142 residues corresponding to the Syrian hamster PrP 27−30 was expressed in *Escherichia coli* and purified (Mehlhorn et al., 1996). rPrP has been refolded into both α-helical and β-sheet structures as well as various intermediates in aqueous buffers. The β-sheet state and two pH-dependent α-helical states were characterized by CD and NMR. The α-helical conformation occurred only after the formation of an intramolecular disulfide bond, whereas the β-sheet form was accessible either with or without the disulfide. Of the different α-helical forms studied, only those refolded in the pH range 5−8 were substantially soluble at physiological pH, exhibiting similar conformations and monomeric analytical sedimentation profiles throughout the above pH range. Furthermore, refolded α-rPrP showed NMR chemical shift dispersion typical of proteins with native conformations, although 2D NMR indicated large segments of conformational flexibility. It displayed a cooperative thermal denaturation transition; at elevated temperatures, it converted rapidly and irreversibly to the thermodynamically more stable β-sheet form. Unfolding of α-rPrP by GdnHCl revealed a two-phase transition with a relatively stable folding intermediate at 2 M GdnHCl. The ΔG values were estimated to be 1.9 ± 0.4 kcal/mol for the first phase and 6.5 ± 1.2 kcal/mol for the second, consistent with a folding core surrounded by significant segments of flexible conformation. By NMR, α-rPrP(acid) isolated at pH 2 without refolding exhibited heterogeneous line widths, consistent with an acid-denatured molten globular state. We conclude that to the extent that rPrP constitutes a relevant folding domain of PrP^{C}, the various conformations exhibited by rPrP suggest that the PrP sequence may be intrinsically plastic in its conformations; indeed, portions of PrP^{C} may possess a relatively open conformation which makes it susceptible to conversion into PrP^{Sc} under appropriate conditions.

Prions cause neurodegenerative illnesses of human and animals (Prusiner, 1991, 1996). Four neurologic disorders in humans have been attributed to prions: kuru, Creutzfeldt−Jakob disease (CJD), Gerstmann−Sträussler−Scheinker disease (GSS), and fatal familial insomnia (FFI) (Gajdusek, 1977; Masters et al., 1981; Medori et al., 1992). Interestingly, familial CJD, GSS, and FFI not only are inherited illnesses due to point mutations in the prion protein (PrP)[1] gene, but also are transmissible to experimental animals. In animals, bovine spongiform encephalopathy (BSE) or "mad cow" disease has caused more than 160 000 cattle deaths in Great Britain and is thought to be caused by prion-contaminated meat and bone meal (MBM) dietary supplement (Wilesmith, 1994). Prion contamination appears to have resulted from a change in the rendering of offal from sheep and cattle used to produce the MBM. Recent reports have raised the possibility that bovine prions from mad cows have been transmitted to humans (Chazot et al., 1996; Will et al., 1996).

In contrast to viruses and viroids, prions do not contain a nucleic acid genome which encodes their progeny. Rather, prions are composed largely, if not entirely, of a modified host-encoded protein denoted PrP^{Sc}. Through a posttranslational process, PrP^{Sc} is formed from the normal cellular PrP isoform designated PrP^{C}. Attempts to identify a post-translational chemical modification responsible for the conversion of PrP^{C} into PrP^{Sc} were unsuccessful, and no chemical differences between the two isoforms have been found (Stahl et al., 1993). Yet the properties of PrP^{Sc} are considerably different from those exhibited by PrP^{C}. While

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[1] Abbreviations: PrP, prion protein; PrP^{C}, cellular PrP isoform; PrP^{Sc}, scrapie PrP isoform; PrP 27−30, proteinase K resistant fragment of PrP^{Sc}; rPrP, recombinant PrP containing residues 90−231; α-rPrP(acid), α-helical form of rPrP from acid without refolding; α-rPrP, α-helical form of rPrP refolded at neutral pH; β-rPrP, β-sheet form of rPrP; SHa, Syrian hamster; Mo, mouse, NMR, nuclear magnetic resonance spectroscopy; CD, circular dichroism spectroscopy; FTIR, Fourier transform infrared spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; GdnHCl, guanidine hydrochloride; SEC, size-exclusion chromatography; RPC, reversed-phase chromatography; TFA, trifluoroacetic acid; GSSG, oxidized glutathione; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); NTSB, 2-nitro-5-thiosulfobenzoate; NTB, 2-nitro-5-thiobenzoate; ΔG, Gibbs free energy difference between two states; MBM, meat and bone meal.

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PrP^C is soluble in nondenaturing detergents, PrP^{Sc} is not. Although PrP^C is readily degraded by proteases, PrP^{Sc} is N-terminally truncated to form PrP 27−30.

Enriching Syrian hamster (SHa) brain preparations for scrapie infectivity led to the discovery of PrP 27−30; early studies showed that this protein polymerized into amyloids which were known to possess a high β-sheet content (Prusiner et al., 1983). Subsequently, spectroscopic studies confirmed the high β-sheet content of both PrP 27−30 and PrP^{Sc} (Caughey et al., 1991; Gasset et al., 1993; Pan et al., 1993; Safar et al., 1993). Spectroscopic studies of PrP^C showed it to be high in α-helical content and virtually devoid of β-sheet (Pan et al., 1993; Pergami et al., 1996). Molecular modeling predicted PrP^C to be a four-helix bundle protein (Gasset et al., 1992; Huang et al., 1994). Synthetic peptides corresponding to each of the putative helices of PrP^C, H1, H2, H3, and H4, were found to exhibit anomalous structural properties (Gasset et al., 1992).

Attempts to produce PrP^C molecules in large quantities for structural studies have been fraught with difficulties. Meanwhile smaller segments of PrP have been studied extensively. A 56 residue SHaPrP peptide spanning H1 and H2 was found to exist either in an unstructured state; as an α-helical structure; or as one with intermolecular β-sheets, depending on the microenvironment (Zhang et al., 1995). In its α-helical form, chemical shift data confirmed the existence of the predicted helices in the H1 and H2 regions. A 111 residue mouse (Mo)PrP polypeptide spanning H2, H3, and H4, but lacking H1, was expressed in *E. coli* and found to contain H3 and H4 as predicted, with the helices stabilized by a disulfide bind joining the only two Cys residues occurring in mature PrP (Riek et al., 1996). Both PrP^C and PrP^{Sc} have also been shown to possess a disulfide bond (Turk et al., 1988). In addition, an α-helix that was not predicted as part of the C-terminal core of PrP^C and two β-strands, each consisting of three residues, were observed (Riek et al., 1996). One of these β-strands corresponds to a portion of H2 that was predicted to participate in a β-sheet of PrP^{Sc} (Huang et al., 1996).

Of the 11 known point mutations that segregate with the inherited human prion diseases, 10 lie either within or adjacent to the regions of secondary structure (Cohen et al., 1994; Prusiner, 1996; Riek et al., 1996; Zhang et al., 1995), whereas amino acid substitutions in the PrP gene due to species variation generally occur outside of these regions (Schätzl et al., 1995). The most conserved region of PrP among all species studied, including the chicken, is the domain which contains H1 and the loop joining this to H2 (Gabriel et al., 1992; Schätzl et al., 1995). Remarkably, experiments seeking to simulate the interaction between PrP^C and PrP^{Sc} by mixing PrP^C with peptides containing H1 and H2 have revealed that PrP^C could be induced to form a protease-resistant intermolecular β-sheet (Kaneko et al., 1995; Nguyen et al., 1995).

Despite the difficulties referred to above, expression in *E. coli* of a 142 residue polypeptide (rPrP) with the SHaPrP 27−30 sequence containing all 4 putative core, α-helical segments has recently been achieved (Mehlhorn et al., 1996). We report here on its folding and unfolding as measured by HPLC, CD, FTIR, analytical sedimentation, mass spectroscopy, and NMR. In aqueous solutions, rPrP could be folded into pH-dependent α-helical conformations, a thermodynamically more stable β-sheet, and various stable or transient intermediates. These results are consistent with the notion that PrP is capable of existing in multiple conformations, and they suggest that PrP^C may be an intrinsically flexible structure prone to structural alteration, such as conversion into PrP^{Sc}.

MATERIALS AND METHODS

Expression and Purification of rPrP. Syrian hamster rPrP was expressed using an alkaline phosphatase promoter in a protease-deficient strain of *Escherichia coli* (27C7), as described previously (Mehlhorn et al., 1996). Insoluble particles containing rPrP were extruded by a microfluidizer and pelleted by centrifugation. The extruded material was solubilized in 8 M GdnHCl/100 mM DTT and subjected to purification by two sequential chromatographic procedures: size-exclusion chromatography (SEC) (Pharmacia Superdex 200) eluted by 6 M GdnHCl/50 mM Tris, pH 8/1 mM EDTA with or without 5 mM DTT, followed by reversed-phase chromatography (RPC) using a C-4 column (Vydac) eluted by a gradient of acetonitrile/TFA/water. Purified rPrP was subsequently lyophilized for refolding. All RPC traces showed substantial peaks at 55−58 min due to non-PrP proteins; there were no other significant peaks.

Oxidation/Reduction of Cysteines. Immediately prior to SEC, the rPrP was in a buffer containing 100 mM DTT; thus, any potential disulfide bond between the two cysteines was reduced. Immediately after SEC separation, the rPrP remained reduced, whether or not the SEC buffer contained DTT. However, on standing in SEC buffer for several days without DTT, the cysteines were slowly oxidized by atmospheric oxygen. As described previously, the retention times in RPC were dependent on the reduction or oxidation of the cysteines (Mehlhorn et al., 1996); thus, it was possible to isolate both forms for subsequent refolding. Early eluting fractions contained the oxidized rPrP with the disulfide whereas late eluting fractions were reduced.

In an attempt to trap folding intermediates by acid quenching, fresh SEC eluent without DTT was allowed to react with various ratios of GSSG (10−25 mM) versus DTT (10 mM). The samples were incubated at room temperature for 2−14 h and quenched by diluting with 2 volumes of 0.1% TFA/H_2O, pH 2. The diluted solutions were subsequently separated by RPC. Fractions were collected and analyzed by mass spectrometry and CD.

For selected samples, aliquots containing 90−270 μg/mL refolded α-rPrP were unfolded in 6 M GdnHCl/50 mM Tris, pH 8, before reacting with Ellman's reagent (Ellman, 1959) to detect residual free thiols by the Pierce procedure, or NTSB to detect the disulfide bond (Thannhauser et al., 1987). In some cases, the molecular masses of oxidized and reduced rPrP were compared by ESI mass spectrometry, the accuracy of which was sufficient to distinguish the two forms that differed in mass by 2 Da. In other cases, the presence or absence of free thiols was probed by carboxymethylation with iodoacetic acid, before and after reduction with DTT, followed by mass spectrometry, as described previously (Mehlhorn et al., 1996).

Refolding by Dilution. Several related strategies were employed for refolding the lyophilized rPrP obtained from the RPC fractions. Initially rPrP in the oxidized form was solubilized at 3−10 mg/mL in 6 M GdnHCl/50 mM Tris,

pH 8, and then rapidly diluted into either (i) 20 mM Tris, pH 8; (ii) 20 mM sodium phosphate, pH 7.2; or (iii) 20 mM sodium acetate, pH 5, to a final protein concentration of 100 mg/mL. The resulting solutions were incubated at 10−25 °C for 2−12 h; then insoluble material was separated by centrifugation, leaving α-helical rPrP in solution, as determined by CD. The yield of soluble α-rPrP as measured by bicinchoninic acid (BCA) assay (Pierce) was ≥85%. For further physical studies, this α-rPrP was dialyzed further against one of the three buffers listed above and concentrated by Centriplus 10 (Amicon).

In a later protocol that also gave α-rPrP, the lyophilized oxidized fractions were dissolved in a small volume of water, assayed for protein content, and then made up to 1 mg/mL in 8 M GdnHCl. This was diluted into 10 volumes of 20 mM Tris−acetate, pH 8.0/5 mM EDTA. This solution was dialyzed against 50 mM HEPES, pH 7.0/0.005% thimerosal, and the protein was purified further by cation exchange chromatography with a HiLoad SP Sepharose Fast Flow 26/10 column using a gradient of 0−0.4 M LiCl at 4 mL/min, monitoring UV absorption at 280 nm. Fractions containing the protein were dialyzed against 20 mM sodium acetate, pH 5.5/0.005% thimerosal. It was noted that over time, α-rPrP refolded by the earlier protocol underwent a degree of proteolysis; in material refolded by the second method, degradation was inhibited by the presence of thimerosal, even when the rPrP solution was maintained at 37 °C for 10 days.

Lyophilized RPC fractions containing freshly purified rPrP obtained under reducing conditions were dissolved in 20 mM MES, pH 6.5, to give *β*-rPrP, as established by CD and FTIR. This material could be concentrated to 10 mg/mL and appeared to remain in solution over an extended period of time without precipitating. At higher pH (7.5), the solubility was reduced substantially. The solubility at pH 6.5 was investigated by ultracentrifugation at 100000*g* for 1 h. The oxidation state of the cysteines was probed by carboxymethylation with iodoacetic acid, before and after reduction with DTT, as described previously (Mehlhorn et al., 1996).

Spectroscopic Measurements. CD spectra were obtained for solutions containing 1−3 mg/mL rPrP. Spectra were collected at room temperature on a spectropolarimeter (Jasco, Model 720) equipped with a stress-plate modulator continuously purged with dry nitrogen. Calibration was carried out using an aqueous solution of (+)-10-camphorsulfonic acid. Spectra of 4−10 scans each were accumulated using 0.01 or 0.05 cm path length cells, and buffer spectra obtained under identical conditions were subtracted. Mean residue molar ellipticities were calculated based on the rPrP concentration, the number of residues in the protein fragment, and the cell path length. For FTIR, α-rPrP solutions and pellets resulting from the refolding procedures were resuspended in D_2O. Spectra were collected with a spectrophotometer (Perkin-Elmer, System 2000) equipped with a microscope continuously purged with dry nitrogen. Samples were contained in a 0.05 mm path length cell with barium fluoride windows. Approximately 100 scans each were accumulated for both the peptide solutions and the buffers under identical conditions.

Thermal and GdnHCl-Induced Denaturation or Refolding. The influence of heat on the structure of α-rPrP was monitored by CD in a 0.01 cm quartz cell, temperature-controlled by a programmable water circulator (Neslab 111). CD ellipticity at 222 nm was recorded for α-rPrP at 1 mg/mL in 20 mM sodium acetate, pH 5, or at 2.4 mg/mL in 20 mM sodium phosphate, pH 7.4, over the temperature range 15−72 °C in increments of 0.2 °C with a gradient of 0.7 °C/min. The signal at 15 °C was taken to represent 100% folded protein, and the data at other temperatures were normalized to this ellipticity to generate the fraction folded. Due to concerns over potential proteolysis at the elevated temperatures, the samples contained a 1:25 dilution of Complete protease inhibitor cocktail (Boehringer Mannheim). Individual CD spectra were recorded under the same conditions at 15, 50, and 70 °C.

Aliquots of α-rPrP from the same stock solutions as above were diluted to a final concentration of 0.5−1 mg/mL in 20 mM sodium acetate, pH 5 with protease inhibitor, containing 0−5 M GdnHCl (8 M stock solution, Pierce) in increments of 0.25 M. The samples were incubated for 2 h at room temperature. Equilibrium was achieved within minutes of mixing, as monitored by CD. The CD spectra were recorded for each concentration, from which the ellipticity at 222 nm was determined. The ellipticity without GdnHCl was taken to represent 100% folded protein, and the values with GdnHCl were normalized to generate the fraction folded. Two sets of data obtained under identical experimental conditions were averaged to yield the curve shown.

The ellipticity at 222 nm as a function of temperature was analyzed using a three-state model (N ↔ I ↔ U) assuming a stable intermediate (I). The relationship between ΔG and the denaturant concentration was assumed to be linear for both transitions, $\Delta G_U = \Delta G(H_2O) - m[D]$. The data were fitted to the following equation (Das et al., 1995; Jackson et al., 1993) using the nonlinear regression analysis program Kaleidagraph (Synergy software):

$$y_{obs} = \frac{y_N}{[1 + e^{-(\Delta G_1 + m_1 \cdot [D])/RT}][1 - e^{-(\Delta G_1 + m_2 \cdot [D])/RT}]} + \frac{y_I}{1 + [e^{-(\Delta G_1 + m_1 \cdot [D])/RT}] + [e^{-(\Delta G_2 + m_2 \cdot [D])/RT}]} + \frac{y_U}{[1 + e^{(\Delta G_2 + m_2 \cdot [D])/RT}][1 + e^{(\Delta G_1 + m_1 \cdot [D])/RT}]}$$

where y_{obs} is the observed ellipticity at 222 nm; y_N, y_I, and y_U are the signal intensities of the native, intermediate, and the unfolded states, respectively; ΔG_1 is the free energy difference between the folded state and the intermediate; ΔG_2 is the free energy difference between the intermediate and the unfolded state; m_1 and m_2 are the proportionality factors of the first and the second transition phases, respectively; R is the gas content; T is the absolute temperature in kelvin; and [D] is the concentration of GdnHCl.

Analytical Sedimentation. Several α-rPrP samples refolded in a number of different buffers at concentrations ranging from 0.33 to 2.8 mg/mL were evaluated by analytical sedimentation in an analytical ultracentrifuge (Beckman Optima XL-A) equipped with a 6-channel cell, using ultraviolet absorption between 220 and 280 nm. The studies were performed for 15−60 h at 25 °C; all samples reached equilibrium after 15 h and did not change thereafter. A solution column of 1.2 mm was centrifuged to equilibrium at either 15 000 or 25 000 rpm. Data for protein concentration versus the center of rotation were fitted to a single-term sedimentation equilibrium equation (IDEAL1), assum-

ing that the protein concentrations were low enough to neglect the virial coefficient term. To yield an association constant, the data from α-rPrP were fitted using nonlinear least-squares routines to a monomer−dimer equilibrium with the program provided by the manufacturer. Similar experiments were carried out with *β*-rPrP in 20 mM MES, pH 6.5, at 0.56 and 2.8 mg/mL.

Mass Spectrometry. RPC eluents as well as refolded solutions were analyzed by electrospray ionization mass spectroscopy (Perkin Elmer-Sciex, API-100B). RPC fractions were analyzed directly from the solutions in which they were eluted, whereas the refolded solutions were desalted by a further RPC procedure. Theoretical molecular masses were calculated by the MacBioSpec program (Perkin-Elmer Sciex) using average rather than monoisotropic atomic masses.

Nuclear Magnetic Resonance Spectroscopy (NMR). Samples for NMR were concentrated to 0.5−1.4 mM. All spectra were obtained on a Varian 600 MHz UnityPlus spectrometer equipped with a 5 mm $^{13}C/^{15}N/^{1}H$ triple resonance Varian probe. Analyses were carried out over the temperature range 10−45 °C. The spectral width was 7000−8000 Hz. For two-dimensional spectra such as NOESY and TOCSY, 2048 data points were collected in the t_2 dimension and 512 transients in the t_1 dimension. The spectra were processed with zero-filling of the t_1 dimension to 2K and apodized by a sine-squared window function shifted by 80° in both dimensions. Mixing times used were in the range 100−200 ms for NOESY and 30−70 ms for TOCSY. Presaturation or symmetrically shifted pulses (Smallcombe, 1993) were used to remove the water signal. All spectra were referenced to an internal standard of 0.2 mM 3-(trimethylsilyl)tetradeuterosodium propionate (TSP).

RESULTS

Cysteine Oxidation/Reduction Affects RPC Retention Times. As was reported previously (Mehlhorn et al., 1996), the elution time in RPC and the conformation of rPrP from lyophilized fractions were dependent on the oxidation state of the two cysteines (Cys 179 and Cys 214). Figure 1 (panels A and B) shows a series of RPC chromatograms obtained under various oxidation conditions. The RPC separation of freshly purified SEC fractions obtained under reducing conditions (A1) gave a late eluting peak at ∼48 min (labeled *β*). Mass spectrometry and carboxymethylation experiments reported previously demonstrated that this material contains free cysteines (Mehlhorn et al., 1996). The minor component (∼5%) at ∼38 min (labeled α) was shown to be oxidized, having an intramolecular disulfide bond. When the SEC eluent was stored at 4 °C for several days, oxygen in the air gradually oxidized the two cysteines to form the disulfide bond, shifting the majority of the rPrP signal to the early eluting peak (B1). After ∼10 days, the early peak contained as much as 90% of the rPrP (data not shown). The oxidation process was shown to be reversible; treatment of the "old" SEC fractions with DTT followed by RPC caused the chromatogram to shift back to the original, with the rPrP eluting in the late fraction (B2). Aerobic oxidization of rPrP was faster if DTT was excluded from the SEC eluent and if a higher temperature was used.

An evaluation was made of controlled oxidation by the addition of oxidized glutathione (GSSG) to the reduced

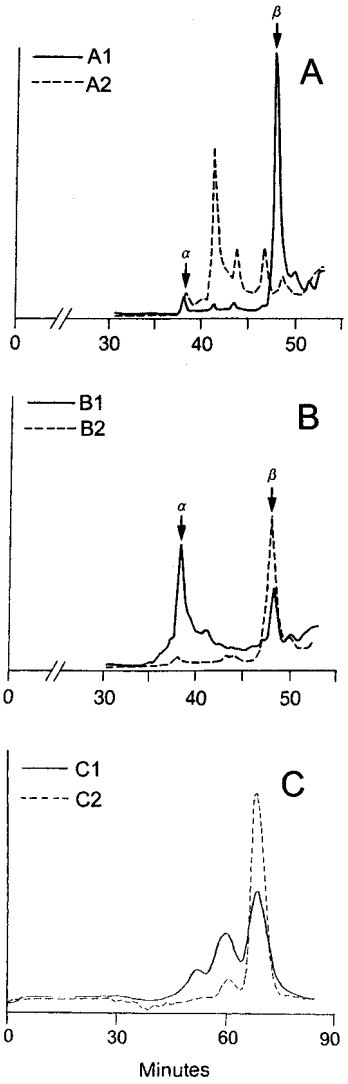

FIGURE 1: (A and B) RPC chromatograms of rPrP monitored by UV absorption at 280 nm: (A1) fresh SEC eluent (eluted without DTT); (A2) after oxidation of the SEC eluent used in A1 by 25 mM GSSG and incubation for 2 h at room temperature; (B1) SEC eluent (eluted without DTT) incubated for 8 days at 4 °C; (B2) after reduction of the SEC eluent used in B2 by 10 mM DTT and incubation for 2 h at room temperature. (C) Ion exchange chromatograms of refolded rPrP: (C1) purified rPrP, refolded then stored in the absence of protease inhibitors; (C2) a similar sample stored in the presence of 0.005% thimerosal.

material. After 2 h at room temperature, this resulted in oxidization of only ∼5% of the rPrP to the desired product having the disulfide bond. Most of the rPrP, however, was trapped in one of three intermediate states that eluted at times between the early and late components (A2). SDS−PAGE under reducing and nonreducing conditions and mass spectrometry revealed that the major intermediate (∼60%) eluting at 41.5 min had glutathione conjugated to both Cys 179 and Cys 214 through two individual disulfide bonds, whereas the 43.5 and 46.5 min intermediates each had one glutathione conjugated to one or the other cysteine through a disulfide bond, leaving the thiol of the other cysteine intact. The presence of the two singly mixed disulfides resolvable by different elution times during RPC suggests that the two cysteines were not equivalent with respect to their chemical environments. The conformations of these intermediates in water at pH 3 were found by CD to be a mixture of random coil and *β*-sheet structure (data not shown). The yield of

oxidized rPrP from GSSG oxidation was increased when this was carried out in the presence of L-cysteine, but GSSG oxidation was not pursued further as aerobic oxidation proved to be highly efficient and reproducible.

Proteolysis of rPrP. Even for the most highly purified fractions, proteolysis occurred in rPrP that was stored in solution for several days or longer, especially at room temperature or above. This is illustrated by the ion exchange chromatogram in Figure 1, trace C1, which shows a number of early eluting peaks that by mass spectrometry were found to correspond to proteolytic fragments. Identification of the cleavage sites suggested an elastase-like activity. We determined that this could be inhibited by a number of agents such as EDTA, sodium azide, 3,4-dichloroisocoumarin (an elastase inhibitor), or thimerosal. Trace C2 shows the separation of a second sample stored under identical conditions except that the buffer contained 0.005% thimerosal, demonstrating a substantial diminution in the degree of proteolysis.

Cysteine Oxidation/Reduction Also Affects Conformation. As reported previously (Mehlhorn et al., 1996), rPrP from lyophilized RPC fractions when dissolved in suitable buffers gave CD and FTIR spectra that showed the reduced rPrP to be predominantly β-sheet whereas the oxidized form was α-helical. We were interested in exploring the α-helical form as a model for PrP^{C}, but as the RPC fractions from which it was derived contained 0.05% TFA and were highly acidic, we were concerned that this material might have different properties than those obtained by denaturation and refolding at a pH closer to physiological conditions. To distinguish the α-helical form obtained directly from acidic RPC material without refolding, it was designated α-rPrP(acid).

The properties of β-rPrP were also of considerable interest as this represented a potential model for PrP^{Sc}. It was known that solubilization of lyophilized RPC fractions containing oxidized rPrP would give α-rPrP(acid), but it had not been established whether the aerobic oxidation of β-rPrP in solution would cause a transition to the α-helical form. RPC fractions from freshly isolated SEC material were lyophilized and dissolved in 20 mM MES, pH 6.5/0.005% thimerosal; CD showed this to be β-rPrP. Aliquots were taken over a period of several days and were separated by RPC. The trends shown in Figure 1 were duplicated; initially >90% of the rPrP was in a reduced form that eluted in late fractions, whereas this changed over the course of ~10 days to the point where ~90% eluted in the early fractions. Carboxymethylation by iodoacetic acid, with or without prior reduction by DTT, as described previously (Mehlhorn et al., 1996), established that the protein had become oxidized and the disulfide bond was formed (data not shown). Nevertheless, the CD spectrum was unchanged, and the protein remained in the β-sheet form.

Refolding rPrP at Neutral pH. As an alternative to α-rPrP(acid) obtained by direct solubilization of RPC-derived oxidized protein, the oxidized protein was denatured in guanidine and refolded by dilution to 0.1 mg/mL in one of several buffers having pHs in the range 5−8. Refolding at pH 8 can facilitate redox shuffling of the cysteines (Darby & Creighton, 1995). In some cases, the refolded protein was centrifuged to determine the yield of soluble material. In the absence of DTT, refolding at any of these pH values generated high yields of soluble α-rPrP (≥85%), whereas addition of 10 mM DTT to the refolding buffer drastically

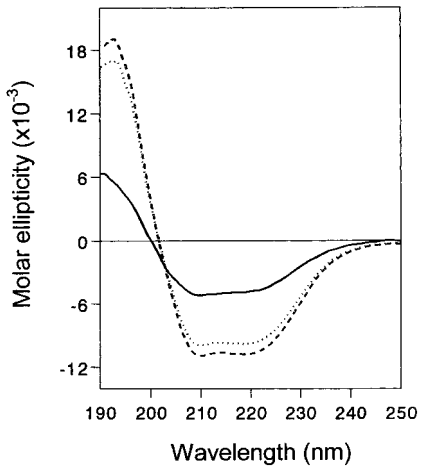

FIGURE 2: CD spectra of rPrP: (—) α-rPrP(acid) in H_2O at pH 3.5; (- - -) α-rPrP refolded in 20 mM sodium phosphate at pH 7.2; and (•••) α-rPrP refolded in 20 mM sodium acetate at pH 5. The CD spectra were recorded at room temperature. The vertical axis is the mean residue molar ellipticity.

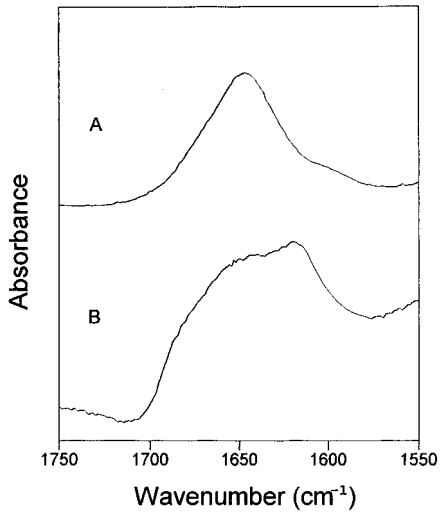

FIGURE 3: FTIR spectra of rPrP at room temperature: (A) refolded α-rPrP (supernatant) in D_2O/20 mM sodium phosphate, pD 7.2; (B) β-sheet (pellet), analyzed as a thin film after repeated addition of D_2O and vacuum-drying.

reduced the yield of α-rPrP (<5%), the majority of the rPrP being in the pellet. Refolded α-rPrP samples gave very similar CD spectra (Figure 2) and FTIR spectra (Figure 3A), irrespective of the pH of the refolding buffer. The pellets from the refolding procedures exhibited FTIR spectra characteristic of intermolecular β-sheets with IR absorption bands at 1621 cm^{-1} (Figure 3B). 1D NMR spectra showed that the supernatant fractions exhibited similar chemical shift dispersion independent of the folding protocol. SDS−PAGE performed under reducing or nonreducing conditions showed that rPrP migrated more slowly during electrophoresis than did the nonreduced form (data not shown), suggesting that the reduced rPrP may possess a less compact structure than the refolded α-rPrP with an intramolecular disulfide bond. Using Ellman's and NTSB assays (Ellman, 1959; Thannhauser et al., 1987) in conjunction with electrospray mass spectrometry, we confirmed that the refolded α-rPrP possessed an intramolecular disulfide bond and that intermolecular cross-linking had not occurred. Similarly, the pellets were also found to possess the intramolecular disulfide bond, but bands corresponding to dimers in SDS−PAGE were

somewhat more pronounced with the pellets than the corresponding supernatants (data not shown).

α-rPrP(Acid) and Refolded α-rPrP Have Different Properties. Both α-helical forms of rPrP proved to be soluble in aqueous buffers at millimolar concentrations but at different pHs. Although α-rPrP(acid) purified from acidic RPC fractions was soluble at pH 2, it was only slightly soluble at pH 5 and insoluble at physiological pH. This form exhibited less helical character as judged by CD; although it gave a mostly α-helical pattern with minima at 208 and 221 nm, the ellipticity was reduced by ∼50% in comparison with the refolded material. By NMR, compared with refolded α-PrP (Figure 4A,B), α-rPrP(acid) gave line widths (Figure 4C) consistent with intermolecular aggregation and smaller chemical shift dispersion, suggesting that its folding was nonnative. Line widths in the 2D NMR NOESY spectra for α-rPrP(acid) reflected a range of oligomeric states that were found to increase with increasing protein concentrations (Figure 5B). These findings suggested that α-rPrP(acid) is likely to be in a partially folded α-helical state that readily forms intermolecular aggregates of heterogeneous size. On the other hand, refolded α-rPrP was readily soluble in the pH range 5−8 and exhibited essentially invariant CD spectra (Figure 2, traces B and C) and NMR features (Figure 4, traces A and B) throughout this range. These observations indicate similar structures in the pH range of 5−8. With overnight dialysis at pH 5, about 50% of α-rPrP(acid) could be converted into α-rPrP, as judged by the characteristic NMR chemical shift dispersion (Figure 4, trace D), suggesting a low energy barrier between the two α-helical forms and the possibility of a common α-helical core.

Under all conditions examined, including varying NaCl concentrations from 0 to 300 mM and pH from 5 to 8, α-rPrP exhibited a range of proton line width intensities (Figure 5A), with some strong and others weak or unobservable. The number of observable 2D TOCSY and NOESY cross-peaks was significantly lower than would be expected for 142 amino acid residues. Detailed assignments and a comprehensive analysis of the NMR data from heteronuclear $^{13}C/^{15}N$ experiments will be presented in a subsequent publication (Farr-Jones et al., unpublished results), but this observation suggested that a substantial portion of the molecule was likely to be in a partially folded state or to exist in a flexible conformation. This is consistent with the results of others who studied a C-terminal fragment of PrP (121−231); they were unable to position residues 121−124, 167−176, and 220−231 in their calculated structure (Riek et al., 1996). It is possible that dynamic exchange among multiple conformations may have contributed to the broad line width distribution.

β-rPrP Is Thermodynamically More Stable than α-rPrP. The constancy of the data from 1D NMR and CD studies carried out between 10 and 40 °C showed that the refolded α-rPrP possessed similar secondary as well as tertiary structures within this temperature range. As monitored by CD at 222 nm, thermal changes began at 45 °C, with gradual loss of the α-helical CD signal, followed by continuous and cooperative transitions centered at 52.2 °C for pH 7.4 and at 55 °C for pH 5 (Figure 6A). However, these were not classical melting curves as they were not reversible, and analysis of entire CD spectra over this temperature range revealed that the rPrP was refolding from the α-helical conformation to one consisting mainly of β-sheet, with a minimum at 218 nm (Figure 6B).

Unfolding of rPrP in GdnHCl Reveals a Stable Folding Intermediate. To examine further the stability of the refolded α-rPrP, unfolding experiments were performed with increasing concentrations of GdnHCl in 20 mM sodium acetate at pH 5, monitoring the secondary structure of the protein by CD at 222 nm. As the GdnHCl concentration increased, the CD signal due to the α-helix decreased (Figure 6C), displaying a two-step unfolding transition with a relatively stable folding intermediate at 2 M GdnHCl. The CD signal decreased substantially in the first transition phase at low GdnHCl concentrations, indicating that a large segment of the refolded α-rPrP possessed partially folded or flexible α-helical structures. Data from the transition curve were fitted to a three-state model using least-squares routines. The free energy change between the folded and the intermediate state (ΔG_1) was calculated to be 1.9 ± 0.4 kcal/mol. The second transition phase was observed when less than 40% of the α-helical CD signal remained; here ΔG_2 was calculated to be 6.5 ± 1.2 kcal/mol. The observation of a folding intermediate such as this is consistent with a relatively stable α-helical core.

α-rPrP Is Monomeric but β-rPrP Is Not. Several α-rPrP samples were evaluated by analytical sedimentation. The datasets for all samples refolded under a variety of different conditions and with concentrations varying by a factor of almost 10 fitted well with a monomeric molecular mass of 16 242 Da, with small residuals. Figure 7A shows a typical example of one of these data sets (circles) and the fitted model (solid line), obtained from a solution at relatively low rPrP concentration. Assuming a monomer−dimer equilibrium, the association equilibrium constant was calculated to be 5.4×10^{-5} M^{-1}. These findings and the random distribution of the residuals in the upper panel A(r) indicate that the refolded α-rPrP was predominantly monomeric.

Similar experiments were carried out with β-rPrP at two different concentrations. The data points in Figure 7B (circles), which correspond to the low concentration data, did not fit well to a monomeric model (solid line), and the marked trend in the residuals in B(r) indicated the discrepancy was due to aggregation. Analysis of the data gave a predicted molecular mass of 1.09×10^5 Da for the dilute solution and 1.03×10^5 Da for the more concentrated solution, suggesting an average particle size corresponding to ∼6−7 rPrP molecules.

Samples of β-rPrP appear to remain in solution for periods of weeks or months under suitable conditions, with no significant change in CD spectra or protein assays. However, samples at 0.5 and 2.8 mg/mL were subjected to ultracentrifugation at 100000*g*. After 45 min, the concentration of protein dropped to 0.2 mg/mL or less, confirming that β-rPrP was aggregated and that it was not truly soluble by this definition. By CD, the remaining protein in the supernatant gave an α-helical spectrum.

DISCUSSION

In spite of considerable skepticism initially, the prion concept has gained widespread acceptance with the convergence of many lines of evidence arguing that infectious prions are composed largely, if not entirely, of PrP^{Sc} molecules (Prusiner, 1991). The underlying event in the

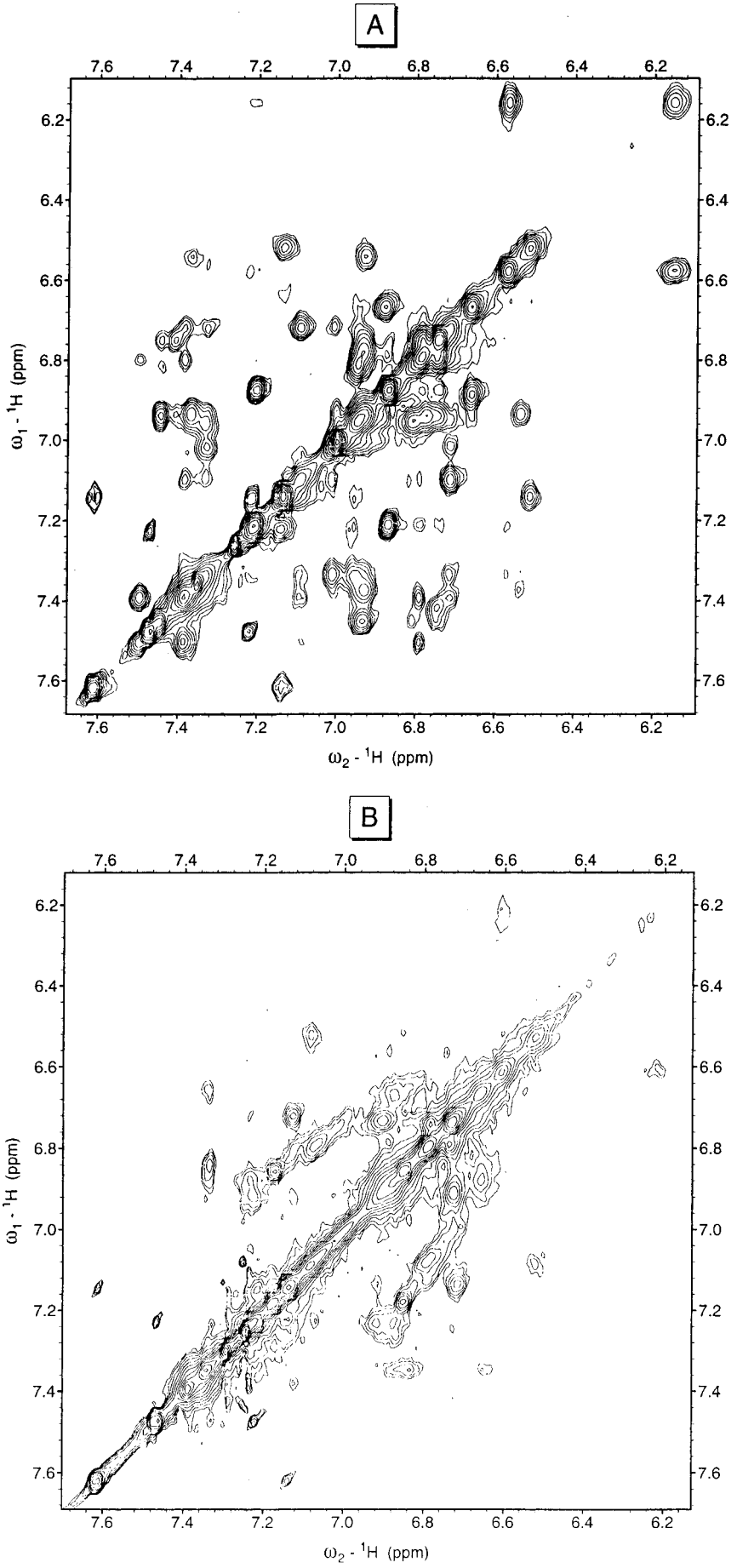

FIGURE 4: Aromatic proton region of 2D NOESY spectra of rPrP: (A) refolded α-rPrP in 20 mM sodium phosphate/D_2O, pD 7.2; (b) α-rPrP(acid) in D_2O, pD 3.5. Mixing times were 200 ms.

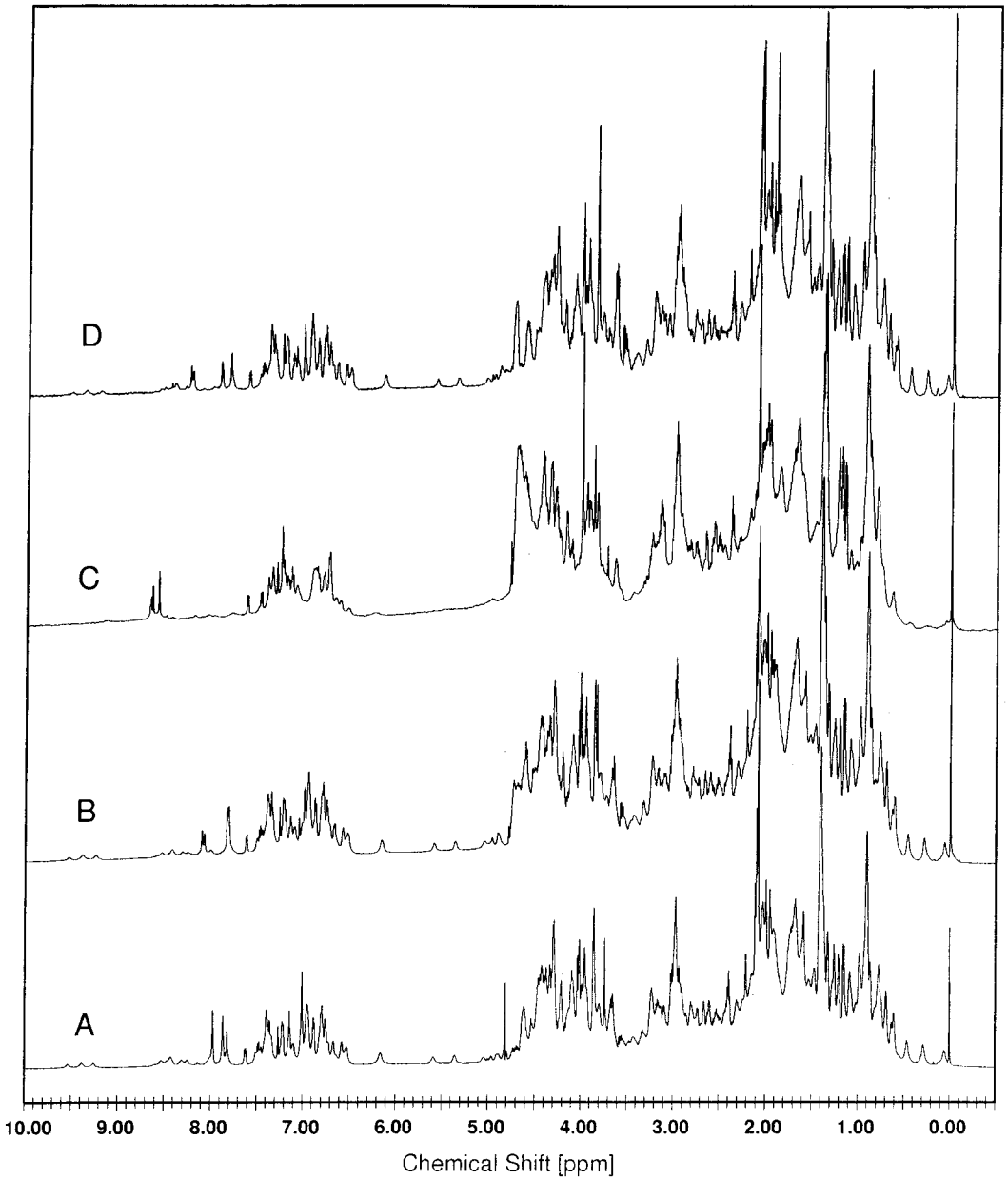

FIGURE 5: 1D proton NMR spectra of rPrP: (A) refolded α-rPrP in 20 mM sodium phosphate/D_2O, pD 7.2; (B) refolded α-rPrP in 20 mM sodium acetate/D_2O, pD 5; (C) α-rPrP(acid) in D_2O, pD 3; and (D) α-rPrP(acid) after dialysis overnight in 20 mM sodium acetate/D_2O, pD 5. All of the 1D spectra were recorded at 35 °C, referenced to internal TSP.

propagation of prions and the pathogenesis of prion diseases seems to be a profound conformational change in PrP^C as it is converted into PrP^{Sc} (Pan et al., 1993). Transgenetic studies argue that PrP^{Sc} formation requires the substrate PrP^C to bind to the product PrP^{Sc} at an intermediate stage of the conversion process (Prusiner et al., 1990). PrP^C is thought to be in equilibrium with a metastable intermediate, designated PrP*, which binds to PrP^{Sc} in the conversion process. It may be possible to simulate PrP* using 0.3 M GdnHCl or another denaturant to destabilize PrP^C; such conditions allow PrP^C to bind to PrP^{Sc} or synthetic PrP peptides (Kaneko et al., 1995; Kocisko et al., 1994; Safar et al., 1994). Recent studies on the transmission of human prions to transgenic mice suggest that a species-specific factor, designated protein X, might function as a molecular chaperone in PrP^{Sc} formation (Telling et al., 1995). It seems likely that the GPI anchor of PrP^C targets it to caveolar-like structures within or adjacent to the plasma membrane where PrP^C either is degraded or is converted into PrP^{Sc} (Taraboulos et al., 1995).

Tinctorial studies and spectroscopic measurements have shown that the conversion of PrP^C into PrP^{Sc} is accompanied by a reduction in the α-helical content of PrP and an increase in β-sheet structure (Pan et al., 1993; Prusiner et al., 1983). Although the molecular mechanism remains to be elucidated, the magnitude of this structural change seems to be unprecedented in biology. Increasing experimental evidence argues that PrP can exist in at least two reasonably stable conformations; the apparent diversity of prions, often referred to as strains, suggests that multiple conformers of PrP^{Sc} may exist, each displaying somewhat different properties which are manifest as incubation times of distinct length (Telling et al., 1996).

Recombinant PrP as a Model for Structural Studies of PrP Isoforms. Defining the atomic structures of PrP^C, PrP^{Sc}, and intermediates along the folding pathway seems critical to determining the mechanism of the conformational transition associated with conversion of PrP^C into PrP^{Sc}. Efforts to obtain large amounts of recombinant PrP for structural

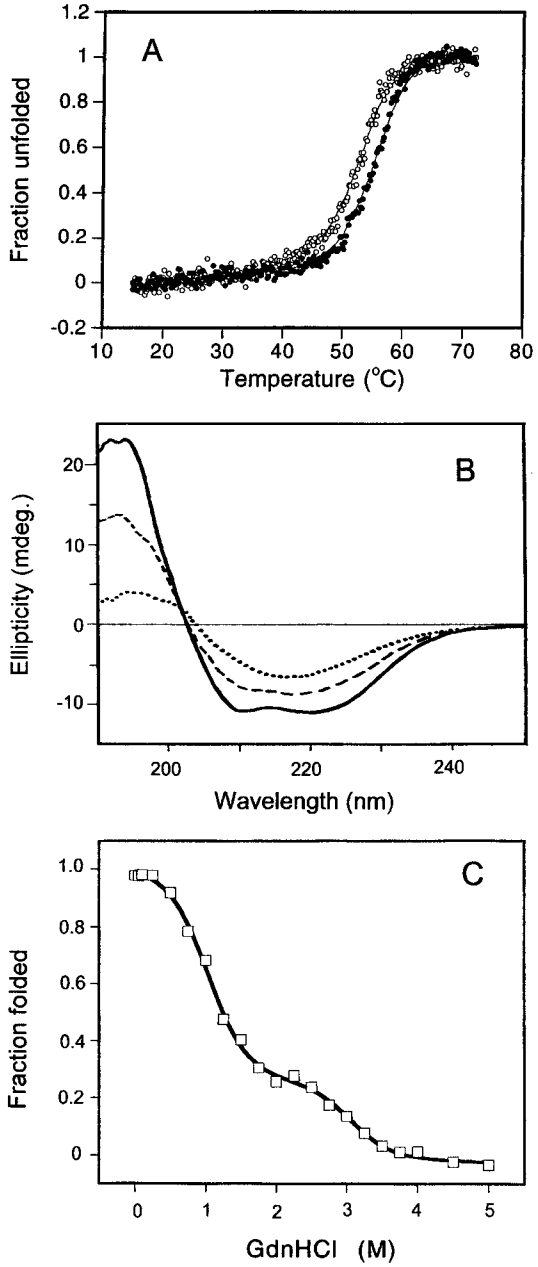

FIGURE 6: Thermal transitions of refolded α-rPrP monitored by CD. (A) In 20 mM sodium acetate, pH 5 (filled circles), and 20 mM sodium phosphate, pH 7.4 (open circles). The ellipticity at 222 nm was recorded for each temperature and normalized to that at 15 °C (100% folded). (B) CD spectra recorded for refolded α-rPrP at 15 °C (solid line), 50 °C (dashed line), and 70 °C (dotted line) as the temperature was raised. (C) Unfolding of refolded α-rPrP by GdnHCl in 20 mM sodium acetate, pH 5. The ellipticity at 222 nm was recorded for each GdnHCl concentration at room temperature and normalized to that without GdnHCl (100% folded).

studies have been impeded by low levels of expression as well as by the instability of the protein. The studies reported here describe the structural characterization of a 142 amino acid polypeptide (rPrP) that corresponds to the sequence of PrP 27−30, residues 90−231 (Mehlhorn et al., 1996). Not unexpectedly, the rPrP polypeptide displays an unusual degree of conformational plasticity. We have demonstrated that rPrP can be folded into α-helical and β-sheet structures as well as various stable or transient intermediates.

Purification of reduced rPrP by SEC and RPC gives a β-sheet form that can retain its secondary structure, even after oxidation of the cysteines to form a disulfide bond.

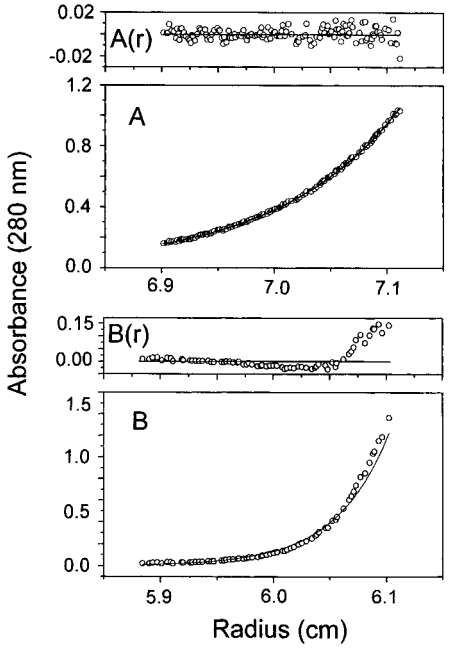

FIGURE 7: Sedimentation equilibrium distributions from analytical ultracentrifugation: (A) refolded α-rPrP at 0.4 mg/mL in 20 mM sodium phosphate/50 mM sodium chloride at pH 7.2; (B) β-rPrP at 0.52 mg/mL in 20 mM MES, pH 6.5/0.005% thimerosal. Data points (circles) were obtained by monitoring the absorbance at 280 nm as a function of the distance from the center of rotation; the solid lines represent models assuming a monomeric distribution. Panels A(r) and B(r) show the differences between the data points and the models (residuals).

However, it can also be oxidized and converted into at least two different α-helical states. The α-rPrP(acid) protein isolated from acidic RPC fractions exhibits less α-helical structure than the refolded α-rPrP molecule. α-rPrP(acid) is heterogeneous in size due to aggregation while refolded α-rPrP is soluble in aqueous buffers. Although α-rPrP(acid) is soluble at low pH, it is insoluble at physiological pH. The refolded α-rPrP exhibits similar conformations over the pH range of 5−8 and is predominantly monomeric under these conditions. It shows native chemical shift dispersion by NMR and cooperativity in its thermal refolding. These properties are consistent with the characteristics manifested by other native proteins (Creighton, 1992) and suggest that refolded α-rPrP is likely to resemble the native conformation of this amino acid sequence under physiologic conditions. The ease of converting α-rPrP(acid) into refolded α-rPrP and the fact that both states are α-helical indicate that both may possess a similar α-helical folding core. It is reasonable to speculate that α-rPrP(acid) may be an acid-denatured, molten globular folding intermediate similar to that observed for some other proteins (Fink, 1995; Goto et al., 1990); molten globules generally exhibit broad NMR line widths, low CD ellipticities, and partially folded structural motifs (Creighton, 1992).

Disulfide Bond in PrP. rPrP has been shown to form predominantly intermolecular β-sheet structure in the absence of the intramolecular disulfide bond (Mehlhorn et al., 1996). We have now demonstrated that rPrP can also retain conformations rich in intermolecular β-sheets after the presence of the intramolecular disulfide bond; this finding may be significant since the intramolecular disulfide seems to be present in both PrP^{C} and PrP^{Sc} (Turk et al., 1988).

The contribution of disulfide bonds to the thermodynamic and kinetic folding stability of proteins has been studied extensively (Creighton, 1992). Our results indicate that the intramolecular disulfide bond is crucial for the folding of α-helical forms of rPrP. In the absence of the intramolecular disulfide bond, little, if any, of the denatured rPrP refolds into the α-helical conformations. This suggests that the packing of the two disulfide-connected segments H3 and H4 is possibly the major stabilizing element in the folding of the entire molecule. By varying the ratio of GSSG versus DTT, we trapped several folding intermediates conjugated with glutathione in singly (two species) and doubly (one species) mixed disulfides. Similar folding intermediates have been observed in other proteins (Mostov et al., 1984; Operskalski & Mosley, 1995) in the process of studying the transition between native structures and acid-denatured molten globules.

Molecular Modeling. Molecular modeling studies predicted that the disulfide bond stabilizes the structures of H3 and H4 in both PrP^C and PrP^{Sc} (Huang et al., 1994, 1996). The results of NMR studies of the C-terminal domain of MoPrP containing amino acids 121−231 show that the residues predicted to form H3 and H4 do indeed form these helices and are stabilized by a disulfide bond (Riek et al., 1996). Whether an additional short helix (144−154) found in this C-terminal domain polypeptide is present in PrP^C or in longer protein fragments such as that studied here remains to be established. The initial modeling studies suggested that this putative helical region was unlikely to contribute to the hydrophobic core of PrP^C. The extreme sensitivity of regions of putative secondary structure in PrP to surrounding residues is illustrated by spectroscopic results collected on synthetic peptides corresponding to the H1 region; for example, the H1 peptide (109−122) acquires a β-sheet structure in aqueous buffers, but extending the N-terminus by five amino acids gives the 104H1 peptide (104−122) that is devoid of β-sheet in water (Nguyen et al., 1995).

A 56 residue peptide spanning H1 and H2 was found to exist in several different conformations, including an α-helical structure and another with intermolecular β-sheets, depending on the microenvironment (Zhang et al., 1995). In an α-helical state, chemical shift data confirmed the existence of helices in the regions predicted earlier (Huang et al., 1994), but this peptide lacks residues 144−154 which were observed to form a helix in the C-terminal fragment (121−231) (Riek et al., 1996). Whether the formation of PrP^{Sc} involves the refolding of H1 and H2 into a β-sheet as we have predicted remains to be determined.

Conformational Plasticity. 2D NMR data revealed substantial conformational flexibility in the refolded α-rPrP protein. Interestingly, studies of PrP epitopes using a battery of anti-PrP Fabs isolated from a phage display library have reached a similar conclusion for PrP^C using FACS analysis of the protein on the surface of neuroblastoma cells (Williamson et al., 1996). Further evidence for this conformational plasticity of PrP is provided here by the unfolding of α-rPrP using GdnHCl; refolded α-rPrP is only marginally more stable ($\Delta G_1 = 1.9 \pm 0.4$ kcal) than the folding intermediate, consistent with α-rPrP having large segments of open or conformationally flexible structures. The free energy difference between the intermediate and the unfolded state ($\Delta G_2 = 6.5 \pm 1.2$ kcal/mol), on the other hand, is comparable to literature values for protein stability of 5−15 kcal/mol (Pace, 1995). Furthermore, ΔG_2 in this study is similar to the free energy change reported for the guanidine-induced monophasic denaturation of MoPrP121−231 (Hornemann & Glockshuber, 1996). Since the disulfide is crucial for α-helical folding and stability, we speculate that the relatively stable folding core represented by the second unfolding transition phase may involve the packing of the amino acid residues containing or adjacent to the disulfide bond, i.e., the H3 and H4 regions of the putative PrP^C structure (Huang et al., 1994; Riek et al., 1996). In summary, our data support the proposal that PrP^C is likely to have a relatively open structure with a stable folded core, which results in considerable conformational flexibility. This flexibility is further manifested in the observation of a thermally induced conversion from isolated α-helical molecules to thermodynamically more stable aggregated structures rich in β-sheet.

Mechanism of PrP^{Sc} Formation. Studies of PrP structure combined with transgenetic studies are beginning to present a fascinating picture of the propagation of prions. Data from transgenic mice argue that PrP^C and PrP^{Sc} form a transient complex during prion replication (Prusiner et al., 1990). The formation of this transient complex in conjunction with molecular chaperones as postulated for protein X may serve to diminish the activation energy barrier between PrP^C and PrP^{Sc} and thereby facilitate formation of PrP^{Sc} (Cohen et al., 1994; Telling et al., 1995). The studies described here demonstrate that rPrP is capable of adopting multiple stable and transient structures that are manifest as proteins with varying amounts of α-helical and β-sheet structure. Whether α-rPrP(acid), which has properties consistent with an acid-denatured molten globular intermediate, or any of the transient intermediates observed in the GSSG/DTT experiments approximate those intermediate structures formed during the conversion of PrP^C into PrP^{Sc} remains to be established.

The stable intermediate of rPrP that is formed in 2 M GdnHCl is of particular interest since destabilization of PrP^C is necessary for it to bind to PrP^{Sc} in vitro (Kaneko et al., 1995; Kocisko et al., 1994). It seems likely that studies of the transient conformational states that PrP is capable of adopting may provide a better understanding of the structural basis for PrP^{Sc} formation. To the extent rPrP constitutes a relevant folding domain of PrP, the conformational pluralism exhibited by rPrP may be biologically significant since one or more of these conformations adopted by rPrP may mimic one or more of the folding intermediates which exist along the conversion pathway from PrP^C to PrP^{Sc} (Telling et al., 1996).

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