Stability and conformational properties of doppel, a prion-like protein, and its single-disulphide mutant

Sheena M. WHYTE*, Ian D. SYLVESTER†, Stephen R. MARTIN*, Andrew C. GILL†, Franziska WOPFNER‡, Hermann M. SCHÄTZL‡, Guy G. DODSON* and Peter M. BAYLEY*¹

*National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K., †Institute for Animal Health, Compton, Berkshire RG20 7NN, U.K., and ‡Gene Center Munich, Feodor-Lynen-Strasse 25, D-81377, Munich, Germany

Both prion protein and the structurally homologous protein doppel are associated with neurodegenerative disease by mechanisms which remain elusive. We have prepared murine doppel, and a mutant with one of the two disulphide bonds removed, in the expectation of increasing the similarity of doppel to prion protein in terms of conformation and stability. Unfolding studies of doppel and the mutant have been performed using far-UV CD over a range of solution conditions known to favour the $\alpha \rightarrow \beta$ transformation of recombinant prion protein. Only partial unfolding of doppel or the mutant occurs at elevated temperature, but both exhibit full and reversible unfolding in chemical denaturation with urea. Doppel is significantly less stable than prion protein, and this stability is further reduced by removal of the disulphide bond between residues 95–148. Both doppel and the mutant are observed to unfold by a two-state mechanism, even under the mildly acidic conditions where prion protein forms an equilibrium

intermediate with enhanced β -structure, potentially analogous to the conversion of the cellular form of the prion protein into the infectious form ($\text{PrP}^C \rightarrow \text{PrP}^{\text{Sc}}$). Furthermore, no direct interaction of either doppel protein with prion protein, either in the α -form or the β -rich conformation, was detectable spectroscopically. These studies indicate that, in spite of the similarity in secondary structure between the doppel and prion protein, there are significant differences in their solution properties. The fact that neither doppel nor its mutant exhibited the $\alpha \rightarrow \beta$ transformation of the prion protein suggests that this conversion property may be dependent on unique sequences specific to the prion protein.

Key words: amyloid protein, prion protein, protein unfolding, thermodynamic stability, urea denaturation, transmissible spongiform encephalopathy.

INTRODUCTION

The prion protein (PrP) in its cellular form (PrP^C) is a glycoprotein, whose normal physiological function is unclear, although it has been associated with copper binding [1,2] and signal transduction [3]. The disease-associated form, PrP^{Sc} [scrapie (protease resistant) form], is characteristic of a range of neurodegenerative amyloid disorders, including scrapie in sheep and goats, bovine spongiform encephalopathy, and Creutzfeldt–Jakob disease, kuru, fatal familial insomnia and Gerstmann–Strausler–Scheinker disease in humans [4–7]. The transition between the normal cellular isoform, PrP^C , and PrP^{Sc} appears to be post-translational [8], and is thought to involve a change in conformation of PrP from a structure containing substantial α -helical content to one with increased β -sheet content [9–11].

The protein doppel (Dpl) is a paralogue PrP, and is encoded by a discrete gene locus *PRND* located 16 kb downstream of the prion protein gene *PRNP* [12]. PrP and Dpl appear to have distinctive specific functions, e.g. ablation of Dpl causes male sterility in the mouse [13]. However, Dpl bears a functional similarity to PrP in that it can also be associated with neuropathological disease. Nonetheless, despite structural similarity between Dpl and PrP, Dpl has not been found to undergo similar conversion *in vivo* to that seen with PrP, which, in pathogenic conditions, results in deposits of β -structured amyloid protein in the brain.

The functional relationship between PrP and Dpl is complex. Ataxia and death of cerebellar neurons was observed in certain

transgenic PrP-knockout mice [14], and in knockouts expressing N-terminally truncated PrP [15]. In the former case, the pathological condition was found to correlate with expression of the Dpl protein in the brain [12] and also could be abrogated by over-expression of the wild-type PrP [16].

Certain evidence suggests that Dpl is not involved in prion disease progression. Genetic variation in *PRND* does not appear to influence the pathogenesis of prion diseases [17], the absence of Dpl does not affect scrapie pathogenesis in neural grafts [18], and expression of Dpl apparently has no influence on the outcome of transmissible spongiform encephalopathy disease in transgenic mice [19,20]. However, recent evidence suggests PrP and Dpl may occur in proximity under normal conditions *in vivo*. Dpl has been found to co-exist with N-terminally truncated isoforms of PrP^C on mature spermatozoa [21]. The two proteins have been reported to co-localize on granule cells of the cerebellum [22]; however, other work indicates that these two raft-associated proteins may occupy distinct lipid microdomains [23]. Recently it has been suggested that mutation in PrP may cause it to act as a weak mimetic of Dpl [24]. Interestingly, truncated PrP (122– or 135–231) itself can produce a pathological state on a PrP null background, which can be rescued by PrP^C [15]. A model was proposed in which the shorter PrP sequence competes with and inhibits the action of an unidentified molecule which can fulfil the function of normal PrP^C in its interaction with a common ligand. In such a model, the action of Dpl could be analogous to that of (truncated) PrP. Significantly, co-expression of PrP

Abbreviations used: C95S, Cys⁹⁵ $\rightarrow$ Ser; [D_{1/2}], urea unfolding midpoint; Dpl, doppel; PrP, prion protein; PrP^C , cellular PrP isoform; PrP^{Sc} , scrapie (protease resistant) PrP isoform; mtDpl, recombinant murine Dpl fragment 25–156, (C95S, C148S); OvPrP(94–233), recombinant ovine PrP fragment 94–233 (ARQ allele); SS 95–148 etc., disulphide bond between residue 95–148 etc.; wtDpl, recombinant murine Dpl fragment 25–156.

¹ To whom correspondence should be addressed (e-mail pbayley@nimr.mrc.ac.uk).

Figure 1 Sequences of (murine) Dpl and PrP showing the relative positions of key structural features

White boxes, α -helices A, B and C; dots, N-terminal and C-terminal cleavable signal peptides; CHO, glycosylation sites; GPI, glycosylphosphatidylinositol addition sites. Disulphide bonds are as indicated. Black boxes, octa-repeat region; TM region, transmembrane region containing the palindromic sequence (112–119).

prevents Dpl-induced neurotoxicity in transgenic mice [16,20], which may imply a potential interaction between PrP and Dpl *in vivo*, or between pathways in which they are involved.

Murine Dpl (Figure 1) is a glycosylphosphatidylinositol-anchored prion-like protein of 179 residues that differs from PrP by lacking the N-terminal PrP octapeptide repeat region and the sequence 106–126 which may be associated with cell toxicity [25]. Dpl shows a striking structural similarity to the C-terminal region of PrP, with two short β -sheets and three α -helices, the second helix being extended and bent in two distinct parts [26]. Murine Dpl contains two disulphide bonds between residues (SS) 95–148 and SS 109–143 which are highly conserved [12]. SS 109–143 aligns with the single SS 178–213 of murine PrP. The two proteins show similar physical and biochemical properties, including solubility and sensitivity to proteinase K [27–29]. The structural similarity raises the question of functional similarity, and the possible direct interaction of the two proteins.

Extensive studies have been made of factors affecting the solution structure of PrP^C and its transformation into a conformation with increased β -sheet structure. This may relate to the pathogenic conformational conversion of PrP^C into the transmissible scrapie-like form PrP^{Sc} [30–33]. The objective of this work is to compare the stability and conformational properties of Dpl and PrP in solution, and to assess the conformational role of the second disulphide bond of Dpl, not found in PrP. We have therefore expressed and purified wild-type murine Dpl (residues 25–156) (wtDpl), and a mutant of Dpl (mtDpl) in which one disulphide bond has been removed by mutagenesis of a pair of cysteine residues [Cys⁹⁵ → Ser (C95S) and C148S], i.e. retaining only the disulphide bond (SS 109–143) analogous to that of PrP. Specifically, we have investigated the role of one intramolecular disulphide bond in the structure and stability of the PrP-like protein Dpl, and the extent to which Dpl resembles PrP in terms of its tendency to undergo an $\alpha \rightarrow \beta$ structural transition in acidic conditions in the presence of a mild denaturant. In addition we have examined whether Dpl and PrP interact under conditions *in vitro* which induce PrP to adopt a β -structured intermediate with a potentially significant function in the mechanism of the transmissible spongiform encephalopathy pathology.

EXPERIMENTAL

Expression, purification and refolding of wtDpl

The coding sequence for murine Dpl(25–156) was amplified by PCR from the expression vector pcDNA3.0.Dpl [12], using the 5' primer IDS 3 (5'-CGGGATCCCATGGGTCACCATCACC-

ATCACCACAAGGCAAGGGGCATAAAGCA-3') and the 3' primer IDS 5 (5'-CGAATTCTTAAGCTCCCCCTTCCAGCCAGA-3'). IDS 3 introduces the amino-acid sequence MGHHHHHH, which acts as a purification tag, directly on to the N-terminus of the Dpl 25–156 sequence, and also an *Nco*I restriction site for cloning. IDS 5 introduces a stop codon and an *Eco*RI restriction site immediately downstream of the Dpl sequence. The amplified gene was cloned into the *Nco*I/*Eco*RI fragment of the expression plasmid pTrcHis (Invitrogen). The resultant expression plasmid was named pMoDpl(25–156) and the integrity of the inserted gene sequence was verified by DNA sequencing. pMoDpl(25–156) was transformed into competent *Escherichia coli* 1B392 cells, and the cells were grown at 37 °C, 250 rev./min in 800 ml of Terrific Broth medium containing 100 μ g/ml ampicillin. When the cells reached a $D_{600nm} = 0.6$, wtDpl expression was induced by the addition of isopropyl β -D-thiogalactoside to a final concentration of 1 mM, and the culture was incubated for a further 5 h. Cells were harvested by centrifugation at 3000 *g* for 10 min and then lysed by resuspending in 30 ml of lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.2 mg/ml lysozyme and 0.1 mM PMSF) and incubating on ice for 15 min. The lysate was then diluted to 1 mg/ml with sodium deoxycholate, 50 μ g/ml with DNase I and 5 mM with MgCl₂, and incubated with stirring for 15 min at 20 °C. Insoluble inclusion bodies were harvested from the lysate by centrifugation at 15 000 *g* for 20 min and then solubilized in 30 ml of buffer A (6 M guanidinium hydrochloride, 100 mM NaH₂PO₄ and 10 mM Tris, pH 8.0). Insoluble material was removed by centrifugation at 15 000 *g* for 20 min. wtDpl was purified from the clarified lysate by immobilized-metal affinity chromatography. The lysate was batch bound to 4 ml of Ni²⁺-nitrilotriacetate-agarose, applied to a gravity flow column, washed and eluted with buffer A at pH 4.5, according to the manufacturer's instructions (Qiagen). The refolding protocol, based on the method of Mo et al. [26], was initially validated in experiments with recombinant murine Dpl(27–155), as reported previously [34]. The solution of purified Dpl in denaturant was diluted to 1 mg/ml protein in 6 M guanidinium hydrochloride, pH 8.0. The protein solution was added dropwise with rapid stirring to refolding buffer (50 mM Tris, pH 8.5, 600 mM L-arginine, 5 mM reduced glutathione and 0.5 mM oxidized glutathione), to give a 20-fold dilution. The mixture was incubated at 4 °C overnight. The refolded wtDpl was dialysed extensively against 10 mM KH₂PO₄, pH 6, spin-concentrated (Vivascience, 10 000 molecular mass cut off) and stored at 4 °C, or in 50 % glycerol at –80 °C.

Production of mtDpl

The mtDpl protein, lacking the SS 95–148, was produced by the simultaneous mutation of both loci using the QuikChangeTM site-directed mutagenesis system (Stratagene). Cys⁹⁵ was mutated to a serine using the primers IDS 14A (5'-CTACTACGAAGGCAGCTCTGAAGCCAACG-3') and IDS 14B (5'-CGTTGGCTTCAGAGCTGCCTTCGCTAGTAG-3'). Cys¹⁴⁸ was mutated to a serine using the primers IDS 15A (5'-CTCCGCCAAGCACAGCGATTTCTGGCTG-3') and IDS 15B (5'-CAGCCAGAAATCGCTGTGCTTGGCGGAG-3'). mtDpl was expressed, purified and refolded as for the wild-type protein described above.

Expression, purification and refolding of recombinant ovine PrP residues 94–233 [OvPrP(94–233) (ARQ)]

cDNA encoding the N-terminally truncated OvPrP(94–233) was amplified from plasmid pARQ#9 PrP using primers

5'-CGGGATCCCATGGGTCACCATCACCATCACACGGTCAAGGTGGTAGCCACAGT, which introduces an N-terminal His₆ sequence, and 5'-AGGGTCCGAATAATGGTTTCCCCGCGAATTCTAGAGCG. After digestion with *Nco*I and *Bgl*II enzymes, the amplified DNA was ligated into a pTrcHis vector (Invitrogen), which had been pre-incubated with *Nco*I and *Bgl*II. The final construct coded for the ARQ allele of OvPrP(94–233) with an N-terminal histidine-tag, and was verified by DNA sequencing. The protein was expressed and purified as described above for Dpl. After overnight incubation with 100 mM dithiothreitol at 4 °C, the Ni²⁺-column eluant was mixed 1:1 in 8 M urea/50 mM Hepes (pH 8.0) and passed through an SP Sepharose fast-flow column, eluting with 8 M urea/50 mM Hepes/1 M NaCl (pH 8.0). The eluant was diluted to approx. 100 µg/ml in 8 M urea/50 mM Hepes (pH 8.0), containing 2 molar equivalents of CuCl₂ per PrP molecule. After overnight incubation at 4 °C the copper was removed by extensive dialysis against 50 mM sodium acetate (pH 5.5). The PrP solution was spin-concentrated as described above and stored at 4 °C. The protein was shown to be > 95 % pure by SDS/PAGE. Oxidation was confirmed by MS analysis.

Capillary LC-MS of recombinant proteins

Recombinant wtDpl, mtDpl and OvPrP(94–233) were analysed for purity and correct expression by capillary HPLC coupled online to MS. Approx. 10 pmol of protein was loaded on to a home-made pre-concentration trap and eluted on to a home-made reversed-phase capillary HPLC column (180 µm internal diameter, 15 cm length; A. C. Gill, M. A. Ritchie and J. Hope, unpublished work) packed with Hichrom C₁₈ sorbent (3.5 µm bead size, 150 Å pore size; Hichrom Chromatography, Theale, Berks., U.K.). Components were eluted by a linear gradient of increasing concentration of acetonitrile, where solvent A was 0.05 % trifluoroacetic acid in 95:5 water/acetonitrile and solvent B was 0.05 % trifluoroacetic acid in 5:95 water/acetonitrile. The eluant from the column was passed through a UV detector (214 nm, Applied Biosystems) equipped with a U-Z view flow cell (LC Packings, Amsterdam, The Netherlands) to a Quattro II mass spectrometer (Micromass UK Ltd, Altrincham, Cheshire, U.K.) equipped with a commercial Z-spray ionization source operated in continuous flow nanospray mode. Positive ion mass spectra were recorded between *m/z* 500–2200 with a scan time of 4.5 s. All proteins were judged to be correctly expressed, to contain the expected number of disulphide bonds and were estimated to be > 95 % pure as determined by UV absorbance.

To check correct disulphide formation in the Dpl samples, proteins were digested with trypsin and the resulting peptides analysed by capillary HPLC–MS. Samples were dialysed into digestion buffer (2 M urea/100 mM ammonium bicarbonate, pH 8.0) and approx. 5 % (w/w) trypsin was added. The samples were incubated at 37 °C overnight. Approx. 20 pmol of each protein was analysed by HPLC–MS (conditions as described above), and positive ion mass spectra were recorded between *m/z* 300–2200. Peptides corresponding to those expected to contain the disulphide bond were detected. No peptides containing other possible disulphide linkages could be detected, confirming correct refolding of the proteins.

Optical spectroscopy

Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter with a protein concentration of typically 0.1 mg/ml in 1-mm pathlength cuvettes using a scan speed of 200 nm/min

and a response time of 0.25 s [35]. Multiple scans were averaged (typically *n* = 20), and the data are reported as such. The closely spaced spectra of Figure 3 (see the Results section) were additionally smoothed numerically with a cubic-quartic function [36]. CD intensities are expressed as mean residue mass ellipticities [θ_{mrw}], in degrees · cm² · dmol⁻¹, based on protein concentrations determined using molar absorption coefficients (ϵ_{280}) 29 400 M⁻¹ · cm⁻¹ for the wtDpl sequence and 29 280 M⁻¹ · cm⁻¹ for the mtDpl sequence calculated, according to Gill and von Hippel [37], and 22 658 M⁻¹ · cm⁻¹ as measured for OvPrP(94–233). For the multi-component secondary structure analysis using the Selcon3 algorithm [38], sets of data were uniformly restricted to the range 195–250 nm for 1-mm pathlength samples containing acetate. For mixing experiments including salt and 3.5 M urea, at higher protein concentration (1.0 mg/ml), a demountable cell of pathlength 0.1 mm was used.

Chemical denaturation with urea [39] was performed as described previously [40]. The process was shown to be effectively reversible in all cases, since > 95 % recovery of the spectroscopic signal of the native protein was observed upon dilution of the unfolded protein to non-denaturing conditions. Unfolding studies were performed using CD intensity at 222 nm, with typically 0.1 mg/ml protein at 22 °C in a 10-mm cuvette in either 20 mM sodium acetate/10 % glycerol (pH 4 or 5) or 20 mM potassium phosphate/10 % glycerol (pH 6 or 7). Aliquots of protein in concentrated denaturant were added to a solution of the protein at the same concentration in aqueous buffer, to achieve the desired denaturant concentration, and to confirm reversibility of the folding/unfolding equilibria.

Thermal denaturation was studied by measuring far-UV CD (222 nm) of protein at 0.05–0.1 mg/ml in 20 mM potassium phosphate/10 % glycerol (pH 7) in a 10-mm cuvette. Protein solutions were heated from 5 to 85 °C at approx. 1 °C/min, and the sample temperature was measured continuously with an immersed thermocouple (Comark). Reversibility of thermal denaturation was assessed to be > 95 %, following rapid heating to 85 °C and cooling to 15 °C. However, prolonged exposure to elevated temperature was minimized to avoid progressive irreversibility.

In studies of the interaction of PrP and Dpl, far-UV CD spectra of mixtures of the proteins were recorded as a function of time (up to 24 h), and compared with corresponding spectra of the individual proteins as controls. Samples of either wtDpl or mtDpl, and OvPrP(94–233) (ARQ) were prepared in buffer (20 mM sodium acetate, pH 4.0). Buffered 7 M urea, 300 mM NaCl or 7 M urea/300 mM NaCl was added to the stock protein to give solution conditions of either 3.5 M urea, 150 mM NaCl or 3.5 mM urea/150 mM NaCl respectively, in 20 mM sodium acetate (pH 4). In mixing experiments with urea, the buffered PrP and Dpl were combined initially, then the buffered salt and denaturant added, to give an equimolar protein concentration at 1 mg/ml total protein.

Data analysis

Chemical denaturation data were analysed by direct non-linear least-squares fitting of the observed far-UV CD signal (*Y*) to a two-state model of a single unfolding transition between native (N) and denatured (D) states:

$$Y = (I_N + S_N[D])f_N + (I_D + S_D[D])f_D$$

where *I_N* (*I_D*) and *S_N* (*S_D*) are the intercepts and slopes for the optical signals of the native (denatured) forms, [*D*] is the denaturant concentration. The fractional populations of native and denatured forms (*f_N* and *f_D* respectively) are determined from

Figure 2 Far-UV CD spectral properties of wtDpl and mtDpl

Spectra of wtDpl (0.14 mg · ml⁻¹; □) and mtDpl (0.11 mg · ml⁻¹; ○) in 20 mM phosphate, pH 7, at 22 °C.

the equilibrium constant for unfolding, K_u [$= \exp(-\Delta G_u/RT)$], with ΔG_u calculated using the linear extrapolation model (LEM) [41]:

$$\Delta G_u = \Delta G^\circ + m[D]$$

where ΔG° is the standard free energy of unfolding in the absence of denaturant and m is the slope which characterizes the change in ΔG_u with $[D]$. The denaturant concentration at the midpoint of the transition, $[D]_{1/2}$, is equal to $-\Delta G^\circ/m$.

Thermal denaturation data were similarly fitted to the following two-state model:

$$Y = (I_N + S_N T)f_N + (I_D + S_D T)f_D$$

where T is the temperature (°C) and the other parameters are defined as above. Free energy values used in calculating the fractional populations were obtained from the modified Gibbs–Helmholtz equation [42]:

$$\Delta G_T^\circ = \Delta H_m(1 - T/T_m) - \Delta C_p[T_m - T + T \ln(T/T_m)]$$

where T_m and ΔH_m are the temperature of the transition midpoint (K) and the van't Hoff enthalpy at T_m respectively, and ΔC_p is the difference in heat capacity between the native and denatured states. ΔC_p is poorly defined by the analysis [43]; a value of 7.5 kJ · mol⁻¹ · K⁻¹ would be appropriate for the full unfolding of a compact protein equivalent to the size of the recombinant Dpl (132 residues plus 7 additional N-terminal residues in the recombinant form). However, far-UV CD spectra show that the thermal transition involves only partial unfolding, and a value of 3.8 kJ · mol⁻¹ · K⁻¹ is more appropriate (see the Results section).

To prevent material being absorbed on to the walls of the cuvette during urea-denaturation titrations, the solvent contained 10% glycerol, which otherwise had no effect on the far-UV CD spectrum of the protein.

RESULTS

Secondary structure of wtDpl and mtDpl

Figure 2 shows that wtDpl and mtDpl exhibit closely similar far-UV CD spectra at pH 7, with minima at 208 nm and 222 nm

Figure 3 Effect of pH change on far-UV CD spectra of wtDpl and mtDpl

Upper panel: wtDpl (0.14 mg · ml⁻¹) and lower panel: mtDpl (0.11 mg · ml⁻¹) in 20 mM phosphate (pH 7 or 6) or 20 mM sodium acetate (pH 5 or 4) at 22 °C.

respectively, and a maximum at 192 nm, indicative of a substantial α -helical content. Spectral deconvolution analysis shows wtDpl contains 32% α -helix and 18% β -sheet, and mtDpl 30% α -helix and 19% β -sheet. These values agree well with those obtained for the wild-type mouse Dpl(27–154) (28–33% α -helix, approx. 15% β -sheet [29]), and for mouse Dpl(24–152) (40% α -helix [27]). The structural similarity between wtDpl and mtDpl indicates that Dpl folds correctly into the wild-type structure, even in the absence of SS 95–148. The CD spectra are similar to those found for the OvPrP(94–233), analysis of which shows 30% α -helix and 18% β -sheet. These values are comparable with those derived by NMR for a similar domain of mouse PrP (31% α -helix and 6% β -sheet) [44], although they differ somewhat from the values (42% α -helix and 5% β -sheet) of the corresponding NMR structures of hamster, bovine and human recombinant PrP [45–47].

Figure 3 shows the effect of pH in the range 4–7 on the spectra of wild-type and mutant Dpl. The secondary structure of wtDpl (Figure 3, upper panel) is relatively insensitive to pH. Over this

Figure 4 Effect of mutation on the thermal unfolding of Dpl proteins

Far-UV CD signal at 222 nm (pathlength, 10 mm) for temperature increase from 18 °C or 5 °C to 85 °C, of samples with concentration 0.05–0.1 mg · ml⁻¹ in 20 mM phosphate, pH 7. (—) measured signal. (○) Fitted data for wtDpl. (□) Fitted data for mtDpl (see the Experimental section).

pH range analysis shows 29–36 % α -helix and 15–19 % β -sheet content.

Figure 3 (lower panel) shows that mtDpl is more sensitive than the wtDpl to changes in pH. The far-UV CD spectra are similar between pH 7 and pH 5, but the spectrum at pH 4 is significantly different. There is a weaker signal at 222 nm, and the minimum at 208 nm has shifted to 205 nm, suggesting some loss of α -helical structure. This effect was verified with three independently prepared samples of the protein. Analysis shows a decrease of α -helix from 30 % to 21 %, with retention of 15 % β -sheet content. Thus, loss of SS 95–148 in the mtDpl causes the loss of a significant fraction of α -helix in mtDpl at low pH, but without obvious increase in β -structure.

Stability of wtDpl and mtDpl: thermal unfolding

Figure 4 shows that both wtDpl and mtDpl give a sigmoidal unfolding curve with increasing temperature at pH 7, consistent with a two-state reversible transition [48]. The amplitude of the transition in both cases indicates that the unfolding is incomplete, and analysis of the high-temperature spectra suggests that approximately half of the compact secondary structure has unfolded. wtDpl at 75 °C shows 12 % α -helix and 20 % β -sheet content, and mtDpl at 65 °C shows 11 % α -helix and 16 % β -sheet content. In both cases, far-UV CD spectra taken at increasing temperatures over this range show an isodichroic point at 203 nm (results not shown), characteristic of a reversible partial transition from α -helix to random coil. wtDpl demonstrates significantly greater thermal stability than mtDpl. Analysis using the modified Gibbs–Helmholtz equation [42] shows that the thermal unfolding transition of wtDpl is characterized by $T_m = 53$ °C and $\Delta H^\circ = 205$ kJ · mol⁻¹, which is in reasonable agreement with the values of $T_m = 58$ °C and $\Delta H^\circ = 192$ kJ · mol⁻¹ for MoDpl (24–152) obtained by Lu et al. [27]. In contrast, significantly higher values have been reported for wild-type PrP, e.g. human PrP(90–231) at pH 7.2 ($T_m = 70$ °C and $\Delta H^\circ = 293$ kJ · mol⁻¹) [49]; ovPrP_{ARQ}(23–234) at pH 5.0 ($T_m = 70$ °C and $\Delta H^\circ = 292$ kJ · mol⁻¹) [50]; and ovPrP_{ARQ}(94–

Table 1 Thermodynamic parameters for urea unfolding of wtDpl (A) and mtDpl (B) as a function of pH

Data in parentheses are approximate, due to partial unfolding occurring in the absence of urea (see the text for details).

(A)

pH	ΔG_{295}° (kJ · mol ⁻¹)	m (kJ · mol ⁻¹ M ⁻¹)	[D _{1/2}] (M)
7	24.7	–4.6	5.4
6	22.6	–4.6	4.6
5	16.7	–4.2	4
4	(4.6)	(–2.5)	(1.8)

(B)

pH	ΔG_{295}° (kJ · mol ⁻¹)	m (kJ · mol ⁻¹ M ⁻¹)	[D _{1/2}] (M)
7	21.3	–5.4	3.9
6	20.5	–5.4	3.8
5	(11.3)	(–4.2)	(2.7)
4	(3.8)	(–2.9)	(1.3)

233) at pH 7.0 ($T_m = 62$ °C, irreversible) (present study). The thermal unfolding curve for mtDpl shows it to be even less stable than wtDpl, and analysis of the thermal transition gives $T_m = 39$ °C, and $\Delta H^\circ = 26$ kcal · mol⁻¹. This lower stability of the mutant protein suggests that the second disulphide bond (SS 95–148), although not essential for correct protein structure formation, contributes substantially to the stability of Dpl, so that, in its absence, sequences adjacent to residues 95 and 148 are selectively destabilized. Consistent with this interpretation, a recent NMR study in which the analogous second disulphide was introduced into the PrP structure shows that this intramolecular linkage significantly reduces the accessible conformational space and restricts the dynamics of the residues of PrP adjacent to the two inserted cysteines [51].

Since the unfolding is incomplete at the upper temperature limit, there is some uncertainty in the calculation of the absolute value of ΔG_{295}° , the stability of either protein at 22 °C. Using $\Delta C_p = 7.5$ kJ · mol⁻¹ · K⁻¹, characteristic of 100 % unfolding of a protein of this size, gives values of $\Delta G_{295}^\circ = 7.9 \pm 3.8$ kJ · mol⁻¹ and 2.5 ± 1.05 kJ · mol⁻¹ for wt- and mtDpl respectively. However, using the theoretical treatment of protein stability described by Becktel and Schellman [42], this large value of ΔC_p predicts the existence of a temperature of maximum stability of 25 °C, with the protein 27 % unfolded at 23 °C, and reduced stability at lower temperatures. None of these features is observed experimentally. On the basis of the similar degree of partial thermal unfolding of both wt- and mtDpl, a value of $\Delta C_p = 3.8$ kJ · mol⁻¹ · K⁻¹ was adopted. This gives values of $\Delta G_{295}^\circ = 13.8 \pm 1.7$ and 4.18 ± 1.05 kJ · mol⁻¹ for wt- and mtDpl respectively. Thus while the absolute values of ΔG_{295}° from thermal unfolding are not fully established, the relative instability (at 22 °C) of mtDpl compared with wtDpl is well defined.

Stability of wtDpl and mtDpl: chemical unfolding

The equilibrium unfolding of the Dpl proteins was also followed by urea denaturation and analysed as described in the Experimental section. The results of the analysis, in terms of a two-state reversible process, are shown in Tables 1(A) and 1(B). wtDpl was studied at 22 °C, and mtDpl was studied at

Figure 5 Urea unfolding of wtDpl and mtDpl

Far-UV CD signal at 222 nm (pathlength, 10 mm) for wtDpl at 22 °C (upper panel), mtDpl at 7 °C (lower panel); concentration 0.04–0.07 mg · ml⁻¹ in 20 mM phosphate, 10% glycerol, pH 7 (□) or pH 6 (◇), or 20 mM sodium acetate pH 5 (△) or pH 4 (○). Fitted data (—) (see the Experimental section).

7 °C in view of its lower stability. The urea unfolding curves are fully reversible for both proteins throughout the pH range studied.

wtDpl (Figure 5, upper panel, and Table 1A) has similar stability at pH 7 and pH 6, with slightly higher values at pH 7 for urea unfolding midpoint [$D_{1/2}$] and for the free-energy difference between the native and unfolded state. wtDpl is considerably less stable to urea denaturation than OvPrP(94–233), which has a ΔG_{295}° value of 35 kJ · mol⁻¹, m of -5.9 kJ · mol⁻¹ · M⁻¹, and [$D_{1/2}$] of 6 M urea (results not shown). wtDpl becomes markedly less stable at pH 5, with a lower free-energy difference, m value and urea unfolding midpoint, whereas at pH 4, although the wild-type protein appears fully structured in the absence of urea (Figure 3, upper panel), the further decrease in stability prevents determination of the thermodynamic parameters, due to the absence of a pre-transitional unfolding slope. Extrapolating the data obtained for the unfolding curves at pH 5–7 this slope could be estimated, allowing approximate values to be obtained.

mtDpl (Figure 5, lower panel, and Table 1B) also exhibits similar stability to urea denaturation at pH 7 and pH 6, although it

is less stable than wtDpl. The stabilization free-energy values for both wt- and mtDpl are larger when determined by urea unfolding than by thermal unfolding. This is attributed to the protein remaining partially folded at high temperatures. Taking this into account, the consistent picture is still of significantly lower thermodynamic stability at pH 6–7 for mtDpl, compared with wtDpl. This decreased stability is even more pronounced at lower pH. Quantitative analysis of the urea unfolding curves becomes unreliable at pH 5 and below; at pH 4, mtDpl is already partially unfolded in the absence of urea (Figure 3, lower panel). Again, only an approximate analysis is possible using data extrapolation.

While the precise quantitation of the lower stability of both wtDpl and mtDpl at pH 4 is precluded due to limited experimental data, both proteins are clearly sensitive to lower pH. Further, mtDpl is more sensitive to urea denaturation than wtDpl, within the pH range studied. Thus removal of SS 95–148 renders Dpl more susceptible to the unfolding transition. However, there is no evidence from these data for an equilibrium unfolding intermediate for wt- or mtDpl either from the urea unfolding curves or from CD spectra at increasing urea concentrations.

Investigation of possible interactions between PrP and Dpl

The neuropathogenic function of Dpl *in vivo* was originally recognized as being abrogated by over-expression of PrP [16,20]. To date, the evidence for a direct interaction between PrP and Dpl has been largely negative (see the Introduction). Yeast two-hybrid techniques were previously used to identify candidate binding partners of PrP in a brain cDNA library, but would not have detected Dpl (since it was not expressed) [20,52]. The more direct assays using PrP as prey and Dpl as bait (and vice versa), as well as attempted co-immunoprecipitation in mammalian cells, both failed to demonstrate interaction of the two proteins (results not shown).

Consistent with these findings, initial CD experiments, mixing PrP and Dpl at concentrations of approx. 0.1 mg · ml⁻¹ at pH 4, showed no evidence of interaction. For either wtDpl or mtDpl mixed with equimolar PrP at pH 4, the far-UV CD spectrum for the protein mixture was effectively the same as that obtained by calculation from the component spectra (Figure 6, upper and lower panels).

However, the most significant implication of a possible interaction of Dpl and PrP would be if this occurs under the destabilizing conditions that are known to induce the $\alpha \rightarrow \beta$ transition in PrP. Incubation of PrP from a number of different species, including human PrP(90–231) [30–32], and murine PrP(121–231) [33], under mildly acidic and mildly denaturing conditions results in the formation of an equilibrium unfolding intermediate with a conformation containing increased β -structure. This intermediate is oligomeric, and has been actively studied as a possible species intermediate in the conversion from normal PrP^C and the pathological scrapie form PrP^{Sc}.

Depending on solution conditions and host species (human and hamster), the formation of the β -intermediate of PrP may be either rapid [33] or slow (minutes to hours) [30]. It is also favoured in rate and extent by increased ionic strength (specifically certain salts, e.g. NaCl), and high protein concentration [30]. In order to investigate whether either wtDpl or mtDpl may also be induced to adopt a similar intermediate conformation, samples of the individual proteins at 1 mg · ml⁻¹ were prepared in 20 mM sodium acetate, pH 4, with 3.5 M urea, and 150 mM NaCl. Buffered urea and NaCl were added to buffered protein to give desired concentrations. Far-UV CD spectra were measured immediately after sample preparation, at 22 °C, and monitored over a 24-h

Figure 6 Far-UV CD spectra of OvPrP(94-233) and wtDpl or mtDpl and of their equimolar mixtures, concentration $0.1 \text{ mg} \cdot \text{ml}^{-1}$ in 20 mM sodium acetate/150 mM NaCl, pH 4, at 22°C

Dpl ($\square$), PrP ($\triangle$), mixture ($\circ$) and calculated combination ($\diamond$). Upper panel: wtDpl mixed with OvPrP(94-233). Lower panel: mtDpl mixed with OvPrP(94-233) (see the text for details).

time course to check for time dependence. Far-UV CD spectra of wt- and mtDpl in 3.5 M urea at pH 4 showed a rapid ($< 1 \text{ min}$) transition of the protein from α -helical to substantially unfolded conformations (cf. Figures 5, upper and lower panels). In addition, the equilibrium unfolding of wt- or mtDpl in urea was not affected by the presence of 150 mM NaCl (results not shown). OvPrP(94-233) at pH 4 shows the typical α -helical conformation, which is unaffected by 150 mM NaCl, but is significantly unfolded by 3.5 M urea. In the presence of both 150 mM NaCl and 3.5 M urea, the spectrum shows the partial $\alpha \rightarrow \beta$ transition (Figure 7, upper panel). All of these changes were rapid, and stable over at least 24 h.

Experiments were therefore designed to investigate whether the presence of Dpl affects this low pH $\alpha \rightarrow \beta$ transition of PrP under controlled conditions for the pure proteins studied *in vitro*. Equimolar samples of either wtDpl or mtDpl and OvPrP(94-233) were prepared in 20 mM sodium acetate, pH 4.0. Buffered urea, NaCl or urea/NaCl were added, to give samples with

Figure 7 Effect of urea and NaCl on the conformational transitions of PrP in the presence of Dpl

Upper panel: far-UV CD spectra (pathlength, 0.1 mm) of PrP at $1 \text{ mg} \cdot \text{ml}^{-1}$ in 20 mM sodium acetate, pH 4, ($\circ$) with 150 mM NaCl ($\square$), 3.5 M urea ($\diamond$), and 150 mM NaCl plus 3.5 M urea ($\triangle$). Middle panel: mixing of wtDpl and OvPrP(94-233) at $0.1 \text{ mg} \cdot \text{ml}^{-1}$ in the presence of urea. Far-UV CD spectra (pathlength 0.1 cm) of wtDpl and OvPrP(94-233) were recorded in 20 mM sodium acetate, 3.5 M urea, pH 4, at 22°C . Spectra were taken immediately after mixing. Dpl ($\square$), PrP ($\triangle$), equimolar mixture ($\circ$) and calculated combination ($\diamond$). Lower panel: mixing of wtDpl and OvPrP(94-233) at $1 \text{ mg} \cdot \text{ml}^{-1}$ with urea and NaCl. Far-UV CD spectra (pathlength, 0.1 mm) of wtDpl and OvPrP(94-233) were recorded in 20 mM Na acetate, 3.5 M urea, 150 mM NaCl, pH 4, at 22°C . Spectra were taken 24 h after mixing. Dpl ($\square$), PrP ($\triangle$), equimolar mixture ($\circ$) and calculated combination ($\diamond$).

$1 \text{ mg} \cdot \text{ml}^{-1}$ total protein in either 3.5 M urea, 150 mM NaCl or 3.5 M urea/150 mM NaCl. Far-UV CD spectra of these 1:1 mixtures of PrP with wt- or mtDpl were compared with those of

the component proteins under identical conditions over a period of 24 h. Figure 7 (middle panel) shows that, in the presence of 3.5 M urea, the far-UV CD spectra of the mixture of wtDpl and PrP is closely similar to that calculated for the sum of the component proteins, and it remains stable for at least 24 h. Figure 7 (lower panel) shows that in the presence of 3.5 M urea/150 mM NaCl, the far-UV CD of the mixture (again stable for at least 24 h) is effectively the same as that of the calculated sum of components. These data, plus similar results obtained with mtDpl and PrP (results not shown), indicate that there is no observable increase in the total amount of β -structured protein in the mixed samples. Thus even under mildly denaturing conditions known to favour the $\alpha \rightarrow \beta$ transition of PrP, there is apparently no observable effect in far-UV CD indicative of an enhancement of the $\alpha \rightarrow \beta$ transition of PrP from interaction with either wtDpl or mtDpl, or for the ability of PrP in the β -rich form to induce either Dpl protein to adopt a more β -like conformation.

DISCUSSION

Despite intensive studies, the mechanism by which PrP transmits disease is largely unknown, but the proposition that PrP^C can be induced to adopt a conformation leading to the formation of pathogenic PrP^{Sc} has become the major working hypothesis. The genetic, structural and pathogenic relationships between PrP and Dpl have prompted the present work. In studying Dpl and its variant, we have attempted to probe similarities and differences compared with the prion protein in order to gain insight into the features which make PrP unique. Although the globular portions of Dpl and PrP have only approx. 25% sequence identity, Dpl shows a strong conformational similarity with the corresponding C-terminal portion of PrP. Dpl lacks the N-terminal octa-repeat domain of PrP, but studies have demonstrated that this region is not essential for prion infectivity [53]. Dpl lacks the sequence of murine PrP(106–126), which has been reported to be neurotoxic [25]. Previous studies of Dpl [26] have suggested two factors which preclude its conversion into a scrapie-like form, namely the absence of the palindromic amino acid sequence AGAAAAGA, (murine PrP) residues 112–119, and the presence of an additional disulphide bond. We have addressed the second of these features, by creating a variant of Dpl with this disulphide bond removed.

We have shown that the absence of the 95–148 disulphide bond does not significantly alter the conformation of murine Dpl from pH 5–7. Below pH 4, the mtDpl shows a greater tendency to unfold than wtDpl. Consistent with this, in the pH range 5–7, wtDpl is more stable than the single-disulphide mutant to both thermal and chemical denaturation, showing that the second disulphide bond has a significant stabilizing effect. In fact, wtDpl with two disulphides is less stable than PrP under comparable [$D_{1/2}$] and lower change in free energy for unfolding at 22 °C. This indicates that, in spite of the similarity in secondary structure, PrP is stabilized by intramolecular contacts to a greater extent than either wtDpl or its single-disulphide mutant.

One distinctive feature of recombinant PrP is that under the conditions of low denaturant insufficient to cause full unfolding, and at acidic pH, the protein can form an equilibrium folding intermediate, undergoing a conformational change with a partial loss of α -helical structure and formation of a conformation with increased β -sheet structure. It has been suggested that this transition may be similar to that of the conversion of PrP^C into PrP^{Sc} *in vivo* [30–32]. Neither wt- nor mtDpl was observed to undergo such a transition in similar conditions of urea denaturation. Although both proteins become increasingly unstable

at acidic pH, the unfolding process for both proteins conforms to a two-state equilibrium model, proceeding directly from the native helical state to the unfolded state. Neither increasing the ionic strength nor allowing an incubation period of up to 24 h in the presence of mild denaturant altered this behaviour. Neither the wtDpl nor the prion-like mtDpl could be induced to exhibit the $\alpha \rightarrow \beta$ conformational variations characteristic of PrP. Thus the greater thermodynamic stability of PrP compared with wtDpl appears to provide greater possibilities for it to adopt, possibly by a favoured kinetic pathway [54], a significantly different secondary structure, namely a species with enhanced β -sheet content. This conformation is generally an aggregated oligomeric form, hence the thermodynamic stability of PrP as a monomeric β -rich conformation cannot readily be derived.

The possibility that PrP and Dpl may interact *in vivo* was examined by mixing the two proteins under a range of conditions that favour the PrP transition process. In no case was there an indication from far-UV CD spectra that Dpl participated in an $\alpha \rightarrow \beta$ conversion itself, or had an effect on the same transition in PrP. It is possible that the interaction may not have involved conformational change, or that there is a requirement for the octa-repeat region of PrP. Alternatively such an interaction (*in vivo*) may depend on an as yet unidentified ligand that is common to both proteins.

Thus in spite of striking common structural features, the prion protein and Dpl variants behave significantly differently in their conformational stability. Previous results [55] showed that variations in the thermodynamic stability of a number of PrP mutants is not necessarily an indicator of an enhanced propensity for $\alpha \rightarrow \beta$ conformational transitions. The presence of the second disulphide (SS 96–148) in wtDpl represents a significant restriction in the range of conformational possibilities for this protein. The results with mtDpl clearly demonstrate that removal of this disulphide further decreases the stability of the protein (relative to PrP), but does not itself increase the potential for the $\alpha \rightarrow \beta$ conformational transformation observable under appropriate conditions with PrP. The greater thermodynamic stability of PrP may allow an increased probability of adopting a stable β -rich intermediate state, further stabilized by intermolecular association. In addition, a number of specific sequences and residues have been potentially implicated in the conversion reaction of PrP [7,56–60]. Taken together, these factors may indicate that the β -structural conformational conversion transformations of PrP are indeed a unique property of this molecule, and consequently are not shared by the related, but distinctive protein, Dpl.

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