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Human prion proteins expressed in *Escherichia coli* and purified by high-affinity column refolding

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Abstract An efficient method is presented for the production of intact mammalian prion proteins and partial sequences thereof. As an illustration we describe the production of polypeptides comprising residues 23–231, 81–231, 90–231 and 121–231 of the human prion protein (*h*PrP)[1]. Polypeptides were expressed as histidine tail fusion proteins into inclusion bodies in the cytoplasm of *Escherichia coli* and refolded and oxidized while N-terminally immobilized on a nickel-NTA agarose resin. This 'high-affinity column refolding' facilitates the preparation of prion proteins by preventing protein aggregation and intermolecular disulfide formation. After elution from the resin the histidine tail can be removed using thrombin without cleaving the prion protein polypeptide chain. The same protocol as used here for *h*PrP has been successfully applied with bovine and murine prion proteins. The protein preparations are stable for weeks at room temperature in concentrated solution and are thus suitable for detailed structural studies. Preliminary biophysical characterization of *h*PrP(23–231) suggests that the C-terminal half of the polypeptide chain forms a well-structured globular domain, and that the N-terminal half does not form extensive regular secondary structures.

Key words: Human prion protein; Prion protein partial sequence; Creutzfeldt-Jakob disease; Transmissible spongiform encephalopathy; High-affinity column refolding

1. Introduction

Mammalian prion proteins (PrP) have a 22-amino acid N-terminal signal sequence [2,3], and a 23-amino acid C-terminal signal sequence encoding attachment of a glycosylphosphatidylinositol (GPI) anchor [4,5]. The mature protein of 208 or 209 amino acids contains one disulfide bond [3], and has two asparagine-linked glycosylation sites [6,7]. Keen interest is focused on PrP since evidence has been presented that post-translational conversion of a ubiquitous cellular isoform (PrP^{C}) into the scrapie isoform (PrP^{Sc}) is the fundamental process underlying transmission and pathogenesis of transmissible spongiform encephalopathies (TSEs), which include kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in humans, bovine spongiform encephalopathy (BSE), and scrapie in sheep (for recent reviews see, for example, [8–10]). This 'protein-only' hypothesis [11,12] provides a rationale for the observation that inherited TSEs can be transmitted to laboratory animals, which is a unique feature in currently known genetic diseases [13] so that its further investigation is of general interest. TSE research will also in the future rely on the availability of high quality preparations of prion protein [10]. This paper describes an efficient procedure for the production of recombinant prion proteins in *Escherichia coli* that is based on 'high-affinity column refolding'.

Alternative procedures for the production of recombinant mammalian prion protein or fragments thereof have previously been reported. Syrian hamster PrP (*sh*PrP) comprising residues 90–231, which corresponds to the protease-resistant PrP 27–30 form [13], was expressed as an insoluble protein in the cytoplasm of *Escherichia coli* [14]. Other *sh*PrP constructs could not be expressed using the same expression system because of protein degradation. After solubilization in 8 M GdmCl *sh*PrP(90–231) was purified by size exclusion chromatography and reversed phase chromatography. Purification under reducing conditions yielded a high content of β-sheet and relatively low solubility, similar to PrP^{Sc}, whereas refolding by oxidation to form a disulfide bond between the two cysteinyl residues produced a soluble protein with a high α-helix content, similar to PrP^{C}.

Recombinant mouse PrP comprising residues 121–231, *m*PrP(121–231), was expressed as a soluble protein in the periplasm of *E. coli* using an OmpA signal sequence [15]. Larger fragments of *m*PrP were amino-terminally degraded during periplasmic expression. *m*PrP(121–231) was purified by anion-exchange chromatography, hydrophobic chromatography and gel filtration. HPLC analysis revealed that the single disulfide bond was quantitatively present in the purified protein. The nuclear magnetic resonance (NMR) structure of *m*PrP(121–231) [16] shows a high content of α-helices and a small β-sheet, which coincides with earlier characterization of the PrP^{C} form of prion proteins by biophysical methods [17,18].

The intact *m*PrP polypeptide was expressed in inclusion bodies in the cytoplasm of *E. coli* [19], and was purified by conventional chromatographic techniques, including ion-exchange chromatography and reversed phase chromatography. Oxidation of the purified protein was performed in basic buffer containing 8 M urea and 1 μM $CuSO_4$. NMR experiments on *m*PrP(23–231) in aqueous solution at pH 4.5 showed that the previously determined globular three-dimensional structure of the C-terminal domain *m*PrP(121–231) is preserved in the intact protein, and that the N-terminal polypeptide segment 23–120 is flexibly disordered [20]. Intact bovine PrP has been expressed as a fusion protein with a histidine tag in *E. coli* [21], which was purified via a nickel-NTA column and refolded by dialysis from 8 M urea into buffer.

The method described here for expression and purification of prion proteins is illustrated with the production of polypeptides comprising residues 23–231, 81–231, 90–231, and

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[1]Sequence positions according to Syrian hamster PrP [1].

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121–231 of human PrP (*h*PrP). The proteins were expressed as inclusion bodies in the cytoplasm of *E. coli* in a construct with an N-terminal histidine tail and an engineered thrombin cleavage site. The critical step for the production of *h*PrP is its renaturation to the correctly folded protein [14,19,21]. Here, refolding of the histidine tail-fused PrPs immobilized to a nickel-NTA column prevented protein aggregation and favored intramolecular disulfide bond formation during refolding. Using thrombin, the N-terminal histidine tail could be cleaved off without hydrolyzing the prion protein polypeptide chain. The purified *h*PrP polypeptides were characterized by NMR, circular dichroism (CD), and fluorescence spectroscopy. The same protocol has been successfully applied with PrP from different species (to be published elsewhere).

2. Materials and methods

2.1. Cloning of prion proteins

The plasmid pRSET A (Invitrogen) for expression of mammalian prion proteins in *E. coli* encodes an N-terminal histidine tail that contains an engineered thrombin cleavage site [22]. The histidine tail is composed of 17 amino acids (MRGSHHHHHHGLVPRGS). The DNA of human prion protein ([23] accession number M13899) was obtained by polymerase chain reaction (PCR) of commercially available cDNA of human brain (Clontech). Two primers flanking the prion protein with *Bam*HI and *Eco*RI restriction sites enabled us to clone the PCR fragment into the polylinker site of pRSET A. PCR was performed with *Pfu* (Stratagene) to reduce the risk of undesired random mutations. The reaction was performed in 25 μl volumes for 25 cycles with 400 nM primer and 200 μM of each dNTP. The annealing temperature was 60°C. The inserted DNA sequence was verified by the dideoxy-mediated chain-termination method.

2.2. Expression and purification of prion proteins

Freshly transformed overnight culture of *E. coli* BL21(DE3) cells (Stratagene) containing the respective plasmid for expression of prion protein was added at 37°C to 2 l of Luria broth (LB) medium plus ampicillin (100 μg/ml). At $OD_{600} = 0.5$, expression was induced with isopropyl β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. For isotope labeling a minimal medium (MM) containing 1 g/l [^{15}N]ammonium chloride, 2 g/l [$^{13}C_6$]glucose, and a vitamin cocktail (5 mg/l thiamine, 1 mg/l D-biotin, 1 mg/l choline chloride, 1 mg/l folic acid, 1 mg/l niacinamide, 1 mg/l D-pantothenic acid, 1 mg/l pyridoxal hydrochloride, 0.1 mg/l riboflavin) was used. Expression in MM was induced at an $OD_{600} = 1.1$. In each medium the cells were harvested 8 h after induction, centrifuged, resuspended in 100 ml buffer G (6 M GdmCl, 10 mM Tris-HCl, 100 mM Na_2PO_4, 10 mM reduced glutathione, pH 8.0), and further processed as outlined in Fig. 1. After sonication and centrifugation, the soluble protein fraction was added to 30 ml of nickel-nitrilotriacetic acid (NTA) agarose resin (Qiagen) and stirred for 10 min. The resin was poured into a column and washed with 120 ml buffer G, and to the immobilized histidine tail-containing fusion protein we applied a 200 ml gradient of buffer G to buffer B (10 mM Tris-HCl, 100 mM Na_2PO_4, pH 8.0). Protein impurities devoid of histidine tails were removed from the agarose resin with 75 ml of 50 mM imidazole in buffer B. The polypeptides *h*PrP(23–231), *h*PrP(81–231), and *h*PrP(90–231) were eluted with buffer E (10 mM Tris, 100 mM Na_2PO_4, 500 mM imidazole, pH 5.8), while the fragment *h*PrP(121–231) was eluted with buffer B containing 150 mM imidazole. After washing the resin with 30 ml of buffer G, oxidative refolding and imidazole elution were repeated to obtain a second batch of soluble prion protein. Histidine tail-fused PrP was dialysed against buffer D (10 mM Na_2PO_4, pH 5.8), and afterwards against water. The histidine tail was removed from the prion protein using thrombin (Sigma; 0.1 units/ml). The cleaving reaction was carried out at RT for 1 h in buffer C (5 mM Tris-HCl buffer, pH 8.5). The protease was removed from the prion protein fragment *h*PrP(121–231) by loading onto a DE 52 column (10 ml resin; Whatman) equilibrated in buffer C, followed by elution with a 200 ml gradient of 0–200 mM NaCl. The polypeptides *h*PrP(23–231), *h*PrP(81–231) and *h*PrP(90–231) were loaded onto a CM 52 resin (Whatman) equilibrated with buffer I (10 mM Na_2PO_4, pH 6.5), and eluted with a 200 ml gradient of 0–500 mM NaCl. The cleaved histidine tail was removed by dialysis against water was using a Spectrapor-membrane (MW 6000–8000).

Purified protein was analyzed by SDS-polyacrylamide gel electrophoresis in the absence of reducing agent (Fig. 2), N-terminal sequencing, and electrospray mass spectrometry. Protein concentration was determined using the molar extinction coefficients $\varepsilon_{276} = 16\,240$ M^{-1} cm^{-1} for *h*PrP(121–231), $\varepsilon_{276} = 21\,640$ M^{-1} cm^{-1} for *h*PrP(90–231), $\varepsilon_{276} = 32\,440$ M^{-1} cm^{-1} for *h*PrP(81–231), and $\varepsilon_{276} = 56\,940$ M^{-1} cm^{-1} for *h*PrP(23–231).

2.3. NMR spectroscopy

NMR experiments are reported here for the uniformly ^{15}N-labeled human prion protein, *h*PrP(23–231), and the ^{15}N-labeled C-terminal fragment *h*PrP(121–231). The protein concentration was 1 mM in a solvent of 90% H_2O/10% D_2O, and 10 mM [d_4]-sodium acetate buffer, pH 4.5. NMR spectra were recorded on a 750 MHz Bruker DRX spectrometer at 20°C. One-dimensional ^{1}H-NMR spectra were acquired with an acquisition time of 0.1 s, and the spectral range was 10 000 Hz. Two-dimensional ^{15}N,^{1}H-COSY spectra [24] were acquired using spectral widths of $\omega_1(^{15}N) = 1700$ Hz and $\omega_2(^1H) = 10\,000$ Hz, $t_{1max} = 74$ ms, $t_{2max} = 105$ ms, and the time domain data size was 128×1024 complex points. Data processing was performed with the program PROSA [25], and the spectral analysis was supported with the program XEASY [26].

2.4. Circular dichroism spectroscopy

Circular dichroism spectra were recorded on a Jasco J720 spectropolarimeter using a thermostatted cuvette (Helma) with 1 mm pathlength. Measurements were carried out at 14°C in 10 mM potassium phosphate buffer, pH 7.0, at a final protein concentration of 8 μM.

2.5. Fluorescence spectroscopy

Fluorescence spectra were recorded on a SPEX FLUOROLOG spectrometer using a 0.4×1 cm cuvette. Measurements were carried out at 23°C in 10 mM potassium phosphate buffer pH 7.0, and at a final protein concentration of 1 μM. The bandwidth of both the excitation and emission slits was 5 nm.

3. Results and discussion

3.1. Expression and purification of hPrP and partial sequences thereof

Human prion protein polypeptides were expressed with an

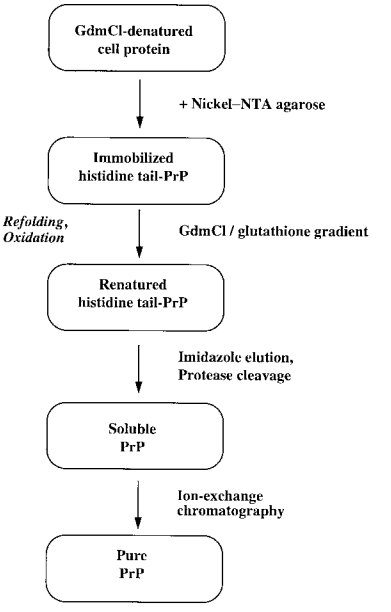

Fig. 1. Scheme summarizing the purification procedure of recombinant human prion protein polypeptides using 'high-affinity column refolding'.

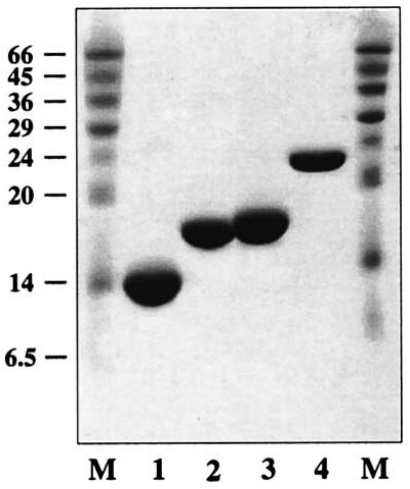

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified *h*PrP polypeptides. M = molecular weight standards (in kDa); 1: *h*PrP(121–231); 2: *h*PrP(90–231); 3: *h*PrP(81–231); 4: *h*PrP(23–231). The calculated molecular weights of the four *h*PrP polypeptides are 13137 Da, 16194 Da, 17156 Da, and 22876 Da. Electrophoresis was carried out under non-reducing conditions. Thus, the single band in all the protein preparations indicates that purified protein does not contain intermolecular disulfide bonds.

N-terminal histidine tail as inclusion bodies in the cytoplasm of *E. coli*. The histidine tail is composed of 17 amino acids comprising the sequence MRGSHHHHHHGLVPRGS, and contains a thrombin cleavage site between Arg-15 and Gly-16. Expression was performed with freshly transformed cells, as PrP inclusion bodies are toxic for *E. coli*, leading to loss of the plasmid. The amount of inclusion bodies was very similar for the various polypeptides, which were all expressed under control of a T7 promoter.

The purification procedure of PrP proteins is summarized in Fig. 1. After sonication and centrifugation of the GdmCl denatured *E. coli* cell lysate, the soluble protein fraction was added to a nickel-NTA agarose resin, which has a high affinity for polypeptides that contain six consecutive histidine residues at their N- or C-terminus [27]. Proteins not containing a histidine tail were removed by washing the resin with GdmCl buffer containing reduced glutathione, to prevent the formation of intermolecular disulfide bonds in the denatured protein. Refolding and oxidation are the critical steps for obtaining high yields of correctly folded prion protein [14,19,21], and renaturation of the protein by simply diluting out the denaturant and the reducing agent leads to considerable protein aggregation and intermolecular disulfide bond formation. However, N-terminal immobilization during refolding via a 100–0% GdmCl/glutathione gradient prevents protein aggregation and favors intramolecular disulfide bond formation. Protein impurities with low affinity for nickel-NTA were removed from the resin with 50 mM imidazole, and monomeric prion protein was then eluted at an imidazole concentration of >125 mM. Oligomeric and/or unfolded prion protein remained bound to the resin, but could in part be converted to soluble protein in a second column refolding step. The N-terminal histidine tail was removed using thrombin. Interestingly, thrombin does not cleave within the polypeptide chain of the prion protein during the incubation time of about 1 h that is necessary for the removal of the histidine tail. In the final purification step for *h*PrP(121–231), thrombin was removed by anion-exchange chromatography. In order to take account of the high content of positive charges in the N-terminal 98-residue peptide segment (the isoelectric point of *h*PrP(121–231) is at pH 5.8, while those of the longer PrP polypeptides range from pH 8.0 to 9.4), intact *h*PrP and the fragments truncated at codons 81 and 90 were subjected to cation-exchange chromatography to remove the protease. Using this procedure, 10–20 mg of prion protein polypeptide was obtained per liter of culture (Fig. 2), also in the minimal media needed for isotope labeling with ^{15}N and ^{13}C.

High-affinity column refolding was also successfully applied for the production of recombinant bovine and mouse prion proteins of various lengths (to be published).

3.2. Preliminary spectroscopic characterization of hPrP

The near-UV CD spectra of the four human prion protein polypeptides (Fig. 3A) indicate a high content of α-helix secondary structure and a low amount of β-sheet structure.

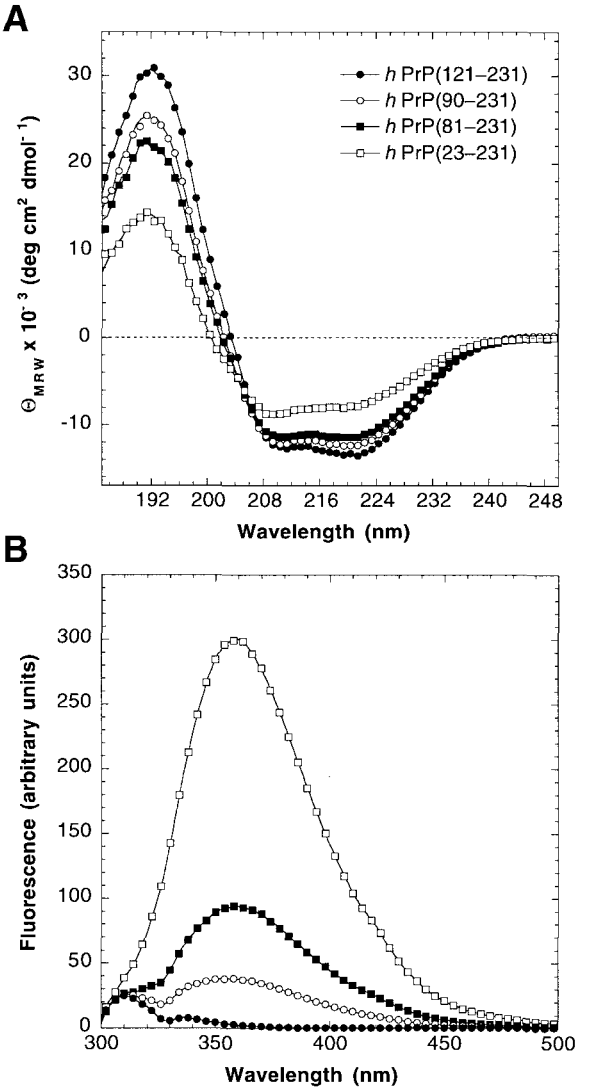

Fig. 3. Near-UV circular dichroism (A) and fluorescence (B) of *h*PrP polypeptides. Circular dichroism spectra were recorded at a protein concentration of 8 μM in 10 mM potassium phosphate buffer, pH 7.0, at 14°C. Fluorescence spectra (excitation wavelength 280 nm) were recorded at a protein concentration of 1 μM in 10 mM potassium phosphate buffer, pH 7.0, at 23°C.

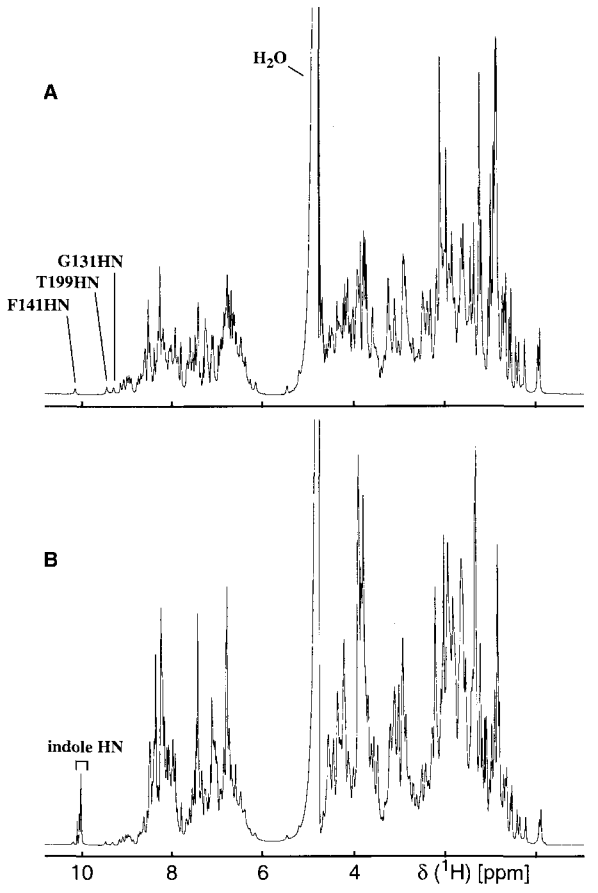

Fig. 4. One-dimensional 750 MHz ^{1}H-NMR spectra of *h*PrP(121–231) (A) and *h*PrP(23–231) (B) (solvent 90% H_2O/10% D_2O, 10 mM [d_4]-sodium acetate pH 4.5). Resonance assignments for selected well-separated resonance signals are indicated in A (R. Zahn, R. Riek, G. Wider and K. Wüthrich, to be published). The signals of the indole groups of the seven Trp residues in intact *h*PrP are indicated in B.

There is an increase in random coil content with increasing length of the polypeptides, indicating that the N-terminal half of *h*PrP does not form extensive regular secondary structures. Interestingly, although the polypeptide sequence 23–120 seems to have little or no regular secondary structure, it was sufficiently resistant against protease cleavage by thrombin to enable use of the aforementioned purification procedure.

After excitation at 280 nm, the fluorescence spectra of *h*PrP(81–231) and *h*PrP(23–231), which comprise 7 and 3 Trp residues, respectively, show a maximal fluorescence intensity at 359 nm (Fig. 3B), indicating that the indole rings are exposed to solvent. The fluorescence maximum of the single Trp residue in *h*PrP(90–231) is slightly blue-shifted to a wavelength of 356 nm, indicating that although the indole ring of Trp-99 is mostly solvent-exposed, it may have some hydrophobic interactions. The C-terminal fragment *h*PrP(121–231) is devoid of Trp residues, and its overall fluorescence is concomitantly low.

The ^{1}H NMR spectra of *h*PrP(121–231) and *h*PrP(23–231) (Fig. 4) show that the protein preparations are homogeneous, and the chemical shift dispersion is typical for globular proteins. Thus, the resonance lines near 0 ppm are representative for ring current-shifted methyl resonances in well-structured

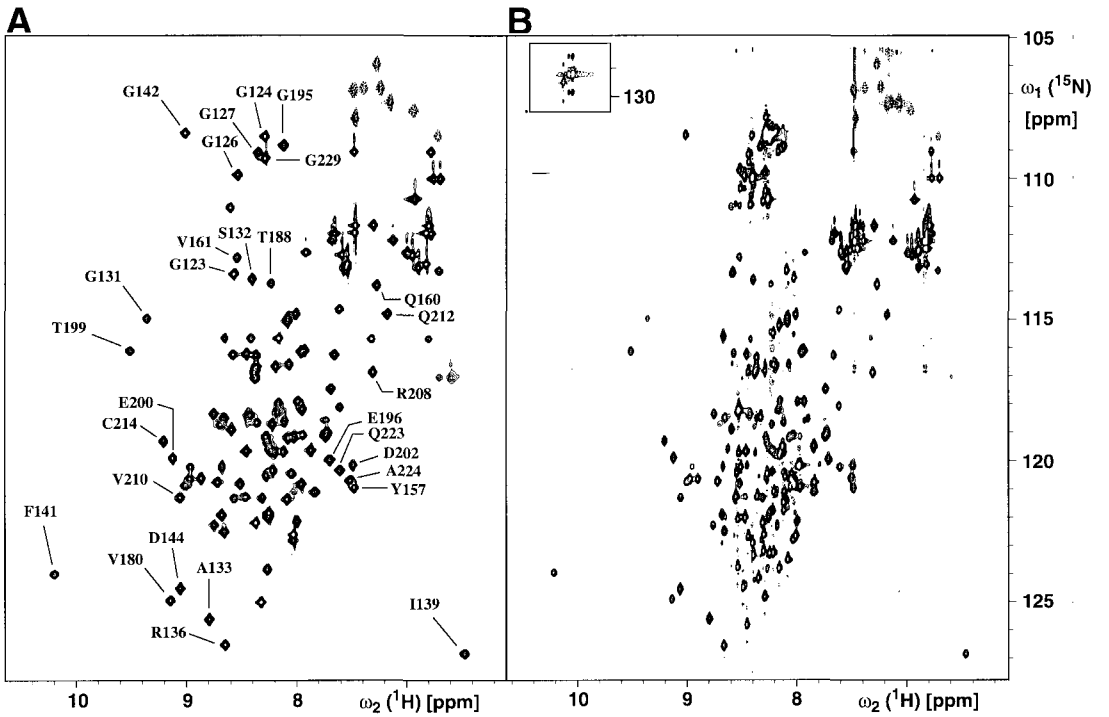

Fig. 5. Two-dimensional ^{15}N,^{1}H-COSY spectra of *h*PrP(121–231) (A) and *h*PrP(23–231) (B). Resonance assignments for selected well-separated resonance signals are indicated in A (R. Zahn, R. Riek, G. Wider and K. Wüthrich, to be published). The rectangle in B contains the Trp indole $^{15}N^{\varepsilon}$-^{1}H cross peaks, which are folded in ω_1.

globular proteins [28]. Near 10 ppm a group of lines (Fig. 4B) represent the resonances of indole NH groups of seven Trp residues in *h*PrP(23–231), all of which are located within the 23–120 segment. The small chemical shift dispersion of these lines suggests that the indole rings are solvent-exposed, which is in agreement with the CD and fluorescence measurements and has also been observed for *m*PrP [20]. The absence of Trp residues in *h*PrP(121–231) is confirmed in the ^{1}H NMR spectrum (Fig. 4A). Similar patterns of the signals near 10 ppm, between 5.0 and 6.5 ppm, and near 0 ppm (Fig. 4A,B) indicate that the globular structure of the C-terminal fragment is conserved in the intact protein. The similar structure of the partial sequence 121–231 within the two polypeptides is clearly apparent in the two-dimensional $^{15}N,^{1}H$-COSY NMR spectra (Fig. 5). In the spectrum of *h*PrP(121–231) (Fig. 5A) we identified 103 out of 108 expected backbone amide resonances (the thrombin cleavage site of the *h*PrP constructs introduces an additional Gly-Ser dipeptide preceding the N-terminus of the prion protein sequence, so that for *h*PrP(121–231) the resonances of Ser and Val-121 are also observed in the $^{15}N,^{1}H$-COSY spectrum). All eight expected Gly backbone amide protons (Fig. 5A), all eight expected side chain guanidino $^{1}H^{N\varepsilon}$ resonances, and 23 out of 28 expected side chain amide resonances have been identified. In the intact protein, cross peaks in identical positions to all outstanding chemical shifts assigned in the C-terminal domain are observed (Fig. 5). The additional cross peaks in the spectrum of *h*PrP(23–231) show little ^{1}H chemical shift dispersion, as would be expected for an extended, 'unfolded' polypeptide [28]. There was no change in ^{15}N and $^{1}H^{N}$ chemical shifts when the *h*PrP polypeptides described here were incubated for several weeks at room temperature, indicating that the purified protein is free of contaminating proteases, and thus suitable for structure determination by NMR.

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