

Recombinant full-length murine prion protein, *mPrP*(23–231): purification and spectroscopic characterization

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Abstract The cellular prion protein of the mouse, *mPrP*^C, consists of 208 amino acids (residues 23–231). It contains a carboxy-terminal domain, *mPrP*(121–231), which represents an autonomous folding unit with three α -helices and a two-stranded antiparallel β -sheet. We expressed the complete amino acid sequence of the prion protein, *mPrP*(23–231), in the cytoplasm of *Escherichia coli*. *mPrP*(23–231) was solubilized from inclusion bodies by 8 M urea, oxidatively refolded and purified to homogeneity by conventional chromatographic techniques. Comparison of near-UV circular dichroism, fluorescence and one-dimensional ¹H-NMR spectra of *mPrP*(23–231) and *mPrP*(121–231) shows that the amino-terminal segment 23–120, which includes the five characteristic octapeptide repeats, does not contribute measurably to the manifestation of three-dimensional structure as detected by these techniques, indicating that the residues 121–231 might be the only polypeptide segment of PrP^C with a defined three-dimensional structure.

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1. Introduction

Transmissible spongiform encephalopathies (TSEs) such as the Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle are believed to be caused by a novel class of infectious pathogens, the 'prions' [1–3]. According to the protein-only hypothesis [4–6], the prion consists of an abnormal oligomeric form, PrP^{Sc}, of the host-encoded cellular prion protein, PrP^C. While mammalian PrP^C is a highly conserved, monomeric cell surface glycoprotein, PrP^{Sc} forms protease-resistant oligomers with amyloid characteristics [1–3,7]. PrP^C and PrP^{Sc} monomers appear to be identical in their covalent structures [7], but an increased β -sheet content has been demonstrated for PrP^{Sc} when compared with PrP^C [8,9]. Different kinetic models, such as the 'nucleation-polymerization' model [10] and the 'template assistance' model [11], have been proposed for the mechanism of self-replication of the infectious oligomer (reviewed in [3]).

The murine prion protein, *mPrP*(23–231), consists of 208 amino acids (residues 23–231 in the numeration of PrP from Syrian hamster, with deletion of residue 55 [12]). It has a

single disulfide bond (Cys¹⁷⁹–Cys²¹⁴), two N-glycosylation sites (Asn¹⁸¹ and Asn¹⁹⁷) and a glycosyl-phosphatidyl-inositol (GPI) anchor at its carboxy-terminal Ser²³¹ [7]. We previously demonstrated that the recombinant segment 121–231 of *mPrP* represents a distinct domain that folds autonomously and reversibly [13] and has a well-defined three-dimensional structure formed by three α -helices and a two-stranded antiparallel β -sheet [14].

The exact size of the domain *mPrP*(121–231) was deduced from the observation that expression of the PrP fragments 95–231 and 107–231 in the periplasm of *Escherichia coli* was accompanied by amino-terminal degradation, with cleavage at multiple sites in the segment 100–120 [13]. In this paper, we report the purification of the complete, recombinant murine PrP^C protein with intact disulfide bond, its spectroscopic characterization, and comparisons of the polypeptide segment 121–231 in full-length *mPrP*^C with the isolated carboxy-terminal domain, *mPrP*(121–231).

2. Materials and methods

2.1. Materials

Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany) and SP-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Tryptone and yeast extract were from DIFCO (Detroit, USA) and isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from AGS (Heidelberg, Germany). 1,4-Dithio-DL-threitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and dithionitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (Deisenhofen, Germany). All other chemicals were of analysis grade.

2.2. Expression and purification of recombinant *mPrP*(23–231)

The gene coding for *mPrP*(23–231) was amplified by the polymerase chain reaction from a plasmid harboring the *mPrP*(23–231) cDNA [15] using the oligonucleotide primers as listed in Table 1.

Table 1

N-terminal primer
5'-GACTGATGTCCATATGTCTAAAAAGCGTCCAAAGCCTGGAGGGTGG-ACACCG-3'
C-terminal primer
5'-AGGAGGGGAGGGGATCCAAGCTTACTAGCTGGAACGACGCCCGTCGT-AATAGCCTGGGACTCC-3'

The amplified gene was cloned into the plasmid pRBI-PDI-T7 [16] via the *Nde*I and *Bam*HI restriction sites. In the resulting expression plasmid, termed pPrP(23–231), the *PrP* gene is under control of the T7 promoter/lac operator sequence. As recombinant *mPrP*(121–231) [13], the polypeptide expressed with pPrP(23–231) contains an additional Ser at the carboxy-terminus. According to the N-end rule in bacteria [17], we also introduced a Ser at the amino-terminus of

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mPrP(23–231) to minimize proteolytic degradation in the cytoplasm. Thus, recombinant *mPrP*(23–231) used in this study consists of 210 amino acids. The correct sequence of the amplified gene in *pPrP*(23–231) was verified by dideoxy sequencing.

For the production of unlabeled *mPrP*(23–231) and uniformly ^{15}N -labeled *mPrP*(23–231), cells of *E. coli* BL21(DE3) [18] harboring *pPrP*(23–231) were grown at 37°C in 10 l LB medium, or in 10 l minimal medium with unlabeled glucose (5 g/l) and $(^{15}\text{NH}_4)_2\text{SO}_4$ (1 g/l), respectively. All media contained ampicillin (100 µg/ml). At an optical density at 550 nm of 1.0–1.3 (rich medium) or 0.9–1.1 (minimal medium), IPTG was added to a final concentration of 1 mM and the cultures were grown for another 16 h. The cells were harvested by centrifugation and suspended in 100 ml 150 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl pH 8.0 supplemented with 1 mM PMSF and two protease inhibitor cocktail tablets ('COMPLETE' (EDTA free); Boehringer Mannheim, Germany). The bacteria were disrupted in a French Pressure cell (18000 PSI) and the lysate was centrifuged (4°C, 39000 × g, 1 h). The insoluble inclusion bodies were washed twice with the above buffer and solubilized in 100 ml 8 M urea, 10 mM Tris/HCl pH 8.0, 1 mM EDTA, 10 mM DTT. After centrifugation (39000 × g, 22°C, 1 h) the pH of the supernatant was adjusted to 7.0 with HCl and applied to a SP-Sepharose column (20 ml) equilibrated with 8 M urea, 10 mM MOPS/NaOH pH 7.0. *mPrP*(23–231) was eluted with a linear NaCl gradient (400 ml, 0–600 mM). Fractions containing *mPrP*(23–231) were combined and the protein was reduced with 10 mM DTT at pH 8.0 (10 mM Tris/HCl) for 1 h at 37°C. After addition of 10% (v/v) acetic acid the solution was applied to a SP-Sepharose column (20 ml) equilibrated with 8 M urea, 100 mM NaCl, 10% (v/v) acetic acid, then washed with 8 M urea, 10 mM MOPS/NaOH pH 7.0, and eluted with a linear NaCl gradient (400 ml, 100–600 mM NaCl).

Oxidation of *mPrP*(23–231) was performed for 3–16 h at 22°C at a protein concentration of 0.1 mg/ml in 8 M urea containing 50 mM Tris/HCl pH 8.7 and 1 µM CuSO_4 . The reaction was analyzed by separation of acid-quenched samples (pH ≤ 2) on an analytical reversed-phase HPLC column (Vydac C18, 4.6 × 250 mm) at 55°C with a linear gradient from 28% to 40% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA). After addition of one protease inhibitor tablet, 10 µM EDTA and 10 µM PMSF, the denaturant was removed by dialysis against distilled water. Unfolded proteins were precipitated by addition of 10 mM sodium phosphate pH 7.0, 10 µM EDTA and 10 µM PMSF, and the precipitate was removed by centrifugation (39000 × g, 30 min, 4°C). The supernatant was applied to a SP-Sepharose column (15 ml) and *mPrP*(23–231) was eluted with a linear NaCl gradient (300 ml; 200–600 mM). Fractions containing homogeneous *mPrP*(23–231) were pooled and dialyzed against distilled water. The preparations were concentrated by ultrafiltration (Amicon YM10) to a volume of 50 ml. For storage, a protease inhibitor tablet was added and the preparation was adjusted to 10 µM PMSF, 0.1 µM pepstatin and 10 µM EDTA. 50 mg of unlabeled and 25 mg of uniformly ^{15}N -labeled *mPrP*(23–231) were obtained with this protocol.

Edman sequencing of *mPrP*(23–231) revealed that the methionine at the amino-terminus had been cleaved off quantitatively (sequence found: SKKRPKPGWNTGGS). The mass of the unlabeled *mPrP*(23–231) was verified by electrospray mass spectrometry (calculated mass: 23107 Da; measured: 23113 Da). Purified *mPrP*(23–231) was completely oxidized, as shown by analytical HPLC and by the lack of free thiols in Ellman's assay [19] after unfolding.

2.3. Protein concentrations

Protein concentrations were measured by the absorbance at 280 nm [20]. Specific absorbances ($A_{280\text{nm}, 1\text{mg/ml}, 1\text{cm}}$) of 2.70 and 1.55 were used for *mPrP*(23–231) and *mPrP*(121–231), respectively.

2.4. Circular dichroism and fluorescence spectroscopy

Spectroscopic measurements were performed at 22°C with filtered buffer solutions (0.2 µm pore size). Far-UV and near-UV circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter at protein concentrations of 0.2–0.5 mg/ml in 50 mM sodium phosphate pH 7.0. The spectra were recorded in a 0.2 mm cuvette in the far-UV region (180–250 nm) and in a 10 mm cuvette in the near-UV region (250–350 nm).

Fluorescence measurements were carried out with a Hitachi F-4500 fluorescence spectrophotometer in 0.4 × 1 cm cuvettes. An excitation wavelength of 280 nm was used in all experiments.

2.5. NMR measurements

^1H -NMR spectra of *mPrP*(23–231) and *mPrP*(121–231) were recorded on a Varian UNITYplus spectrometer at a ^1H frequency of 750 MHz with 0.8 mM protein concentration in a mixed solvent of 90% $\text{H}_2\text{O}/10\%\text{D}_2\text{O}$, pH 4.5 at 20°C. The acquisition time was 0.2 s, and the spectral range was 10000 Hz.

3. Results and discussion

3.1. Cytoplasmic expression, oxidative refolding and purification of *mPrP*(23–231)

To obtain the recombinant murine prion protein *mPrP*(23–231) with intact disulfide bond, we initially tried to express the protein in the periplasm of *E. coli* using the OmpA signal sequence. Although this expression system allows the production of soluble *mPrP*(121–231) in the *E. coli* periplasm [13], we were not able to detect a protein band corresponding to *mPrP*(23–231) in SDS-polyacrylamide gels, neither in the periplasmic fraction nor in the insoluble fraction of induced *E. coli* cells. Similar observations have recently been reported for the periplasmic expression of hamster PrP(23–231) [21].

Eventually, *mPrP*(23–231) was expressed in the cytoplasm of *E. coli* BL21(DE3) under the control of the T7 promoter/lac operator system [18]. We thus obtained large amounts of reduced recombinant *mPrP*(23–231), which accumulated in insoluble inclusion bodies. The inclusion bodies were solubilized in 8 M urea, and *mPrP*(23–231) was purified by cation exchange chromatography in the presence of urea. Oxidation of the single disulfide bond by air oxygen was achieved at pH 8.7 in 8 M urea with 1 µM Cu^{2+} as a catalyst, using low protein concentration to avoid formation of intermolecular disulfide bonds. The reaction was monitored by analytical HPLC and the denaturant was subsequently removed by dialysis. *mPrP*(23–231) with intact disulfide bond was then purified to homogeneity by cation exchange chromatography at pH 7.0 under non-denaturing conditions (Fig. 1). Overall, 5 mg of homogeneous *mPrP*(23–231) were obtained per liter of bacterial culture with rich medium, and 2.5 mg per liter for growth in minimal medium with ^{15}N -ammonium sulfate as the sole nitrogen source. *mPrP*(23–231) proved to be soluble in H_2O at concentrations of 1.5 mM between pH 4.0 and 7.0.

Recombinant *mPrP*(23–231) was found to be rather sensitive towards proteolytic digestion. Therefore, protease inhibitors were added during the purification of the protein. In the absence of protease inhibitors, we found proteolytic cleavage after residues 116, 118, and 120 (Fig. 1B). Long-term incubation of *mPrP*(23–231) at 20°C and pH 4.5 in the absence of protease inhibitors resulted in complete degradation of the amino-terminal polypeptide segment 23–120, while the carboxy-terminal domain was not degraded.

3.2. Circular dichroism and fluorescence spectra

The amino-terminal region of mammalian prion proteins comprising residues 23–120 contains a segment of 40 amino acids (residues 51–91 in *mPrP*) that consists of 5 octapeptide repeats with the consensus sequence PHGGG(S)WGQ [22]. Due to the high content of Gly residues, regular secondary structure predictions for residues 51–91 of PrP did not yield conclusive results [23]. To analyze the contents of secondary and tertiary structure in the amino-terminal segment 23–120 of recombinant *mPrP*(23–231), we compared its far-UV and near-UV circular dichroism and fluorescence spectra with

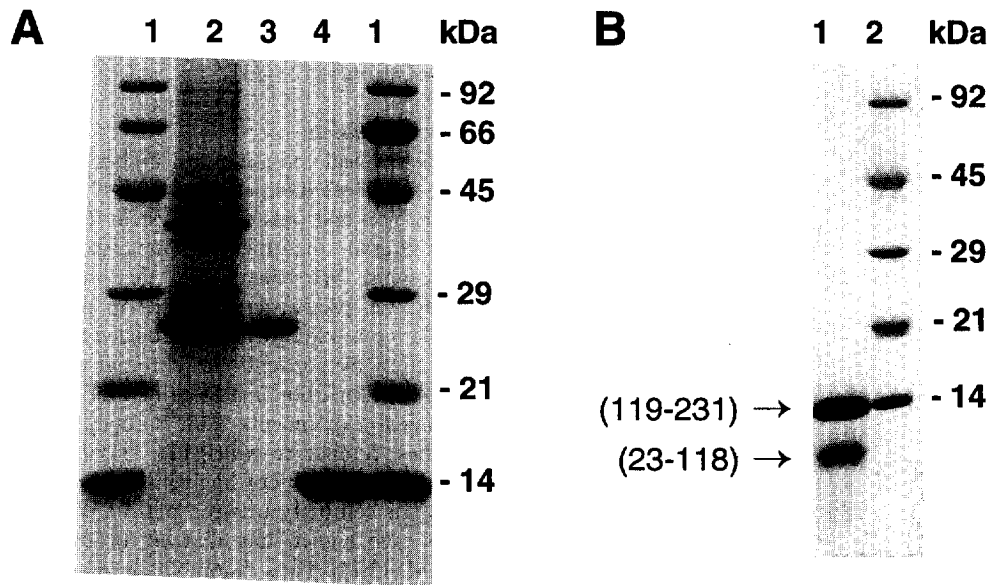


Fig. 1. Purification of *mPrP*(23–231). A Coomassie-stained 15% polyacryl-amide/SDS gel is shown in (A). Lanes: 1, molecular mass standard; lane 2, solubilized inclusion bodies prepared from *E. coli* BL21(DE3)/pPrP(23–231); 3, recombinant *mPrP*(23–231) after oxidative refolding and purification; 4, purified *mPrP*(121–231). (B) Proteolytic cleavage of *mPrP*(23–231) after prolonged incubation at pH 4.5 and 20°C at a protein concentration of 1 mM in the absence of protease inhibitors. Lanes: 1, Cleavage products of PrP(23–231) (the main cleavage site is after residue 118); 2, Molecular mass standard.

those of *mPrP*(121–231). Both *mPrP*(23–231) and *mPrP*(121–231) exhibit typical α -helical far-UV CD spectra, with minima at 222 and 208 nm (Fig. 2A). Although the shapes of the spectra are very similar, the mean residue ellipticities of full-length *mPrP* are significantly less negative than those of its carboxy-terminal domain, indicating that the percentage of residues located in regular secondary structures is higher in *mPrP*(121–231) than in the full-length protein. In accordance, the recently reported mean residue ellipticities of the segment

90–231 of hamster PrP are also less negative in the far-UV region when compared with *mPrP*(121–231) [24].

Corresponding observations were made for the near-UV CD spectra of *mPrP*(23–231) and *mPrP*(121–231) (Fig. 2B), where spectra of similar shape but lower mean residue ellipticities were obtained for *mPrP*(23–231) when compared to *mPrP*(121–231). Since the near-UV CD data relate to the tertiary structure of a protein [25], this would be compatible with the assumptions that the structure of the carboxy-terminal

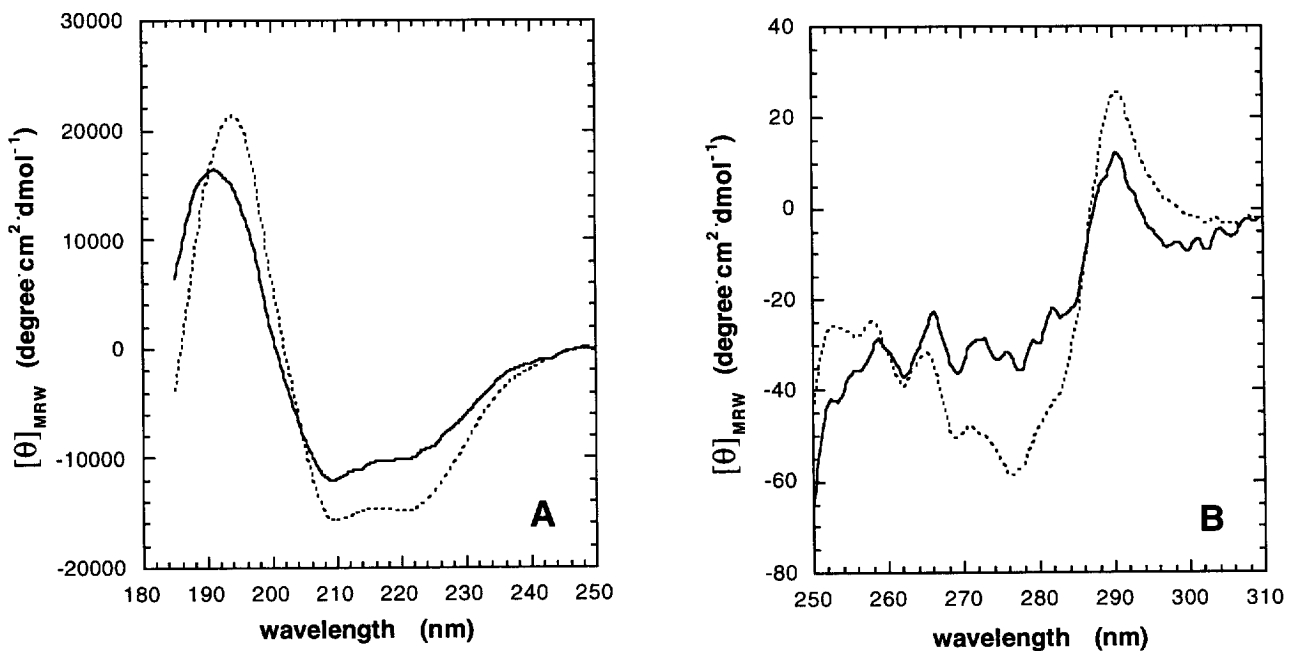


Fig. 2. Far-UV and near-UV circular dichroism spectra of *mPrP*(23–231) (solid line) and *mPrP*(121–231) (dashed line). Spectra were recorded at 22°C in 50 mM sodium phosphate pH 7.0. (A) Far-UV CD spectrum. (B) Near-UV CD spectrum.

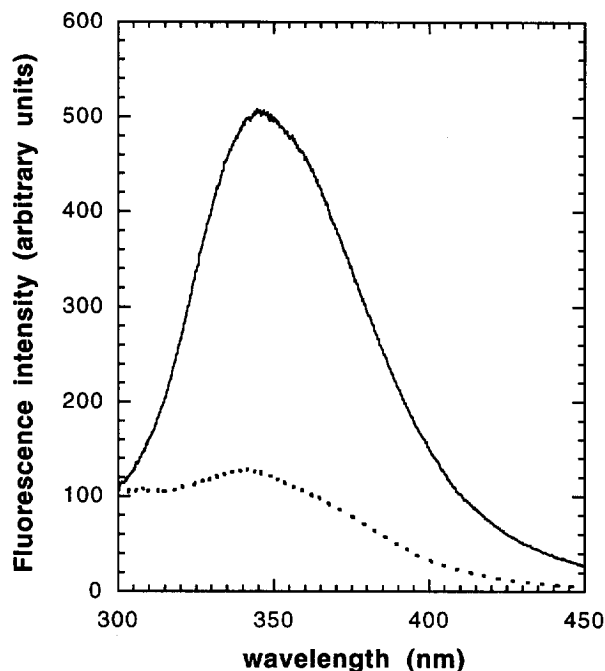


Fig. 3. Fluorescence spectra of *mPrP*(23–231) (solid line) and *mPrP*(121–231) (dashed line) at 22°C in 50 mM sodium phosphate pH 7.0. Identical protein concentrations of 0.75 μ M were used. The excitation wavelength was 280 nm.

domain observed in *mPrP*(121–231) is retained in the full-length protein, and that the amino-terminal segment 23–120 is not significantly involved in tertiary structure formation.

The emission maximum at 345 nm in the fluorescence spectra of *mPrP*(23–231) (Fig. 3) indicates that most of the 7 Trp residues in the segment 23–120 are solvent exposed and thus not in a hydrophobic environment. Reference values are 320–335 nm for tryptophans in the hydrophobic protein core, and 348 nm for free tryptophan [25]. The single tryptophan of the carboxy-terminal domain, Trp¹⁴⁵, which is also solvent exposed in the three-dimensional structure [14], has an emission maximum of 340 nm in *mPrP*(121–231).

3.3. One-dimensional ¹H-NMR spectra

A ¹H-NMR spectrum of *mPrP*(23–231) at pH 4.5 in a mixed solvent of 90% H₂O/10% D₂O (Fig. 4A) shows a chemical shift dispersion and resonance linewidths that are typical for a monomeric protein of size about 200 residues. The lines near 0 ppm are representative for ring current-shifted methyl resonances in well structured globular proteins [26]. The 30 glyceryl residues in the segment 23–120 of *mPrP*(23–231) give rise to narrow, intense lines around 4 ppm. Near 10.2 ppm a group of lines represents the resonances of the indole NH-groups of the 8 Trp residues in *mPrP*(23–231). In accordance with the fluorescence properties of *mPrP*(23–231), the small chemical shift dispersion of these lines suggests that the indole rings are not in the interior of a densely packed structural element. The Fig. 4 also affords a comparison of the ¹H-NMR spectrum of *mPrP*(23–231) (Fig. 4A) with that of the isolated carboxy-terminal domain *mPrP*(121–231) (Fig. 4B), for which some well separated resonances are labeled with the previously obtained assignments [14]. Similar patterns of resonance lines near 0 ppm and from 5.0 to 6.3 ppm are seen in *mPrP*(23–231) and *mPrP*(121–231), which would

be compatible with a situation where the structure observed for the isolated domain *mPrP*(121–231) would be largely retained in *mPrP*(23–231).

3.4. Conclusions

Biophysical and structural studies on the cellular prion protein have been hampered in the past by the lack of an efficient expression and purification system that allows the production of milligram quantities of homogeneous PrP with intact disulfide bond. We believe that the protocol presented in this study, i.e. production of *mPrP* in the cytoplasm of *E. coli*, oxidative refolding from solubilized inclusion bodies and purification by conventional chromatography, provides a suitable tool for obtaining large quantities of pure PrP^C for biochemical experiments and isotope-labeled PrP^C for NMR studies. Similar to *mPrP*(121–231) [13,14], *mPrP*(23–231) is soluble and does not aggregate irreversibly in aqueous solution. Its solubility between pH 4 and pH 7 is ≥ 1.5 mM and thus even higher than that *mPrP*(121–231), which is around 1 mM [13,14]. Thus, recombinant *mPrP*(23–231) is suitable for structural studies by NMR, as evidenced by the one-dimensional ¹H-NMR spectrum in Fig. 4A. The following paper [27] presents a NMR study of the conformational state of the segment 23–120 in the full-length protein and a compar-

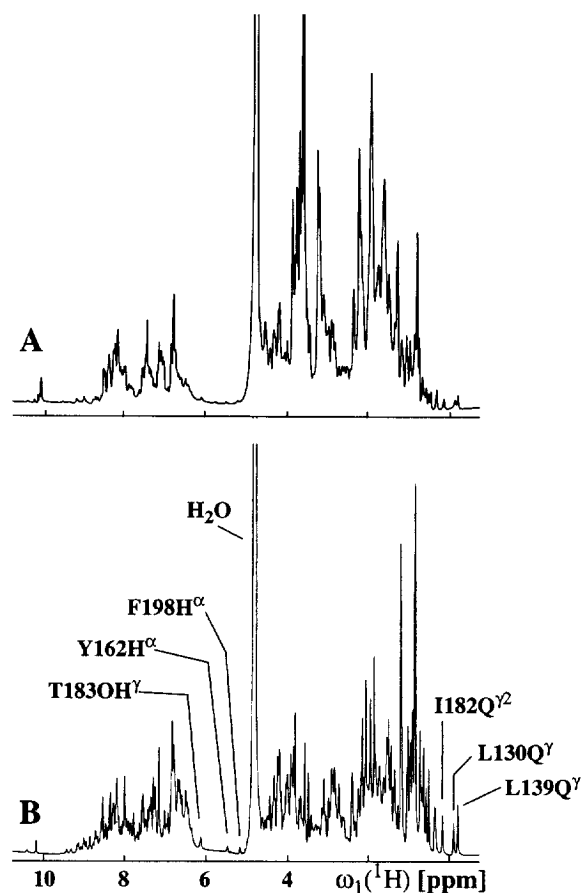


Fig. 4. One-dimensional 750 MHz ¹H-NMR spectra of *mPrP*(23–231) (A) and *mPrP*(121–231) (B). The previously established resonance assignments [14] for selected well-separated peaks are indicated in (B).

ison of the isolated carboxy-terminal domain *mPrP*(121–231) with the corresponding polypeptide segment in *mPrP*(23–231).

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