The complete mature bovine prion protein highly expressed in *Escherichia coli*: biochemical and structural studies

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Abstract According to the 'protein only' hypothesis, modification of the 3-dimensional fold of the constituent cellular protein, PrP^C, into the disease-associated isoform, PrP^{Sc}, is the cause of neurodegenerative diseases in animals and humans. Here we describe the high-level synthesis in *Escherichia coli*, and purification in the monomeric form, of a histidine-tagged full-length mature PrP (25–249) of bovine brain, termed His-PrP. Based on biochemical and spectroscopic data, His-PrP displays characteristics expected for the PrP^C isoform. The reported expression system should allow the production of quantities of bovine PrP^C sufficient to permit 3-dimensional structure determinations.
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Key words: Spongiform encephalopathy; Scrapie; Prion; Recombinant bovine prion protein

1. Introduction

Intense interest is nowadays focussed on a group of transmissible neurodegenerative diseases among which are spongiform encephalopathy of ox, scrapie of sheep, and Creutzfeldt-Jakob disease of humans. These diseases can be transmitted by an infectious agent, called prion, which appears to be of a proteinaceous nature [1]. The tenet of the 'protein only' hypothesis formulated by Prusiner [2] to explain prion diseases holds that a post-translational structural change converts the cellular constituent protein, PrP^C, to the pathogenic isoform, PrP^{Sc} [3–5], which is chemically identical to the native protein [6] but with an altered sensitivity to proteases [7,8], different solubility and tinctorial properties [9] as well as a distinct cellular distribution [10]. Elucidating the structure of prion isoforms is fundamental to understanding the molecular mechanism of the hypothesised structural transition. In spite of much effort devoted to the 3-dimensional analysis of PrP, PrP^{Sc} insolubility or the low yield of purified PrP^C [3,11], have hindered progress. Results have been equally unsatisfactory when the heterologous expression of several constructs of

PrP^C was probed in a variety of systems [11–15]. Recently, by exploiting fusion proteins expressed in the periplasm of *Escherichia coli*, spectroscopic or NMR studies have been carried out on hamster [16] and mouse [17,18] PrP fragments, which reside within the protein core of infectivity [19].

To approach this problem, we have now expressed intracellularly in bacteria the full-length mature part of bovine PrP (25–249) fused at its N-terminus to a hexahistidine extension (His-PrP). The bovine protein was chosen in view of the important health concerns raised by bovine spongiform encephalopathy and because its structure has been less extensively studied among prions. A three-step procedure, consisting of extraction from inclusion bodies with guanidinium chloride, and purification by immobilised Ni(II) chromatography and reverse-phase HPLC was used to recover monomeric His-PrP in high yields. Biochemical and spectroscopic evidence identified (the prion sequence of) His-PrP with the naturally occurring PrP isoform.

2. Materials and methods

2.1. Materials

Restriction enzymes and T4 DNA ligase were purchased from Gibco-BRL and proteolytic enzymes from Boehringer. Oligonucleotides were synthesised with an Applied Biosystems 380B DNA synthesiser. Plasmid pRSETA was purchased from Invitrogen, while host cell lines *E. coli* BL21 (DE3) LysE and HB101 were obtained from Novagen. Plasmids were constructed in *E. coli* HB101 and verified by double-stranded dideoxynucleotide sequencing using a Sequenase kit (United States Biochemical). Cloning procedures were essentially as in [20].

2.2. Construction of plasmid pRSETA-PrP

Oligonucleotides derived from the 5′ and 3′ coding regions of the bovine *PrP* gene were used to amplify the mature sequence by PCR, using cDNA templates generated from bovine brain mRNA. Oligonucleotide primers (5′-cgctcgactcaAAGAAGCGACCAAAACCTG-GA-3′ (forward sequence); 5′-cccatatgTCGTTGGTAATAAGCCT-GGGATTCT-3′ (reverse sequence)) were placed between bp 72 and 93, and bp 693 and 717, respectively, of the bovine PrP sequence [21]. After a 5-min incubation at 94°C, samples were subjected to 32 cycles of PCR as in [20]. PCR products were purified [20], digested with *Sal*I and *Nde*I, and subcloned between the same restriction sites in pGEM5 to give the pGEM-PrP plasmid. pGEM5-PrP was finally digested with *Sac*I and *Eco*RV and subcloned between *Sac*I and *Pvu*II in pRSETA to produce plasmid pRSETA-PrP (Fig. 1A).

2.3. Synthesis and purification of His-PrP

Freshly prepared cultures of *E. coli* BL21 (DE3) LysE cells transformed with pRSETA-PrP were grown at 37°C in Luria's broth containing 0.1% ampicillin, until reaching an absorbance (at 590 nm) of 0.5 [20]. Protein synthesis was induced by addition of 0.4 mM iso-propyl-β-D-thiogalactopyranoside, and 3 h later bacteria were harvested. Recombinant protein was extracted from inclusion bodies by resuspending bacterial cells (0.1 g of wet pellet weight per ml) in 6 M

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Abbreviations: PrP, prion protein; PrP^C, cellular form of the prion protein; PrP^{Sc}, pathogenic form of the prion protein; HPLC, high-performance liquid chromatography; CD, circular dichroism; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride

guanidine-HCl, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, pH 8.0, and son-ication [22]. The lysate was cleared by centrifugation at $28\,000 \times g$ for 20 min, and then filtered through a 0.45 µm (Millipore) membrane. The filtrate was applied to a column (1×4 cm) of Ni(II)-nitriloacetate agarose (Ni(II)-NTA; Diagen) [23]. After exhaustive washing with 8 M urea, 0.1 M NaH_2PO_4 and 10 mM Tris-HCl (pH 8.0), His-PrP was eluted by shifting the pH to 4.5. Protein refolding was accomplished by a 3-day dialysis against 50 mM sodium acetate, pH 4.0. Further purification was achieved by reverse-phase HPLC using a 0.46×15 cm C_4 column (Vydac) equilibrated with 0.05% (v/v) aqueous TFA. The sample was eluted using a 5–25% acetonitrile gradient (in 0.05% TFA) in 10 min and 25–50% acetonitrile in 40 min, at a flow rate of 0.6 ml/min. His-PrP eluted at 37% acetonitrile and was then lyophilised. Stock solutions of His-PrP (1 mg/ml) in 10 mM sodium acetate, pH 4.0, were stored at $-80°C$.

2.4. SDS-PAGE and Western blotting

SDS-PAGE was performed according to [24]. Proteins were stained with Coomassie Blue. For Western blotting, after SDS-PAGE samples were electro-transferred overnight (40 mA) to Immobilon (PVDF) membranes (Millipore). The membranes were saturated for 1 h with 3% gelatin in Tris-Tween buffer solution, then incubated 2 h with 1:500 rabbit polyclonal antibodies against PrP (see below). Bound antibodies were visualised by use of the Auroprobe kit (Janssen). Antibodies were raised against the following bovine PrP amino acid sequences: 102–119 (GQGGTHGQWNKPSKPKTN) and 153–168 (GSDYEDRYYRENMHRY). Peptides were synthesized using a Millipore Milligen 9050 synthesiser (Milford, MA), purified by re-verse-phase HPLC, and verified by amino acid analysis. Antisera were produced by immunizing female New Zealand white rabbits. Antibody titer was evaluated by standard ELISA techniques using peptide-coated microwells. The IgG fraction was precipitated with caprylic acid and purified by affinity chromatography [25].

2.5. N-terminal sequencing

Proteins were separated by SDS-PAGE and electrotransferred to PVDF membranes (ProBlott, Applied Biosystems) and visualised with Coomassie Blue. Sequencing was then performed by automated Edman degradation using an Applied Biosystems 477A sequencer, according to the manufacturer's protocols.

2.6. Protein concentration

Concentrations of His-PrP solutions were determined by UV ab-sorbance spectroscopy, using a double-beam Perkin-Elmer Lambda 2 spectrophotometer and 1-cm path length quartz cuvettes. An extinc-tion coefficient for His-PrP of 2.68 mg^{-1} cm^2 at 280 nm was calcu-lated according to Gill and von Hippel [26].

2.7. Protein digestion

Proteinase K (0.6 µg) was added to 10 µg of His-PrP in 25 µl final volume of 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1% octyl-β-D-glycopyranoside. Digestion was carried out as in [8]. Endoproteinase LysC (1 µg) was added to 100 µg of His-PrP in 200 µl final volume of 50 mM sodium acetate, pH 6.0. Digestion was carried out at room temperature and was stopped by adding 0.1% TFA and heating in a boiling water bath for 5 min. In either case, digestion products were examined by SDS-PAGE; endo LysC products were also controlled by mass spectrometry.

2.8. CD spectroscopy

His-PrP samples were dialysed 24 h against 10 mM sodium phos-phate buffer (pH 3.5 or pH 6.5) and adjusted to 300–600 µg protein/ml. A JASCO J-710 spectropolarimeter, fitted with a thermostatted cell holder and interfaced with a Neslab RTE-110 water bath (New-ington, NY), was calibrated with (+)-10-camphor sulfonic acid [27]. CD spectra were recorded at 25°C using 0.2-cm (190–250 nm region) or 0.5-cm (250–320 nm region) path length quartz cuvettes. Values are expressed as mean residue ellipticity ($[\Theta]_{MRW}$) [28]. Four consecutive accumulations were averaged after subtraction of the corresponding buffer baseline.

2.9. Mass spectrometry

Mass spectra were accumulated using a Voyager Elite MALDI TOF mass spectrometer (Perseptive Biosystems, Framingham, MA). The starting material for all mass spectrometric determinations was

His-PrP purified by reverse-phase HPLC. Protein solutions were ad-justed to pH 2.0 with 10% TFA. Test samples (0.5 µl) were mixed with an equal volume of matrix solution (5 mg/ml α-cyano-4-hydroxy-cin-namic acid in 50% acetonitrile, 0.1% TFA in water) and spotted directly onto the MALDI sample target. Samples were analysed in pulsed extraction linear mode using an accelerating voltage of 25 kV, a pulse delay time of 100 ns, a grid voltage of 91% and guide wire voltage of 0.1%. Spectra were accumulated for 64 lasershots. Cyto-chrome c (horse heart, average mass 12 361) was the internal cali-brant. Samples were also analysed in pulsed extraction reflector mode.

3. Results

3.1. Expression and purification of His-PrP

The expression plasmid pRSETA-PrP (Fig. 1A) codes for a fusion protein of 265 amino acids, in which the bovine PrP sequence 25–249 was linked into a 40-amino-acid N-terminal extension containing a hexahistidine stretch (Fig. 1B), thereby facilitating purification by immobilized Ni(II) chromatogra-phy [23]. Nucleotide sequencing of pRSETA-PrP verified its identity with the bovine PrP cDNA sequence of Goldmann et al. [21]. Fig. 2A (left gel) shows an SDS-PAGE of E. coli BL21 (DE3) LysE cells transformed with either pRSETA-PrP (lane 1), or with two other plasmids lacking the bovine PrP sequence (lanes 2 and 3). Only the former cells synthe-sised a protein of $M_r \approx 30$ K, a value close to the molecular mass of the putative bovine His-PrP (calculated average M_r: 29089.8). This 30 kDa band accounted for approximately 5% of total bacterial protein. Test of the same samples of the left gel for crossreactivity with antibodies against PrP peptides 153–168 (Fig. 2A, middle gel) and 102–119 (Fig. 2A, right gel) further supports the identity of the 30 kDa band as His-PrP.

His-PrP extraction from inclusion bodies was carried out with 6 M guanidine-HCl. The cell extract was then loaded onto a Ni(II)-agarose affinity column. His-PrP eluted as a major fraction when the pH was lowered from 8.0 to 4.5, and was refolded upon urea removal by dialysis at pH 4.0. Elution and dialysis buffers were kept acidic, as neutral or alkaline pH caused substantial precipitation of His-PrP.

Reducing SDS-PAGE of the refolded protein showed a major, single band of around 30 kDa (Fig. 2B, right gel, lane 1). Conversely, under non-reducing conditions His-PrP

Table 1

Molecular masses of His-PrP and of the 60 min endo LysC digests

Residues[a]	Observed mass[b]	Calculated mass[c]
1–265	29 094	29 089.8
1– 31	33 547.2	3 546.8
1–133	13 858.7	13 858.8
1–137	14 325.1	14 333.4
32–133	10 329.7	10 331.0
42–133	9 155.5	9 156.8
134–265	15 252.1	15 250.1
138–212	8 413.3	8 414.3
222–265	5 379.9	5 381.0

[a]His-PrP (1–265), present in the fraction eluting from reverse-phase HPLC at 37% acetonitrile, was subjected to proteolysis by endo LysC as described in Section 2.

[b]Masses were measured by a Voyager Elite MALDI TOF mass spec-trometer on samples treated as described in Section 2 (desalted). pep-tides with mass up to 1500 were considered as monoisotopic, while for peptides above 1500 mass the average isotopic mass was taken. All peptides were considered as single protonated. Quoted values are the means of at least five different spectra.

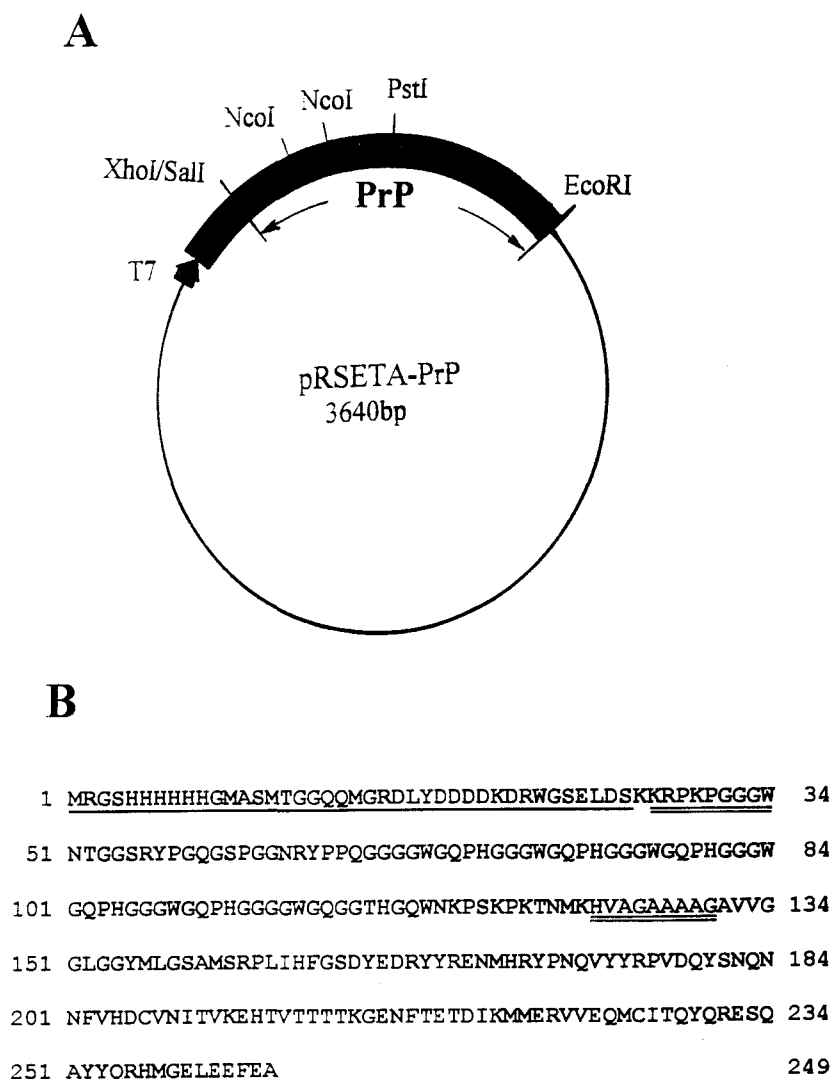[c]Given masses of the (desalted) peptides were calculated as above.

A

B

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  1 MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSELDSKKRPKPGGGW   34

 51 NTGGSRYPGQGSPGGNRYPPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGW   84

101 GQPHGGGWGQPHGGGWGQGGTHGQWNKPSKPKTNMKHVAGAAAAGAVVG  134

151 GLGGYMLGSAMSRPLIHFGSDYEDRYYRENMHRYPNQVYYRPVDQYSNQN  184

201 NFVHDCVNITVKEHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQ  234

251 AYYQRHMGELEEFEA                                      249
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Fig. 1. A: Structure of the expression vector coding fusion protein His-PrP. Note insertion of the bovine prion sequence between restriction sites XhoI and EcoRI. T7 promoter indicates the direction of transcription. B: Deduced amino acid sequence translated by pRSETA-PrP. Single underlining indicates the N-terminal extension which was fused into the bovine 25–249 PrP sequence. Residues identified by Edman degradation are double underlined. The numbering on the left indicates the amino acid sequence of His-PrP, whereas that on the right refers to the deduced bovine prion sequence only.

yielded bands representing multiples of 30 kDa (Fig. 2B, left gel, lane 1), most probably the result of interchain S–S bond formation. As our aim was to isolate monomeric His-PrP, the refolded protein mixture was further purified by reverse-phase HPLC, producing three peaks (Fig. 2C). Only the first peak (eluting at 37% acetonitrile) was utilized for all subsequent studies, given its purity (Fig. 2B, lanes 2) and average molecular mass of 29 094 determined by mass spectrometry (Table 1). Although a cleavage site specific for enterokinase (DDDDK) was placed within the N-terminal extension of His-PrP (Fig. 1B) to permit later removal of this tag, the expected proteolysis failed to occur (see also [29]).

3.2. Protease sensitivity of His-PrP

A major difference between PrP^C and PrP^{Sc} is the sensitivity of PrP^C to proteinase K [7,8]. Incubation of His-PrP with proteinase K produced complete protein digestion, supporting the notion that His-PrP contains the normal cellular isoform, PrP^C (data not shown).

His-PrP was next incubated with endoproteinase LysC to evaluate accessibility of the 11 lysine residues in His-PrP some of which, adjacent to prolines (Lys[45]; Lys[128]; Lys[131]), were expected to have a limited reactivity. A 15-min digestion of His-PrP with endo LysC produced a major band (M_r: ≈15 kDa), along with a fainter band (M_r: ≈9 kDa) on SDS-PAGE (Fig. 3, lane 2). A 60-min digestion, however, markedly increased the ∼9 kDa band, while causing the ∼15 kDa band to decrease in intensity with the concomitant appearance of a new band just below 15 kDa (Fig. 3, lane 3). N-terminal sequencing of this last polypeptide (HVAGAAAAG) and of the ∼9 kDa one (KRPKPGGGW), together with mass spectrometric analysis of the 60-min digestion products (Table 1), identified these two species with His-PrP peptides 138–265 and 42–133, respectively. These data, and the other peptides identified by mass spectrometry (Table 1), suggest that Lys[133] is the first lysine attacked by endo LysC (after 15 min). Of the two peptides so formed, 1–133 and 134–265, only the latter is clearly visible (Fig. 3, lane 2), because peptide 1–133 is imme-
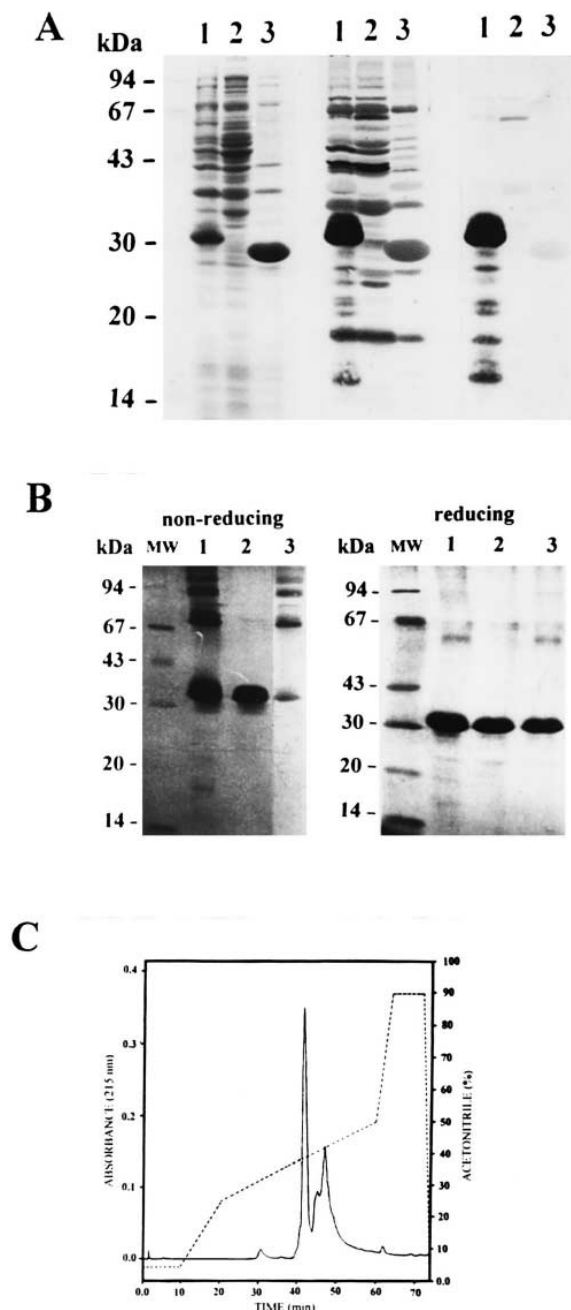
Fig. 2. Verification of synthesis of His-PrP by *E. coli* by SDS-PAGE and Western blotting (A) and of His-PrP purification by SDS-PAGE (B) and reverse-phase HPLC (C). A: Lanes were loaded with 10 μg protein from *E. coli* BL21 (DE3) LysE cells transformed with pRSETA-PrP (lanes 1) or with plasmids pRSETA (lanes 2) and pT7.7hCNTF [36] (lanes 3), which lack the bovine PrP sequence. Proteins were separated on a 12% SDS-PAGE gel (left gel) and then tested for crossreactivity with antibodies against the bovine PrP peptides 153–168 (middle gel) and 102–119 (right gel). B: His-PrP (5 μg/lane) was separated on a 15% SDS-PAGE gel under non-reducing and reducing (plus 5% β-mercaptoethanol) conditions. Lanes 1: His-PrP purified by Ni(II)-affinity chromatography and refolded. Lanes 2 and 3: fractions eluted by reverse-phase HPLC at 37% and 41% acetonitrile, respectively. C: Reverse-phase HPLC chromatogram of refolded His-PrP. Only the peak at 37% acetonitrile was used further.

diately processed at Lys^{41} thus producing the ~9 kDa band (peptide 42–133) (Fig. 3, lane 2). While peptide 1–133 is con-

tinuously digested at Lys^{41}, Lys^{137} of peptide 134–265 is attacked only at later times along with other lysines (Lys^{31}, Lys^{212}, Lys^{221}), as shown by mass determination of the 60-min digests (Table 1) and the electrophoretic patterns of the 60- and 120-min products (Fig. 3, lanes 3, 4). Prolonged (24 h) digestion resulted in the formation of several other fragments, some of which were already present after 120 min (Fig. 3, lane 4).

3.3. Spectroscopic studies of His-PrP

Far-UV CD spectra of His-PrP (Fig. 4A), recorded at pH 3.5 and pH 6.5, showed a maximum at 190 nm and two minima centered at 208 nm and 220 nm. His-PrP may thus contain a substantial amount of α-helical structure at either pH [28], although decrease of the 220 nm signal at pH 3.5 suggests a diminishing α-helix contribution with decreasing pH (from approximately −8000 deg·cm^2·dmol^{-1} at pH 6.5 to −7000 deg·cm^2·dmol^{-1} at pH 3.5). The overall features at near-UV (at pH 3.5) (Fig. 4B) point to the presence of a tertiary structure of the protein. Indeed, in the 275–285 nm region the spectrum is dominated by tyrosine absorption, and in the 260–270 nm region by phenylalanine absorption, a profile diagnostic for fine structures. The distinct positive peak at 296 nm suggests that some tryptophanyl residues are located in a rigid and asymmetric environment [28].

4. Discussion

The 'protein only' hypothesis of prion diseases critically depends on an understanding of the molecular mechanism leading to the generation of PrP^{Sc} from PrP^C which, in turn, requires a knowledge of the 3-dimensional structure of both protein isoforms. Structural studies of PrP^C, however, have been hampered by low levels of expression in mammalian cells [11–14,30] and by difficulties encountered in adopting heterologous expression systems [15]. For example, the recent periplasmic expression in *E. coli* of prion constructs linked to secretion vectors produced PrPs lacking mature N-terminal sequences of various lengths, either because of proteolysis in the periplasmic space [17,18] or because of poor expression of the full-length mature protein, alone or as a fusion protein [16]. At variance with previous heterologous expression attempts, the present study reports on the intracellular synthesis in bacteria of an histidine-tagged mature bovine prion protein, fully resistant to cellular degradation.

Recovery of the recombinant protein was maximized by use of guanidinium chloride for inclusion body extraction, a low pH to guarantee protein solubility, and a hexahistidine tag to permit purification by immobilized Ni(II) affinity chromatography. These conditions, coupled with maintaining the protein in an oxidised environment throughout, yielded 22 mg of monomeric His-PrP per liter of bacterial broth. The construct was designed to code for the mature part of the bovine prion protein (Fig. 1), i.e. lacking the N-terminal first 24 amino acids and the C-terminal last 16 amino acids [31,32]. Crossreactivity with antibodies against bovine PrP peptides, (partial) Edman sequencing and mass of endoproteinase LysC products, electrophoretic behaviour and molecular mass of the recombinant protein, all indicate that His-PrP is correctly expressed. Moreover, susceptibility of the protein to proteinase K, from within the inclusion bodies to the final stage of
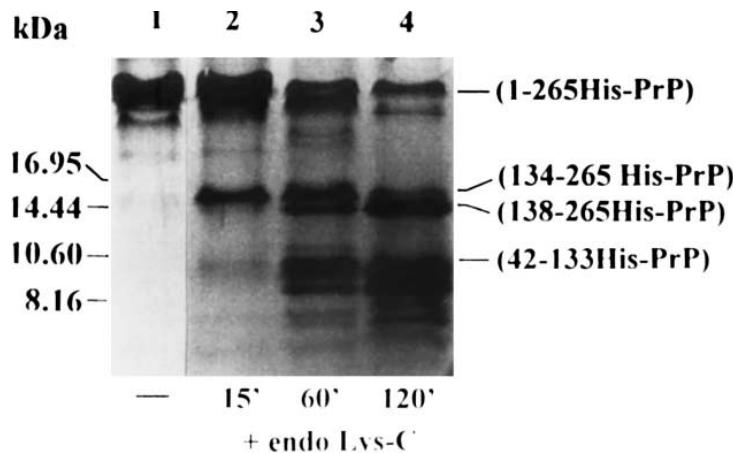
Fig. 3. Digestion of His-PrP by endoproteinase LysC. 15% SDS-PAGE of His-PrP treated with endo LysC (His-PrP: endo LysC, 100:1) for 15 min (lane 2), 60 min (lane 3), or 120 min (lane 4). Lane 1: Untreated His-PrP. Peptides (shown at right) were identified on the basis of the determined average mass (Table 1) and of N-terminal sequencing for peptides 138–265 and 42–133 (see text).

purification (not shown), strongly argues in favour of the identity of His-PrP with the PrP^C isoform.

Near- and far-UV CD analysis of His-PrP showed the presence, under the acidic conditions employed, of a tertiary structure with substantial α-helical content. A comparison with CD data for other oxidised prion proteins is intrinsically difficult for several reasons. Unlike native hamster PrP^C, His-PrP contains an additional octapeptide repeat and an extended N-terminal tail, either of which may contribute to, or diminish, overall secondary structure content. Secondary structure determinations of full-length hamster PrP^C were carried out in the presence of the detergent Zwittergent 3-12 [3] which may increase ellipticity [33]. Lastly, literature reports until now have dealt with shorter PrP sequences (hamster 90–231 sequence [16] and mouse 121–231 sequence [18]).

The experiments with endoproteinase LysC may shed additional light on the structure of PrP. Of the 11 LysC peptide bonds in His-PrP, some are clearly more enzyme-accessible than others. Since digestion was carried out under non-de-

naturating, oxidised conditions, the sensitivity to cleavage of Lys^{41}, Lys^{133} and Lys^{137} may indicate these amino acids to occur in regions lacking defined secondary structure [34]. In particular, the lysine in other PrP sequences corresponding to Lys^{137} of His-PrP was predicted to belong to an α-helix domain [35], a model questioned by Hornemann and Glockshuber [18] based on the extensive proteolysis that takes place in this region of mouse PrP when periplasmically expressed. Attempts to express bovine PrP as a secretory protein were likewise unsuccesful (our unpublished observations). Together, these data support the hypothesis that this region lacks a definite secondary structure in either mouse or ox PrP. Conversely, the higher resistance to proteolysis of His-PrP Lys^{231} could reflect its belonging to an α-helix, as predicted by Huang et al. [35] and confirmed by NMR structure determinations [17].

In conclusion, we have developed a protocol whereby the mature part of bovine PrP (25–249), linked to a polyhistidine tag, is expressed intracellularly in bacteria and can be recov-
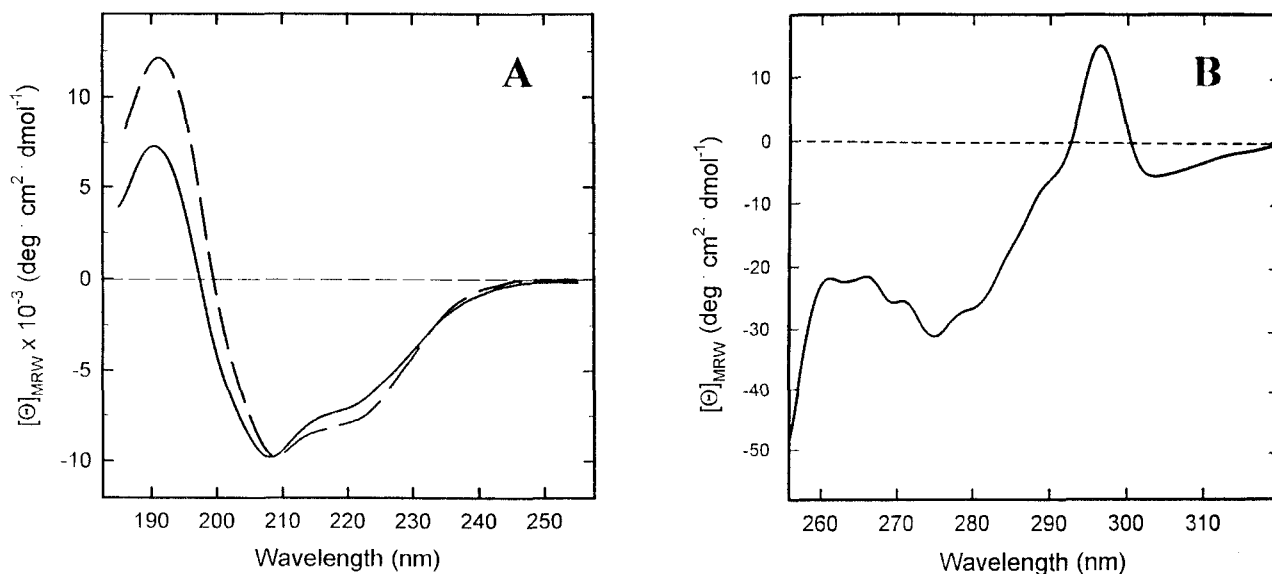

Fig. 4. Far- and near-UV CD spectra of His-PrP. A: Far-UV CD spectra of purified His-PrP (300 μg/ml of 10 mM sodium phosphate) at pH 3.5 (continuous line) or pH 6.5 (dashed line) at 25°C. B: Near-UV CD spectrum of His-PrP with conditions as in (A) but at pH 3.5.

ered intact. The intrinsic stability to proteolysis of the fused protein is most probably a consequence of the storage environment (inclusion bodies) and/or of the extremely rapid synthesis of large protein amounts induced by the T7 expression system. In spite of the extended N-terminus, His-PrP retained several characteristics expected for the non-infectious PrP isoform. The ideal recombinant protein, in which the N-terminal tag is removed, was not obtained although the plasmid was constructed for this purpose. The present expression system should, in every case, spur efforts at producing large amounts of intact PrP, thus overcoming difficulties in obtaining sufficient quantities of PrP^C for structural determinations.

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