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Lysine hydroxylation and O-glycosylation in the globular, C-terminal region of mammalian-expressed, recombinant PrP

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ABSTRACT

Conversion of PrP^C, the prion protein, to a conformationally altered isoform, PrP^{Sc}, is the major pathogenic event in the transmissible spongiform encephalopathies, a family of neurodegenerative diseases including bovine spongiform encephalopathy, Creutzfeldt-Jakob disease and scrapie. Known post-translational modifications to the protein include disulfide bridge formation, addition of a membrane anchor and N-linked glycosylation. We have previously identified the pro-collagen-like hydroxylation of proline 44 in a murine, recombinant prion protein expressed in Chinese hamster ovary cells and herein report the identification of a second pro-collagen-like modification in this protein. In a proportion of the molecules, Lys193, within the C-terminal, folded domain of the protein, is specifically modified to hydroxylysine with subsequent addition of two hexose units, assumed to be the collagen-like disaccharide modifier Gal-Glu. Proof of the existence of these modifications has been obtained by means of tandem mass spectrometry and Edman degradation. Molecular dynamics simulations show that these modifications lead to a pronounced stabilising effect on the β_2–α_2 loop, a region of PrP crucial for the disease-associated conversion. If present *in vivo*, these modifications may have important implications in PrP structure, interactions with ligands or may modulate PrP aggregation.

1. Introduction

The transmissible spongiform encephalopathies (TSEs) are a group of progressive, neurodegenerative disorders that may be acquired genetically, arise spontaneously or may be transmitted by ingestion or inoculation of diseased tissues. This family of fatal diseases includes bovine spongiform encephalopathy, scrapie of sheep and Creutzfeldt-Jakob disease in humans [1]. The fundamental molecular event in pathogenesis of all TSE diseases is the conversion of a normal, cell-surface glycoprotein (PrP^C – cellular prion protein) to a partially protease-resistant isoform (PrP^{Sc} – prion protein scrapie isoform) [2]. PrP^{Sc} is deposited in insoluble aggregates and this form of the protein is partially resistant to degradation with proteases. Since there appear to be no differences between the primary structures of PrP^{Sc} and PrP^C, the conversion is believed to be purely conformational [3].

The prion hypothesis suggests that PrP^{Sc} is the only or, at least, major component of the infectious agent and the conformational change of host PrP^C is believed to proceed catalytically through the use of PrP^{Sc} as a template [2]. Such a mechanism requires the interaction of PrP^{Sc} and PrP^C, either directly or through intermediary molecules such as polyanions or lipids, which may also act as cofactors aiding conversion [4–7]. The advent of several seeded misfolding assays, in which PrP^{Sc} can convert recombinant PrP (recPrP) to a protease-resistant isoform *in vitro* [8–11], suggests that a mechanism involving templated, conformational conversion occurs *in vivo* and that disease is indeed passed by ingestion or inoculation of PrP^{Sc}-containing tissues. However, various sporadic forms of TSE disease exist in humans [12], and possibly in cattle [13] and sheep [14], and it is unclear what factors initiate the conformational cascade in such diseases, in the absence of an exogenous infectious agent. Whilst sporadic diseases are believed to be caused by somatic mutations to the *PRNP* gene in dividing cells, which produces PrP^C protein that can spontaneously misfold, it remains possible that a small proportion of PrP^C molecules may undergo covalent post-translational modifications (PTMs) that cause misfolding and drive the disease process.

PrP^C has between 208 and 220 residues, depending on species, and the primary structure is highly homologous across mammals. A

Abbreviations: CAD, collisionally activated dissociation; CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *PRNP*, the gene encoding the prion protein; PrP^C, cellular prion protein; PrP^{Sc}, scrapie isoform of the prion protein; PrP GPI–, prion protein lacking the GPI anchor; PTM, post translational modification; recPrP, recombinant prion protein; TSE, transmissible spongiform encephalopathy.

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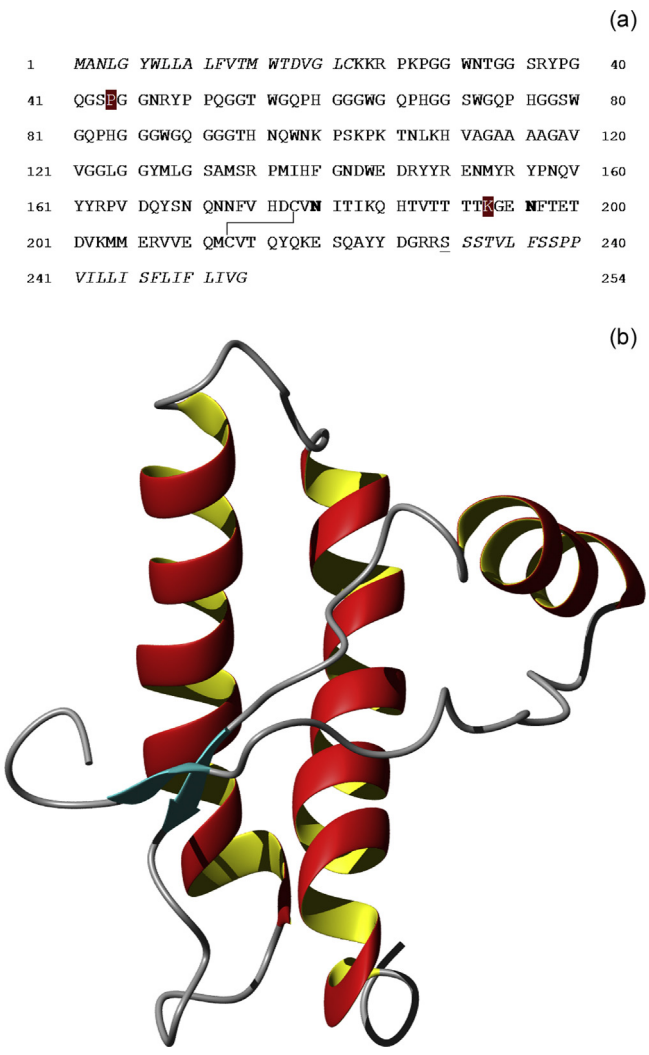

Fig. 1. (a) The full sequence of murine PrP^C. N- and C-terminal signal peptides are shown in italics and the site of attachment of the GPI membrane anchor is underlined. A single disulfide bond links cystines 178 and 213. Sites of N-linked glycosylation are in bold whilst the sites of proline hydroxylation and lysine hydroxylation (this work) are highlighted with a dark background. (b) Ribbon diagram of the structured, C-terminal domain of murine PrP^C (residues 124–226). The structure was based on that of murine PrP determined by Riek et al. [24] (PDB code 1AG2) and rendered using MolMol and PovRay.

number of physiological PTMs have been identified, which appear common to all mammalian prion proteins [15,16]. The sequence of the murine prion protein is shown in Fig. 1(a), on which sites of PTMs are highlighted. These include cleavage of N and C-terminal signal sequences leaving a mature protein spanning residues 23–230 (murine numbering, which is used throughout) followed by attachment of a glycosylphosphatidylinositol (GPI) membrane anchor at the mature C-terminus. In the ER/golgi complex, further modifications involve addition of two N-linked carbohydrate chains to Asn180 and Asn196 [17,18] and formation of a single, internal disulfide bond. We have also identified the pro-collagen-like hydroxylation of proline 44 in both recPrP expressed in Chinese hamster ovary (CHO) cells and, at least at low levels, in PrP^{Sc} purified from the brains of scrapie infected mice [19], a modification that demonstrates that the N-terminal region of PrP can adopt polyproline II structure [20]. There have been reports of modifications to arginine residues in the N-terminal region, although initial suggestions that these are converted to citrulline remain to be confirmed [21,22]. There are further reports of modifications to arginine or lysine residues in the N-terminal region by advanced glycation end products [23]. The effects of N-terminal amino acid modifications on the structure of PrP have largely not been studied.

The N-terminal region of PrP is rather flexible but, by contrast, the C-terminal domain of PrP^C possesses typical globular structure. NMR studies of recombinant PrP expressed in bacteria have shown that the C-terminal region is composed of a 3-helix bundle with two short β-strands, depicted in Fig. 1(b) [24]. Conversion of PrP^C to PrP^{Sc} is associated with an increase in the β-sheet content of the protein involving at least part [3], or potentially all of the helical region of the protein [25]. Thus, post translational modifications in this region of the protein may be more likely to impact on PrP folding/misfolding. There have been reports of oxidation of specific methionine residues [26] and a suggestion that asparagine/aspartate residues may be altered in PrP^{Sc} [27,28]. In proof of principle studies, it has also been shown that conditions that cause oxidative or nitrative modifications, or that deiminate PrP, can lead to misfolding [29,30] and it is clear that any PTMs that can be detected in the C-terminal domain of PrP are candidates to impact adversely on protein folding.

Following our previous identification of 4-hydroxyproline in the N-terminal region of mammalian-expressed recPrP and in PrP^{Sc} purified from mouse brains [19], herein we report the discovery of a second novel modification to the recPrP. Lys193 was found to be specifically converted to δ-hydroxylysine with subsequent addition of a di-hexose unit. Both Edman degradation and mass spectrometry have been used to characterise these modifications by comparison to complement component C1q, a protein known to contain similar modifications in its collagenous domain, which have previously been shown to constitute the addition of the disaccharide galactose-glucose to hydroxylysines. Molecular dynamics simulations show that disaccharide modification of Lys193 of PrP leads to little effect on the motion in the loop around the modification, but that the loop joining β-sheet2 with α-helix2 becomes more stable. The β_2–α_2 loop region has been postulated to be implicitly involved in modulating the susceptibility of PrP to misfold, hence modification to Lys193 may be a candidate post-translational modification initiating PrP misfolding.

2. Methods

2.1. Protein production, purification and digestion

The expression, production and purification of aglycosyl recombinant PrP lacking its GPI anchor is as described previously [31]. Briefly, CHO cells were grown on Cytodex 1 microcarriers (Pharmacia) in Techne spinner flasks for 3 days in serum-containing medium, followed by a recPrP production phase in serum-free medium. The protein accumulated in the culture medium and, after 3 days production, soluble protein was purified by means of cation exchange chromatography eluting with a sodium chloride gradient. Fractions containing recPrP, as determined by Western blotting techniques, were pooled and further purified by immobilised metal affinity chromatography, eluting by means of an imidazole gradient. The final fractions contained mature length recPrP, in addition to a 9 kDa N-terminal fragment resulting from a single proteolytic cleavage that has previously been characterised [31].

CHO cell recPrP in 6 M urea, pH 8.5, was reduced with 5 mM dithiothreitol and alkylated with 20 mM iodoacetic acid then was dialysed against 3 M urea, 200 mM ammonium bicarbonate pH 9.0. 10% (w/w) trypsin was added and the recPrP was digested overnight. For selected analyses, detailed in the results section, recPrP was partially digested by the addition of 1% (w/w) trypsin without prior reduction or alkylation. Complement component C1q from human serum (Sigma) was dissolved to 1 mg/ml in 0.2 M ammonium bicarbonate pH 8.5, 2 M urea and