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Citation for published version:

Young, DS, Meersman, F, Oxley, D, Webster, J, Gill, A, Bronstein, I & Dear, DV 2009, 'Effect of enzymatic deimination on the conformation of recombinant prion protein', *BBA - Proteins and Proteomics*, vol. 1794, pp. 1123-1133.

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

BBA - Proteins and Proteomics

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Effect of enzymatic deimination on the conformation of recombinant prion protein

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ARTICLE INFO

Article history:

Received 26 September 2008

Received in revised form 19 March 2009

Accepted 20 March 2009

Available online 31 March 2009

Keywords:

Prion protein

Post-translational modification

Conformational change

Deimination

Citrulline

Arginine modification

Amyloid

ABSTRACT

Deimination is the post-translational conversion of arginine residues to citrulline. It has been implicated as a causative factor in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis and more recently, as a marker of neurodegeneration. We have investigated the effect of the post-translational modification of arginine residues on the structure of recombinant ovine prion protein. Deiminated prion protein exhibited biophysical properties characteristic of the scrapie-associated conformer of prion protein viz. an increased β -sheet secondary structure, congophilic structures indicative of amyloid and proteinase K resistance which could be templated onto normal unmodified prion protein. In the light of these findings, a potential role of post-translational modifications to prion protein in disease initiation or propagation is discussed.

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1. Introduction

The group of neurodegenerative diseases known as prion diseases is frequently associated with the occurrence of plaques containing prion protein (PrP) in an altered conformation, PrP^{Sc}. PrP^{Sc} is purported to be infectious or at least, self-propagating [1]. We have been interested in determining processes that might lead to a conformational change in prion protein *in vitro*, as this might indicate possible therapeutic strategies. Earlier studies indicated that oxidative modifications to amino-acid residues had an impact on conformation of recombinant PrP [2]. In this study we were interested in observing the effect on the conformation of recombinant ovine PrP (rec ovPrP) as a result of deimination.

Deimination is the conversion of arginine residues to citrulline and has several implications for the structure of a protein. For example, the

ureido group of citrulline has a destabilising effect on protein structure due to its urea-like properties; it can also bind Zn²⁺ and increases the solubility of lipid hydrocarbon tails [3]. This destabilising effect of citrulline has been seen on citrullination of several proteins including filaggrin, trichohyalin, and myelin basic protein (MBP) and resulted in a loss of the organised secondary structure of these proteins [4,5]. Loss of the positive charge associated with deimination can also be expected to have a large impact on structure. Basic proteins such as MBP bind to negatively-charged lipid membranes *via* electrostatic interactions so modification of positive residues can disrupt this interaction [6,7].

Peptidylcitrulline is produced from arginyl residues by the action of a group of enzymes known collectively as peptidylarginine deiminases (PADs). PADs catalyse the irreversible conversion of the arginine guanidino group to an ureido group – a process known as deimination – with concomitant loss of ammonia and the positive charge on the residue [3]. This family of PAD enzymes is distinct from other enzymes that produce citrulline such as nitric oxide synthase and arginine deiminase because of the selectivity for peptidylarginine over free arginine. There are several different classes of PAD. A bacterial form has been identified in species such as *Pseudomonas aeruginosa* and *Porphyromonas gingivalis* that allow the bacteria to adapt to acidic environments by producing ammonia to lower the local pH [8,9]. There are five known categories of mammalian PAD, all requiring calcium ions for activity [10–14].

Abbreviations: BSE, Bovine Spongiform Encephalopathy; CJD, Creutzfeldt–Jakob disease; PrP, prion protein; PrP^C, cellular form of PrP; PrP^{Sc}, scrapie (pathological) form of PrP; huPrP, human PrP; ovPrP, ovine PrP; recPrP, recombinant PrP; PAD, peptidylarginine deiminase; MBP, myelin basic protein; BD, 2,3-butanedione; SDS-PAGE, sodium dodecyl sulphate polyacrylamide electrophoresis; CD, Circular Dichroism; PK, Proteinase K; PMSF, phenylmethylsulphonyl fluoride; CR, Congo Red; ANS, 1-anilinonaphthalene-8-sulfonic acid; ThT, Thioflavin T

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In the case of prion protein, early amino acid sequencing data reported the possibility of post-translationally modified arginyl residues in the N-terminus of cellular PrP [1]. Gas-phase sequencing of the N-terminus of Syrian hamster PrP^C could not detect the predicted arginine residues at position 3 (R25) in PrP^C and positions 3 (R25) and 15 (R37) in PrP^{Sc} [15]. This suggested a modification of arginyl residues might be present. In addition, different forms of PrP^{Sc} isolated from scrapie-infected mice cells were N-terminally sequenced to confirm their identity. It was noted that an unidentified amino acid derivative of arginine was present at position 3 (R25), which may facilitate a conformational change of PrP^C to PrP^{Sc} [16].

In a review of mass spectrometric analysis of PrP, the theoretical presence of citrulline instead of arginine in the N-terminus was discussed [17]. The mass of citrulline is 1 Da more than that of arginine. Therefore, especially in crude, heterogenous preparations, peptides containing the different residues are difficult to distinguish from unmodified, native peptides. Analysis of endoproteinase Lys-C and tryptic digests of PrP could not confirm the presence of any arginine modification, although the authors did not discuss the possibility of a citrulline modification *per se* it was acknowledged that a low level of modification, or a labile modification would not be observed [18]. Trypsin cleaves proteins C-terminal of arginyl residues (unless the subsequent residue is proline), and thus would not cleave if citrulline were present instead. Although the tryptic peptide containing R25 gave the mass expected for arginine rather than citrulline, the authors noted that the M + 1 ion for the peptide was stronger than expected and that a small fraction containing citrulline could have been present [18–19].

In this study, we provide data on the effects of deimination of recombinant ovine PrP including the ability to aggregate into large insoluble amyloid fibrils and plaques, an increased resistance to protease digestion, a high β -sheet secondary structure content, and the potential to template its properties onto unmodified rec ovPrP.

2. Materials and methods

2.1. Recombinant ovPrP (Met₂₅–233)

The protein used in this study was produced as described previously [20]. Briefly, full-length ovine protein was produced in *E. coli* using a dual origin expression vector, and purified using two sequential chromatographic steps, immobilised metal ion affinity chromatography and cation exchange chromatography. The protein was purified under denaturing conditions from inclusion bodies and refolded in non-reducing conditions by incubation with copper ions followed by dialysis into sodium acetate storage buffer, pH 5.5. Mass spectrometric analysis was used to confirm the presence of full-length protein and the oxidation of the two key cysteine residues.

2.2. Arginine modification of ovPrP

Rec ovPrP was diluted in glycine–NaOH buffer (50 mM, pH 7.6), containing calcium chloride (5 mM) and the deimination reaction carried out using PAD at 0.66 U/ml. Samples were incubated at 52 °C for 2 h or at times specified in the figures. The reaction was stopped by dialysis of the protein back into sodium acetate buffer (pH 5.5) to remove the calcium prior to further analysis [3].

2.3. SDS-PAGE analysis

Protein samples were separated by SDS/PAGE under reducing conditions using pre-cast 4–20% (w/v) tris–glycine gradient gels (Invitrogen, Holland). Bands were visualised using the Coomassie[®]-based protein stain, SimplyBlue™ (Sigma, UK) according to the manufacturer's protocol. Gels were destained overnight, and then dried at 80 °C for 1 h for storage. The gels displayed in this work are

representative of at least 2 experiments. Gel densitometry was carried out using the Scion Image 1.63 software available at <http://www.ScionCorp.com>.

2.4. Circular Dichroism spectroscopy

The CD spectra were acquired using a cell of 0.02 cm path length in a spectropolarimeter (JASCO). Measurements were taken between 250 and 200 nm using a temperature-controlled cell, with a step resolution of 0.1 nm and sensitivity of 10 mdeg. Twenty scans were acquired and averaged for each sample. Spectra were normalised for protein concentration and amino acid length to give a reading in mean residue weight ellipticity [θ mrw], degrees cm² dmol⁻¹.

2.5. Protease assays

Protein samples were incubated with proteinase K (PK) or trypsin at a final dilution, of 20 or 100 μ g/ml, respectively. Incubations were terminated at different times (default 1 h) by addition of PMSF to 10 mM to inhibit the protease and then boiled in SDS-PAGE sample buffer at a 1:1 volume ratio. In some PK studies, copper sulphate (1 mM) was also included in the protease reaction. Samples were analysed by SDS-PAGE followed by Coomassie staining, as described previously. β -breaker peptides (BACHEM, UK) were preincubated with rec ovPrP (0.1 mM final concentration) for 2 h with shaking, prior to PK digestion and the results were analysed by SDS-PAGE.

2.6. Dye affinity studies

Precipitates of modified rec ovPrP were spotted onto gelatin-coated slides and stained with the dye Congo red (CR) using a modification of a previous protocol [21]. Stained protein was then examined under a polarising microscope (Olympus, London, UK) for birefringence. Soluble forms of the modified protein were incubated with CR (25 μ M) in phosphate buffer solution (0.1 M, pH 7.4) with sodium chloride (150 mM), for 30 min at room temperature with shaking, and the absorbance measured on a UV/Vis Spectrometer Lambda 8 16 (PerkinElmer, Bedfordshire, UK) at 540 and 477 nm and the amount of bound dye was calculated, following a previously reported protocols [22]. ANS binding studies were carried out by incubating rec ovPrP (0.1 mg/ml) with ANS in sample buffer for 10 min at 37 °C and the fluorescence was measured on a Fluoroskan Ascent (ThermoLabsystems, Basingstoke, UK) with excitation at 355 nm and emission 485 nm with a 20 ms integration time. Thioflavin T (ThT) measurements were obtained by incubating rec ovPrP with the dye (10 μ M) in glycine–NaOH buffer (50 mM, pH 9), for 10 min before the fluorescence was measured with excitation at 450 nm and emission 485 nm with a 20 ms integration time.

2.7. β -breaker peptides

A stock solution (1 mM) of the 13-residue peptide DAPAAPAG-PAVPV, denoted iPrP13, was prepared in 50% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid, and stored in aliquots at –80 °C until use. The standard protocol for PK digestion of modified and unmodified rec ovPrP was then followed, but including a preincubation of the protein with iPrP13 (0.1 mM final concentration) for 2 h at 37 °C with shaking, and the results assayed by SDS-PAGE. During titrations of the iPrP13 peptide, the void volume was made up with corresponding volume of a solution (1 mM) of a 10 residue peptide corresponding to the helix 1 region of ovPrP (DYEDRYREN).

2.8. Fourier-transform infra-red (FTIR) spectroscopy

To investigate the structure of templated protein, FTIR spectra were recorded at room temperature on a Bruker Equinox 55 spectrometer

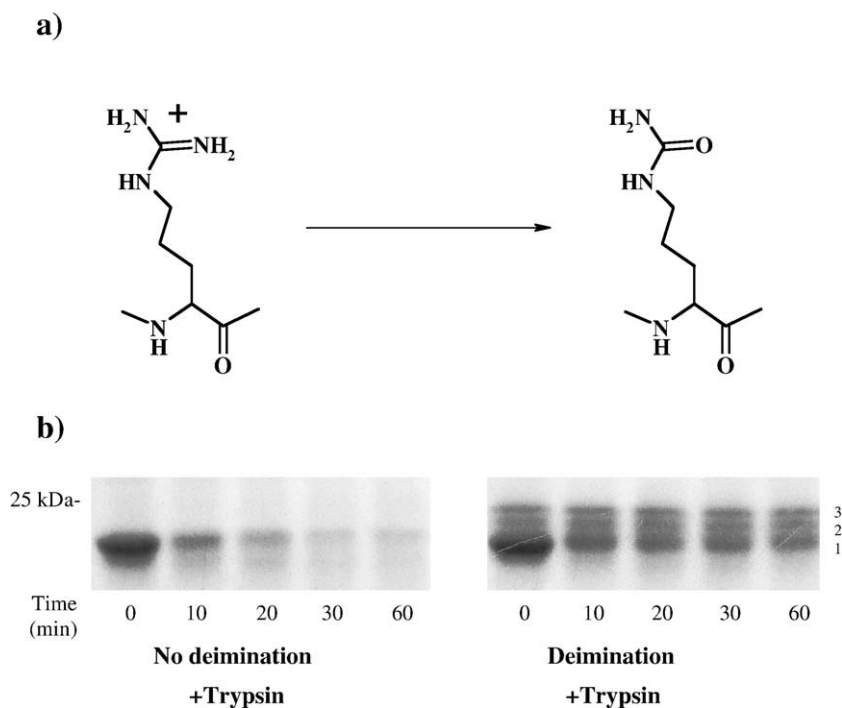


Fig. 1. SDS-PAGE analysis of deimination of rec ovPrP by peptidylarginine deiminase (PAD). (a) Conversion of peptidylarginine to peptidylcitrulline. The figure details the deimination of a positively-charged arginine residue to a neutral citrulline residue, such as that catalysed by PAD. The guanidino group of peptidylarginine is converted to an ureido group by nucleophilic attack by the enzyme at the carbon of the guanidino group with concomitant protonation and loss of the imino group as ammonia. This is followed by nucleophilic attack by water to displace the enzyme and generate free peptidylcitrulline [11]. (b) SDS-PAGE analysis of deiminated recombinant ovPrP. The gel shows analysis of the trypsin digestion of untreated (control) and PAD-treated recombinant ovPrP (0.75 mg/ml) over an hour. At $t = 0$, deiminated ovPrP showed the presence of three clear bands (labels 1, 2, and 3 on gel) clustered between 23 and 25 kDa whereas the control samples did not. Samples were removed at timed intervals and stored at $-20\text{ }^{\circ}\text{C}$ prior to SDS-PAGE analysis and coomassie staining.

equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The sample compartment was continuously purged with dry air. For each sample, 256 interferograms were collected at a spectral resolution of 2 cm^{-1} . Sample and buffer spectra were measured separately and subsequently subtracted using Protein Dynamics software (Bruker). Thin films of hydrated rec ovPrP were obtained by depositing $200\text{ }\mu\text{g}$ of protein in a $30\text{ }\mu\text{l}$ volume on the crystal and subsequent removal of excess water under a gentle N_2 flow.

2.9. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) of modified rec ovPrP

Deiminated rec ovPrP was reduced, carbamidomethylated and digested with trypsin. A 10% (w/v) stock solution of trypsin was prepared in sodium acetate buffer (50 mM, pH 5.5). Prior to use, this was diluted 100-fold and incubated with deiminated and control samples of rec ovPrP (0.75 mg/ml) at $37\text{ }^{\circ}\text{C}$ with shaking for 1 h. The peptide products were analysed by on-line LC-MS/MS. The tryptic peptides were separated on a reversed-phase column ($0.1 \times 100\text{ mm}$; Vydac C18) with a gradient of 0–30% (v/v) acetonitrile (containing 0.1% (v/v) formic acid) over 30 min, at a flow rate of 500 nL/min. Peptides were eluted via a nanoelectrospray interface into a quadrupole time-of-flight mass spectrometer (Applied Biosystems). The mass spectral data were matched to the protein sequence using Mascot software (Matrix Science) and confirmed by manual interpretation of MS/MS spectra.

3. Results

3.1. SDS-PAGE analysis and mass spectrometry of deiminated rec ovPrP populations

To investigate the effects of conversion of arginyl residues to citrulline, rec ovPrP was incubated with PAD for increasing time

periods (data not shown). The product was largely soluble, although a small amount of an insoluble white precipitate formed with each reaction. The latter increased with longer incubations and increasing concentrations of rec ovPrP. After PAD treatment, the modified protein sample could be resolved as a series of bands by electrophoresis. The separate bands were expected to represent distinct populations of modified protein, each with a different level of modification, and thus different charge, pI, and electrophoretic mobility. Using rec ovPrP that separated as a single monomeric species at 23 kDa on gel electrophoresis, a sample of PAD-treated protein was prepared that ran as 3 bands between 23–25 kDa on electrophoresis (Fig. 1).

The single band from the untreated protein and the three bands from the treated sample were excised from the electrophoresis gel and digested with trypsin. The separate samples were then analysed by reverse-phase HPLC-coupled mass spectrometry. Trypsin cleaves peptide bonds C-terminal of lysine and arginine residues so modification of these residues may interfere with proteolysis. Unmodified protein was readily digested by trypsin, whereas the PAD-treated rec ovPrP was resistant to digestion. Increased concentrations of trypsin digested both modified and unmodified proteins (data not shown). The extent of modification, as judged by the increase in apparent molecular weight, had little effect on the degree of resistance to proteolysis, as each band remained at a similar intensity throughout the incubation with trypsin.

Extracted ion chromatograms from LC-MS analysis of tryptic peptides are shown in Fig. 2. Several peptide fragments were identified in the untreated protein as those containing arginine residues (Fig. 2): m/z values of 939.71 (R159YPNQVY165), 968.72 (Y¹⁶⁶RPVDRY¹⁷²), 1044.67 (Q²²²RESQAYY²²⁹), 1102.70 (R¹⁵⁹YPNQVYY¹⁶⁶), 1428.62 (I¹⁴²HFGNDYEDR¹⁵¹) and 1591.59 (I¹⁴²HFGNDYEDRY¹⁵²). On treatment of the protein with PAD, the intensity of the signal from the peptides containing arginine decreased dramatically,

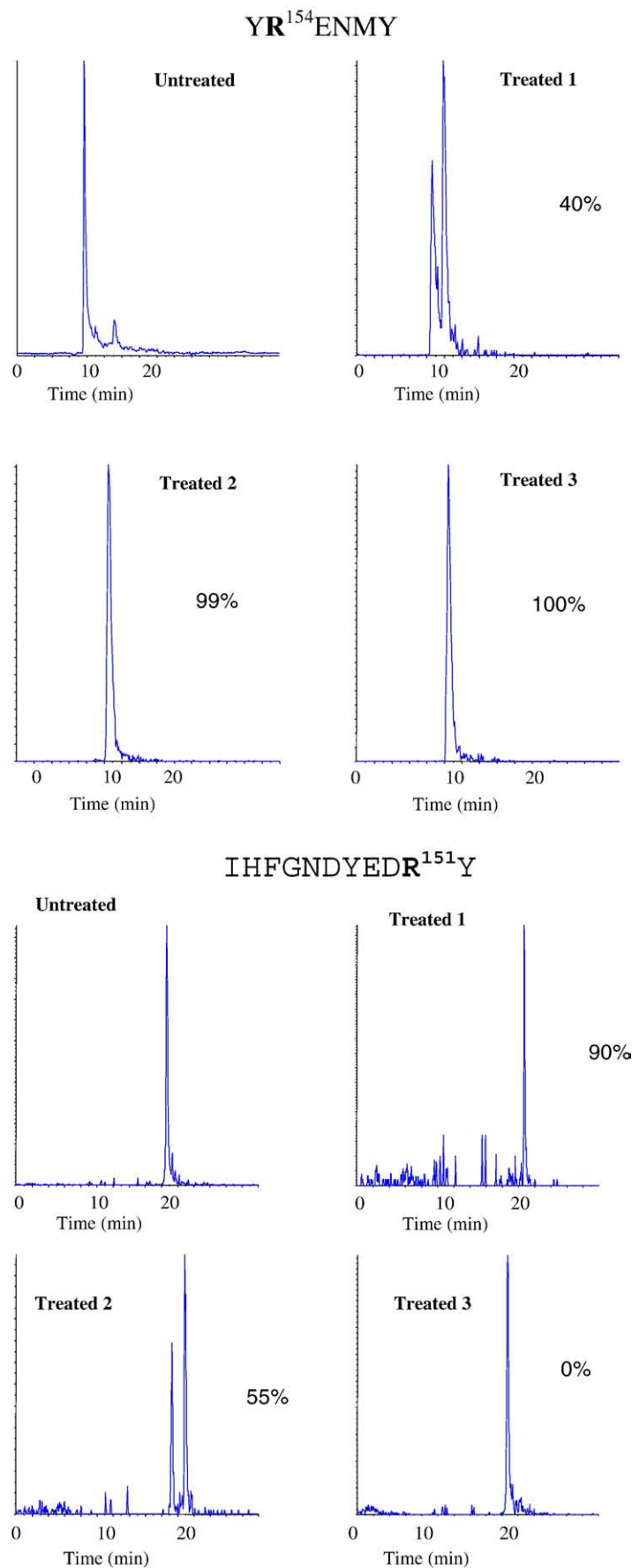


Fig. 2. Extracted ion chromatograms for the peptides MLGSAMSRPL and IHFGNDYEDRY. Chromatograms of the material eluting at the specific masses for the arginine-containing peptides were collected for each of the untreated control and PAD-treated samples. The chromatograms show the % of modifications present in bands 1, 2 and 3 (see Fig. 1b) of modified samples as compared to an untreated control. Modified and unmodified peptides had a different retention time and thus the relative amount of conversion of arginine to citrulline was estimated by the relative peak height.

Table 1

The table indicates the percentage of arginine residues that are deiminated in the tryptic peptides in each of the samples.

Peptide	Percentage arginine modified			
	Untreated	Treated 1	Treated 2	Treated 3
MLGSAMSR ¹³⁹ PL	0	34	87	97
IHFGNDYEDR ¹⁵¹ Y	0	0	55	90
YR ¹⁵⁴ ENMY	0	40	99	100
R ¹⁵⁹ YPNQVY	0	18	89	98
YR ¹⁶⁷ PVDR ¹⁷¹ Y	0 mono/0 di	49/9	59/29	4/95
QR ²²³ ESQAYY	0	6	16	23

The percentage modification was calculated from the peak heights in the extracted-ion chromatograms of the respective monoisotopic peptide molecular ions. The peptide YRPVDRY contained two arginine residues, and the relative amount of mono- and di-modification is indicated.

further suggesting that deimination had occurred since the loss of the positive charge on conversion to citrulline would decrease detection of arginine-containing peptides by the mass spectrometer.

However, several signals were still evident suggesting that not all the arginine residues had been converted. The peak at m/z 969.72 is 1 Da more than that seen in untreated protein at 968.72 for the peptide YRPVDRY, which would agree with one of the two arginine residues (R¹⁶⁷ or R¹⁷¹) having been deiminated; the persistence of the second arginine residue would also explain the only slight decrease in the intensity of the signal. Similarly, signals at m/z 1429.64 and 1592.62 suggest that deimination has occurred at R¹⁵¹. The signal at m/z 1044.68 is similar to that in the untreated protein, suggesting that modification at R²²³ is less favoured.

Correlation of the chromatographic trace and the mass spectrum allowed the ratio of modified and unmodified arginine in each of the peptides to be estimated. These values are summarised in Table 1. Modification of arginyl residues was found to give a clear increase in the retention time of the peptides on reverse-phase chromatography, which would be expected with the loss of charge and increase in mass and hydrophobicity concomitant with deimination. Therefore, the relative amount of modified and unmodified peptides can easily be visualised from the peak heights in the ion extracted chromatograms. No evidence of a background level of citrullination in the untreated protein was observed in any sample. Peptides containing the arginine residues in the N-terminal region of the protein were not distinguishable for analysis.

Because of the difficulty in achieving 100% mono- or di-modification under the conditions used, the mixture of treated protein rather than separated mono- or di-modified protein was investigated further.

3.2. Circular Dichroism (CD) analysis of deiminated ovPrP reveals a β -sheet structure

The solubility of the deiminated product allowed CD spectroscopy analysis of the samples (Fig. 3). The averaged spectra from the deiminated sample showed a marked change in the secondary structure. The untreated protein gave a spectrum indicative of a predominantly α -helical secondary structure with a small amount of β -sheet, as expected. In contrast, the deiminated protein contained mostly β -sheet structure. (CD analysis of α -helical structures gives a strongly positive band at 192 nm and two negative peaks at around 208–210 and 222 nm, whilst β -sheet structures give a moderate positive peak at 198 nm and a negative peak at 215 nm.)

3.3. PK sensitivity of rec ovPrP is altered on deimination and in the presence of copper

The protease resistance of PAD-treated rec ovPrP was investigated in order to determine whether the modification of arginine residues had altered the susceptibility of the protein to PK (Fig. 4a). PK

digestion was carried out either in the presence or absence of copper ions. In the absence of copper ions, both the untreated and deiminated protein digested to give bands between 7 and 11 kDa. However, in the presence of copper ions, untreated PrP was digested whereas deiminated PrP was not.

As copper binding at the N-terminal octarepeats of PrP has been shown to have an effect on the PK sensitivity of different forms of PrP in several studies [23–25] the effect of copper ions on the protease resistance of PAD-treated rec ovPrP was investigated (Fig. 4b). In the presence of copper, the protease resistance of the deiminated protein dramatically increased, whereas that of the untreated control was unchanged.

3.4. PK sensitivity of deiminated protein can be restored with β -breakers

The potential of a 13-residue β -sheet breaker peptide, denoted iPrP13 [26], which could promote the conversion of the deiminated protein from a β -sheet conformation to a predominantly α -helical state was investigated (Fig. 4c). At millimolar concentrations of iPrP13, the PK sensitivity of the modified protein in the presence of copper could be restored at higher protease concentrations.

3.5. Deiminated ovPrP displays birefringence and an increased CR affinity

The insoluble fraction of the rec ovPrP after PAD treatment was pooled from several reactions and dried onto gelatin-coated slides for analysis with CR. The white precipitate, unlike the soluble unmodified protein, was seen to be CR-positive, and gave a characteristic apple-green birefringence under polarised light (Fig. 5a). Interaction of CR with amyloid in solution is known to induce a hyperchromic shift, from a maximal absorbance around 480 nm to around 540 nm [22]. By monitoring the change in intensity of the two absorbance maxima, the amount of CR bound to the PAD-treated protein was estimated (Fig. 5b). The PAD-treated protein was found to bind more molecules of CR than an equivalent amount of untreated protein. This corresponds to an increased proportion of amyloid structure in the modified protein.

3.6. Fluorescent amyloid dyes bind deiminated rec ovPrP

The ability of the dyes ANS and ThT, both used in protein folding and amyloidogenesis studies, to bind soluble deiminated rec ovPrP was investigated (Fig. 6). The deiminated sample demonstrated a

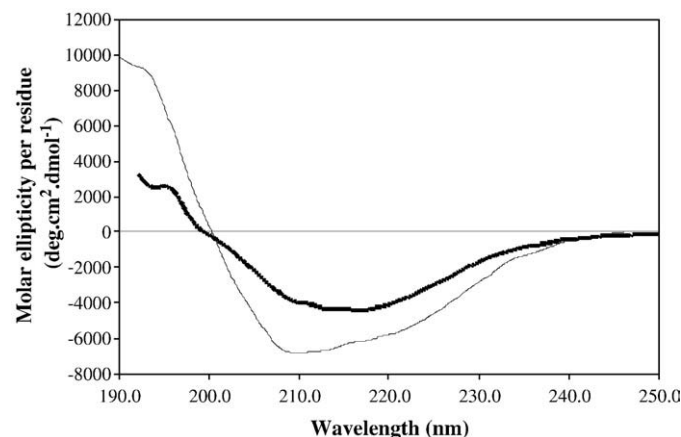


Fig. 3. Secondary structural analysis of deiminated rec ovPrP by Circular Dichroism (CD). Unmodified (thin line, control) and PAD-treated (bold line) rec ovPrP were analysed by CD from 250 to 190 nm. Spectra were normalised for protein concentration and amino acid length (208 nm for rec ovPrP) to give a reading in mean residue weight ellipticity ($[\theta]_{\text{mrw}}$, degrees $\text{cm}^2 \text{dmol}^{-1}$). The unmodified protein gave a spectrum indicative of a predominantly α -helix with 20% β -sheet whereas the deiminated protein had a predicted 40% of β -sheet, as estimated using JFIT (JASCO) software.

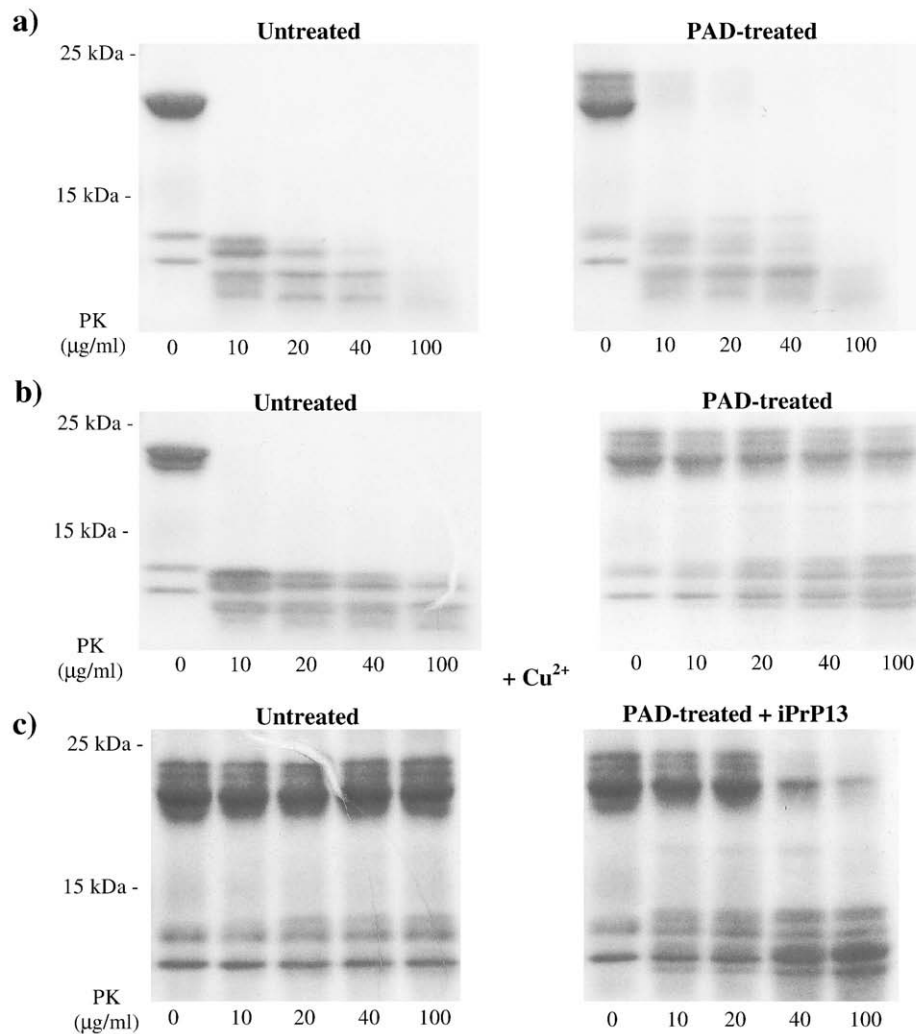


Fig. 4. Deimination of rec ovPrP confers PK resistance in the presence of copper. (a) PAD-treated ovPrP in the presence of Cu²⁺ was incubated either with 50% acetonitrile (untreated control) or with 50% acetonitrile containing iPrP13 (1 mM) for 2 h with shaking at room temperature. Untreated (control) and PAD-modified recombinant ovPrP (0.75 mg/ml) were incubated with proteinase K at the dilutions shown for 1 h at 37 °C with shaking, and the reaction stopped by addition of PMSF. The samples were then analysed by SDS-PAGE and Coomassie staining. (b) Proteinase K resistance of PAD-treated recombinant ovPrP in the presence of copper ions. Control and PAD-treated rec ovPrP (0.75 mg/ml) were incubated with Proteinase K at the dilutions shown in the presence of copper sulphate (1 mM) for 1 h at 37 °C with shaking, and the reaction stopped by addition of PMSF. The samples were then analysed by SDS-PAGE and coomassie staining. (c) Restoration of protease sensitivity to deiminated rec ovPrP on incubation with a β -breaker peptide. PAD-treated rec ovPrP was incubated with 50% acetonitrile as a control, and 50% acetonitrile containing iPrP13 (1 mM) for 2 h with shaking at room temperature. The samples were then assayed for PK resistance in the presence of copper(II) sulphate (1 mM) as described above, and the results assayed by SDS-PAGE and Coomassie staining.

markedly stronger fluorescent signal compared to the control when incubated with ANS, increase in ANS fluorescence was observed to follow a linear trend with both untreated ($r^2 = 1.00$) and PAD-treated ($r^2 = 0.98$) samples.

Both the untreated and deiminated samples gave fluorescent signals of the same order of magnitude with ThT, with similar responses. However, the fluorescence of the deiminated samples was consistently stronger than that of the untreated sample. Therefore, under these experimental conditions, the affinity of the dye for the modified protein was only marginally increased and was not statistically significant ($P = 0.6$). Different solvent conditions were tested, with little apparent difference (data not shown).

3.7. Templating ability of deiminated recombinant ovine rec ovPrP

The templating ability of the deiminated protein to promote a change in conformation to untreated protein was investigated. The deiminated protein was diluted back into unmodified rec ovPrP and incubated, before aliquots were assessed for PK resistance to ascertain whether *in vitro* conversion had occurred. Incubation of the untreated

sample overnight with more untreated rec ovPrP had shown some increase in protease resistance of the sample. However, when deiminated protein was incubated with the untreated rec ovPrP, a more significant increase in the protease resistance was observed (Fig. 7a and b). Furthermore, this increased affinity for CR could be templated onto unmodified protein. Untreated rec ovPrP that had been incubated with PAD-treated protein overnight displayed an increased affinity for CR compared to untreated protein incubated with the untreated control (data not shown).

To determine whether the nature of the templated acquisition of PK resistance might be mediated through the transfer of an altered secondary structure, samples of PAD-treated rec ovPrP were incubated with untreated rec ovPrP and the structure assessed by FTIR spectroscopy after set time intervals (Fig. 7c). After 6 h, little change in the secondary structure of the protein was observed. However, after 12 h incubation, a shoulder appeared in the amide I spectrum at approximately 1620 cm^{-1} , indicating the presence of increased β -sheet structure [27]. This was enhanced further on incubation for 24 h. The intensity of the signal at 1620 cm^{-1} in the deconvoluted spectra was 11.2 after 6 h, 12.9 after 12 h, and 23.6 after 24 h. This suggests that

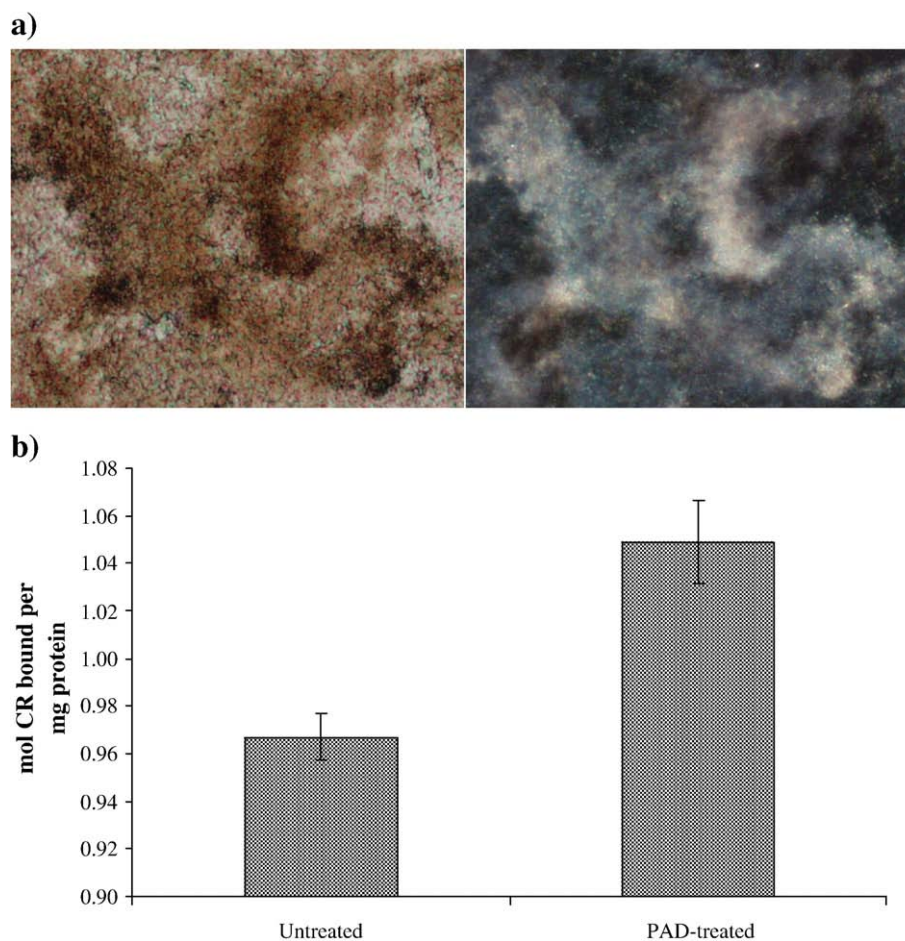


Fig. 5. PAD-treated rec ovPrP shows birefringence and Congo Red affinity. (a) Representative images of PAD-treated rec ovPrP after CR staining. The insoluble fraction of the deimination reaction was dried on gelatin-coated slides and stained with CR for the presence of amyloid fibrils. The left and right panels show the stained protein under normal and polarised light respectively. Magnification is 40 \times . (b) Quantification of the CR affinity of the modified protein. Samples of untreated and PAD-treated ovPrP were corrected for concentration and incubated with CR in potassium phosphate buffer solution (0.1 M, pH 7.4) with sodium chloride (150 mM), for 30 min at room temperature. The absorbance was monitored at 540 and 477 nm with a baseline correction taken at 600 nm, and the number of moles of dye bound to the protein calculated using the method of Klunk et al. [22]. The figure shows the results from 3 independent assays.

the mechanism for templating the properties of PAD-treated rec ovPrP onto untreated rec ovPrP involves the transfer and acquisition of a structure with increased β -sheet content.

4. Discussion

These observations that deimination of rec ovPrP leads to the rapid acquisition of PrP^{Sc}-like characteristics have provided some interesting and novel insights into the possible impact and importance of arginine modification on PrP structure. The recent report regarding the accumulation of citrullinated proteins in the brains of scrapie-infected mice makes this discovery even more pertinent [28]. Arginyl residues in different locations in PrP might be expected to have a different impact on the protein structure and function when modified. The investigation into deimination of prion protein was prompted by the brief mention in several references of an unknown modification occurring with a low incidence in the N-terminus of PrP [17–18]. Modification in the latter case was seen *in vivo* therefore, but only in heterogeneous samples. This could suggest a possible regulatory role for the modification, or a low level of aberrant modification caused by altered enzyme activity in the cell. Deimination of positive arginyl residues in this region (R27, R40 and R51 in rec ovPrP) to neutral citrulline could be expected to alter the copper-binding affinity of the octarepeats, and so disrupt the native function of PrP. Whether this

would be a means of regulating PrP activity, or simply be an indicator of aberrant PAD activity is not clear.

The N-terminus of PrP has also been suggested to play a role in trafficking of the protein to the cell surface, specifically to lipid rafts [29–30]. N-terminal deletions were found to disrupt internalisation and increase the cellular half-life of PrP, and could be restored by addition of regions distinct from the copper-binding elements [31]. If arginyl residues are important in the recognition of the N-terminus during trafficking, or for the lipid association itself, it is possible that modification of these would abrogate the internalisation and allow pathogenic forms of the protein to persist for longer, increasing the risk of transmission of the infectious characteristics.

Several lines of evidence implicate helix 1 as playing a significant role in the structure of PrP^C and in disease propagation. Helix 1 is believed to be stabilised by salt-bridges between two aspartate–arginine pairs; D147–R151 and D150–R154 in rec ovPrP. Deimination of such key arginyl residues could interfere with these salt-bridge interactions and would also be expected to destabilise the native fold. The helix 1 region has also been implicated in dimerisation of PrP [32,33]. This could suggest a role for deimination of helix 1 arginyl residues in reducing the energy barrier to dimerisation and promoting initial aggregate formation. Within Helix 1, R151 appeared less accessible to modification by PAD than R154, and was not modified at all in the PAD-treated protein of lowest apparent molecular mass.

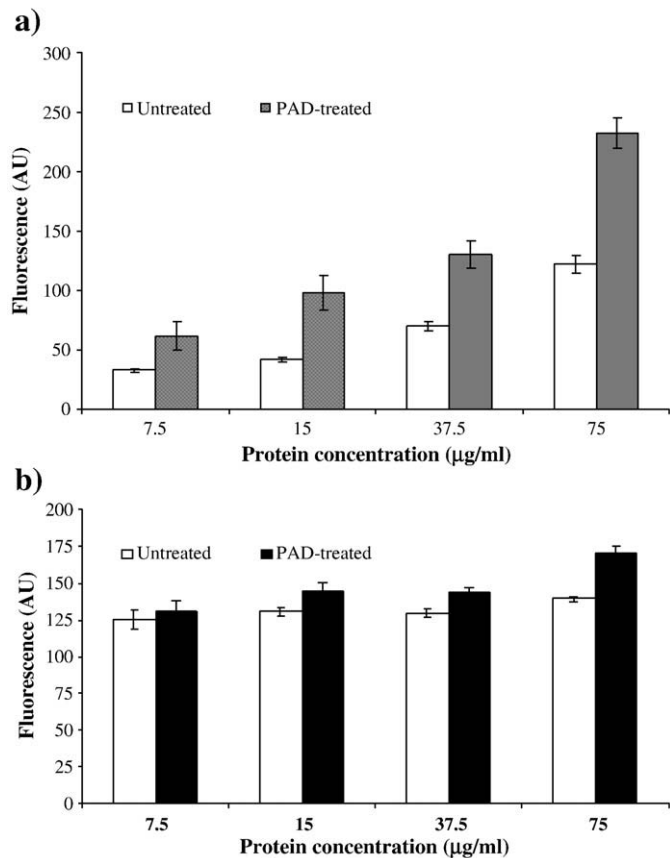


Fig. 6. Binding of fluorescent dyes to deaminated rec ovPrP. Demonstration of (a) ANS and (b) ThT binding by samples containing deaminated rec ovPrP. Graphs showing data taken from six and eight samples from two batches of protein respectively, error bars correspond to one standard deviation.

R151 was modified in the protein of increased apparent molecular mass and was not recognised by the antibody 6H4 (data not shown) [34]. This would suggest that this residue is important in the epitope recognised by 6H4. Both R151 and R154 are also in a helical region of the protein in close proximity to glutamate residues, and are thought to be involved in salt-bridge stabilisation of the structure [35]. Therefore, these residues might not be expected to be readily modified by PAD. However, helix 1 is also thought to be structurally plastic, and modification of other residues in the protein might induce structural rearrangement around helix 1 that increases the rate of modification of R151 and R154. This is suggested by the poor modification of R151 in the population of protein with the least modification.

The peptide YRPVDRY contained two arginine residues R167 and R171, and thus the relative amounts of mono- and di-modification could be estimated. R167 is thought to become more exposed to the solvent on the conformational change from PrP^C to PrP^{Sc} [35]. The NMR structures of rec ovPrP from various species indicate that arginine residues equivalent to R167 are the least solvent exposed arginine residues (Table 1). While deimination of both these residues appear to happen readily, it was not possible to determine the different reactivities of the two residues, although the fragmentation spectrum of the mono-modified peptide showed evidence of modification of both arginine residues i.e. each arginine residue can be independently modified.

By mass spectrometry, each peptide showed an increase in arginine modification which correlated with the increased apparent molecular mass (decreased electrophoretic mobility) of the sample. The extent of modification was high for most residues (at least 90% for six of the seven arginine residues analysed, in the band of highest apparent molecular mass), although the C-terminal residue R223 was less well modified. Although thought to be highly exposed to the

solvent, this residue is within a putative α -helical region and followed by a glutamate residue, and previous studies have shown such an environment not to favour deimination by PAD [3].

Because of the difficulty in achieving 100% mono- or di-modified arginine residues in the protein, studies were carried out on the reaction mixture of treated protein. However, it would be of interest to carry out further studies to determine conditions which allow for partial or total modification of arginine residues and to separate out the mono- and di-modified proteins for further structural studies. By doing this, it should be possible to identify the pivotal role of defined arginine residues on the conformation of prion protein.

The CD analysis of deaminated rec ovPrP revealed a β -sheet structure and this characteristic has been used previously to monitor changes in PrP conformation under various conditions [36–39]. Although the protein sample could not be modified to homogeneity under the conditions used in this study, the results from the CD spectroscopy showed a change to a predominantly β -sheet conformation. CD spectroscopy of deaminated filaggrin (predominantly β -turn secondary structure) and trichohyalin (α -helical) has shown previously that the protein secondary structure can be disrupted by deimination, but in the cases examined so far, the structure has been lost rather than converted [3]. This change was shown to be analogous to the addition of urea, and it was suggested that the ureido group of citrulline was acting in a similar manner. Thus, in the case of prion protein, the presence of the ureido group on the citrulline may act to destabilise the native fold enough to allow the protein to partially unfold and so adopt a more stable scrapie-associated conformation with a higher β -sheet content.

Protease resistance has long been established as a key characteristic by which to distinguish PrP^C and PrP^{Sc} [40–43], where PrP^{Sc} is distinguished by the presence of a 17 kDa protease-resistant core. However, despite an apparent conformational change in the protein as determined by CD spectroscopy, the deaminated protein is still largely susceptible to PK digestion, except in the presence of copper (Fig. 1b). The fact that it was the full-length protein that was PK-resistant is of interest as, in general, PrP^{Sc} is characterized by a PK-resistant core of 17 kDa. This finding may be explained by the decrease in the pI of the deaminated protein altering the interaction of copper ions and PrP; whether this causes deaminated rec ovPrP to adopt a conformation less accessible to proteinase K, or is otherwise more stable, is unclear. The fact that the untreated PrP was still sensitive to PK in the presence of copper ions either argues against the recent finding that PK activity can be inhibited by copper ions or was carried out at a concentration that was not inhibitory [44]. Increased PK resistance of rec ovPrP in the presence of manganese ions has been reported previously, although the authors only mention a possible alteration in secondary structure as the cause [45,46]. Therefore, it seems possible that the creation of ureido groups on citrulline within the protein may create alternative copper complexation sites. Alternatively, the ureido groups may destabilise the protein structure and reveal hidden or cryptic sites able to bind copper ions. An internal copper-binding site was found in recombinant human PrP (huPrP), formed by histidines 96 and 111, with a similar affinity to that of the octarepeat region [47]. It may be that destabilisation of the protein structure by the presence of citrulline residues alters the accessibility and/or affinity of this site. The same authors also found that copper bound to the reduced β -sheet conformation of the recombinant huPrP with a marginally higher affinity than the oxidised α -helical form. This would correlate with our finding that the deaminated rec ovPrP, has an increased PK resistance in the presence of copper. In addition, the fact that PK sensitivity of deaminated protein could be restored with β -breakers correlates with the work of Soto et al. [26]. The latter found that incubation of protease-resistant PrP with iPrP13 restored the PK sensitivity by promoting the conversion of the protein back to its α -helical state from the resistant β -sheet conformation. Alternatively, it may be that the β -breaker peptide binds copper more efficiently and that this decrease in copper ions allows reactivation of the PK enzyme.

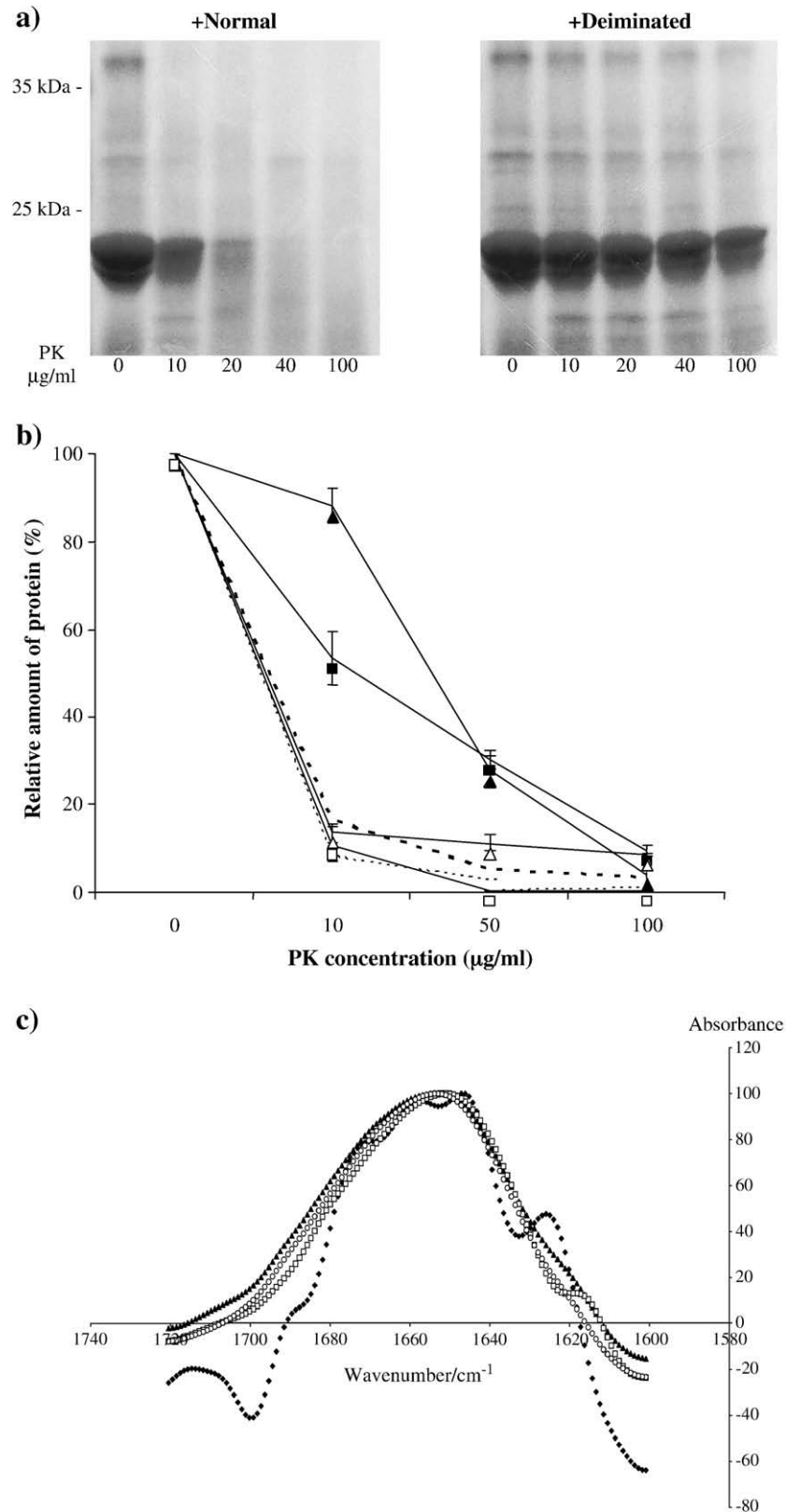


Fig. 7. (a) *In vitro* conversion of rec ovPrP monitored by the acquisition of PK resistance. Samples of untreated control (left panel) and PAD-treated (right panel) rec ovPrP were diluted (1:20 sample:stock protein) into the stock ovPrP (1.5 mg/ml) and incubated at room temperature overnight, with shaking. The samples were assayed for PK resistance in the presence of copper and analysed by SDS-PAGE and coomassie staining. (b) Templating of PK resistance in the presence of copper. Samples of PAD-treated rec ovPrP were diluted (1:20 sample:stock protein) into the stock rec ovPrP and incubated for 12 (triangles) and 24 h (squares) in the presence (black symbols) and absence (white symbols) of copper (500 µM). Samples were then assayed for protease resistance as described previously, and the relative level of protein at 23 kDa was assessed by densitometry. Δ – incubation for 12 h in the absence of copper; \square – incubation for 24 h in the absence of copper; \blacktriangle – incubation for 12 h in the presence of copper; \blacksquare – incubation for 24 h in the presence of copper. Dashed lines represent the levels of protein after 0 h in the absence (light line) and presence (bold line) of copper. Error bars depict the standard deviation for 2 independent samples. (c) FTIR spectroscopy of untreated rec ovPrP after incubation in the presence of deiminated rec ovPrP seed. PAD-treated rec ovPrP was diluted 20-fold into untreated rec ovPrP in the absence of copper and incubated for 0, 6, 12 and 24 h at 37 °C. Spectra show Fourier self-deconvoluted absorbance spectra of untreated templated rec ovPrP samples at $t=0$ (○), $t=6$ h (▲), $t=12$ h (□) and $t=24$ h (◆). Similar changes on storage of untreated protein were not seen.

However, the fact that in our study untreated protein remained PK-sensitive in the presence of copper ions argues against this.

The increased affinity of deaminated rec ovPrP for the amyloid-specific dye Congo red was interesting. Although the exact mechanism of CR binding to amyloid is not known, studies using PrP^C suggested that, as well as the planar aromatic aspect of the dye, the presence of the sulphate groups are vital to form the electrostatic interactions required for binding of the dye to the protein [48,49]. Therefore, it might be expected that deamination, and the loss of positive charge, might decrease the affinity of the dye for the protein. However, the presence of birefringence implies there is perhaps a more suprastructural or conformational reason behind the altered dye interaction with the modified protein. In addition, fluorescent amyloid dyes also bound more strongly to deaminated rec ovPrP indicating an increased proportion of exposed hydrophobic regions in the modified protein. This would correspond with the conformational changes indicated by the apparent shift to a predominately β -sheet conformation, which would be expected to expose areas of the protein buried in the native configuration.

A key finding of these studies was the fact that deaminated rec ovPrP could template its change in PK sensitivity to unmodified rec ovPrP. As residual PAD activity is inhibited, how this *in vitro* conversion might occur is intriguing. If it is the conformation that is crucial for the increased protease resistance of rec ovPrP in the presence of copper, then it is possible that the conformation of the deaminated rec ovPrP can act as a template for the conversion of the unmodified protein. The fact that the protein remains predominantly monomeric, suggests that any templating effect would not necessarily be similar to the mechanism proposed for amyloid fibril formation and growth. In the future, discrimination between the added deaminated seed and unmodified protein could be carried out in studies by western blotting using an anti-citrulline antibody. In this instance, however, we relied on the fact that deaminated protein separated as three bands on SDS-PAGE. Detection of the deaminated seed prior to the experiment was negligible as shown in the right hand panel in Fig. 7a. This suggested that the PK resistance observed in the templating experiment must be due to the templating effect of the added seed rather than PK resistance of any deaminated seed.

Combined, this evidence suggests that post-translational modification may have the potential to play a significant role in the initiation or propagation of prion disease. Whilst deamination could form the basis of future studies into possible causes, treatment or identification of novel biomarkers of prion diseases, the fact that its presence causes a change in protein conformation is of interest in itself. Recently, we have also shown that production of post-translational modifications within recombinant prion protein due to oxidative stress conditions can have a similar effect on conformation [2]. The fact that the residues involved are different but lie within a similar area of the molecule supports observations on the critical role of charged residues in the helix 1 region in changing prion protein's conformation [50].

Acknowledgements

This work was funded by the Department of Health (DVD and IB) and a Biotechnology and Biological Sciences Research Council (BBSRC) to DSY studentship.

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