Separation of native prion protein (PrP) glycoforms by copper-binding using immobilized metal affinity chromatography (IMAC)

Henrik MÜLLER*, Alexander STROM†, Gerhard HUNSMANN† and Andreas W. STUKE†¹

*Institut für Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, D-40225 Düsseldorf, Germany, and †German Primate Centre (DPZ), Department of Virology and Immunology, Kellnerweg 4, D-37077 Göttingen, Germany

The conformational conversion of the normal cellular prion protein (PrP^C) into the pathology-associated PrP^{Sc} isoform is a key event in TSEs (transmissible spongiform encephalopathies). The host PrP^C molecule contains two N-linked glycosylation sites and binds copper under physiological conditions. In contrast with PrP^C, PrP^{Sc} is insoluble in non-ionic detergents and does not bind to Cu²⁺ ions. Hence, we utilized copper binding to separate and characterize both PrP isoforms. Infected and uninfected murine brain and bovine stem brain specimens were treated with the mild non-ionic detergent n-octyl- β -D-glucopyranoside (octylglucoside) to maintain the native PrP conformations during isolation. The solubilized homogenates were loaded on to Cu²⁺-saturated IMAC (immobilized metal affinity chromatography) columns and eluted using the chelating agent EDTA. Fractions were separated by SDS/PAGE and analysed by immunoblotting using anti-PrP

monoclonal antibodies for glycosylation profiling. Whereas native PrP^C and denatured PrP^{Sc} were retained by a Cu²⁺-loaded resin, native PrP^{Sc} and PrP^{res} [PK (proteinase K)-resistant PrP] passed through the column. We demonstrate here that the IMAC technique is appropriate to isolate and partially purify PrP^C from healthy brains in its native-like and biologically relevant glycosylated copper-binding forms. The IMAC technique is also well suited for the separation of native PrP^C from aggregated PrP^{Sc} in infected brains. Our results indicate that in contrast with PrP^{Sc} in uninfected as well as infected brains, PrP^C is predominantly present in the glycosylated forms.

Key words: copper, glycosylation, glycotyping, immobilized metal affinity chromatography (IMAC), prion protein (PrP).

INTRODUCTION

Prion diseases or TSEs (transmissible spongiform encephalopathies) are lethal neurodegenerative disorders, such as scrapie in sheep, BSE (bovine spongiform encephalopathy) in cattle, and CJD (Creutzfeldt–Jakob disease) and FFI (fatal familial insomnia) in humans. In contrast with other neurodegenerative diseases that are characterized by aggregated proteins, TSEs are infectious. Histopathologically, prion diseases are accompanied by spongiform modifications of the grey matter and the appearance of proteinaceous plaques [1]. These plaques are mainly built of fibrils referred to as SAFs (scrapie-associated fibrils), comprising aggregated PrP (prion protein) molecules [2]. The post-translational conversion of the normal cellular form (PrP^C) encoded by the *PRNP* gene into the pathology-associated isoform (PrP^{Sc}) involves non-covalent modifications, leading to the aggregation of PrP^{Sc} into SAFs. The protein-only hypothesis postulates that these SAFs are the infectious agent. This concept is extended by the appearance of different pathology-associated isoforms referred to as prion strains [3].

In contrast with PrP^C, the PrP^{Sc} isoform has a higher content of β -sheets than α -helices in the secondary structure. In addition, PrP^{Sc} is partially proteinase K (PK) resistant (PrP^{res}) and insoluble in non-ionic detergents [1]. These biochemical properties have been widely used for the detection of PrP^{Sc} and the diagnosis of TSEs [4]. PrP^C is an evolutionarily highly conserved molecule. It may play a role in synaptic function, signal transduction or apoptosis [5]. In recent years, an increasing number of different putative PrP receptors have been described, but up to now this

has not led to a convincing explanation of the biological function of PrP^C. PrP^C contains two N-linked glycosylation sites and is embedded in membranes via a GPI (glycosylphosphatidylinositol) anchor [6]. Similar to other GPI-anchored proteins, the host PrP^C is mainly incorporated into cholesterol-rich microdomains (rafts) found at the plasma membrane of neurons [7,8]. PrP^C passes through a subcellular cycle between plasma membrane and endosomal compartments, and seems to recycle to the interior of the cell with a transit time of approx. 60 min [9]. In contrast, PrP^{Sc} is deposited in cytoplasmic vesicles and extracellular plaques [2]. The subcellular sites where PrP^{Sc} is formed and the trafficking pathways leading to these sites are currently under investigation [6].

Distinct glycosylation differences between PrP^C and PrP^{Sc} have been described [10]. Glycosylation is able to protect large regions of the PrP surface from proteases and non-specific protein–protein interactions [11], and also influences the glycosylation of other putative interacting proteins [12]. Two molecular features of PrP^{Sc}, i.e. the molecular mass and the glycosylation profile, have been used to describe different prion strains [13,14] by a technique referred to as glycotyping. In this method the extent of glycosylation of the di-, mono- and un-glycosylated PrP^{res} is examined by immunoblotting after SDS/PAGE. However, a detailed study has shown that glycotyping of PrP isoforms is difficult to perform and to interpret [15]. Recently, it was reported that pH variations in brain homogenates influence the characteristics of PrP^{res} [16].

PrP^C binds copper under physiological conditions [17] via its octarepeat region [18]. This metal-binding property has been used

Abbreviations used: BSE, bovine spongiform encephalopathy; ECL, enhanced chemiluminescence; IMAC, immobilized metal affinity chromatography; mab, monoclonal antibody; NC, nitrocellulose; PK, proteinase K; PrP, prion protein; PrP^C, normal cellular PrP; PrP^{res}, PK-resistant PrP; PrP^{Sc}, pathology-associated conformation of PrP; recbPrP, recombinant bovine PrP; TSE, transmissible spongiform encephalopathy.

¹ To whom correspondence should be addressed (email astuke@dpz.gwdg.de).

to purify PrP^C from normal hamster brains using a Co²⁺-charged column [19] or recombinant PrP from *Escherichia coli* by Ni²⁺-nitrilotriacetate agarose [20]. In contrast with PrP^C, PrP^{Sc} does not bind to copper [21]. Thus in the present study we utilized copper binding to separate the PrP isoforms from each other. To maintain the native PrP conformation during isolation we used the mild non-ionic detergent octylglucoside (n-octyl- β -D-glucopyranoside; Sigma) [22] for sample solubilization. By using IMAC (immobilized metal affinity chromatography), we isolated and partially purified PrP^C from healthy mouse and cattle brain in its native glycosylated copper-binding forms. Furthermore, using TSE-infected brains we separated native PrP^C from aggregated PrP^{Sc}. ECL (enhanced chemiluminescence) immunoblotting experiments were conducted to characterize both isoforms by densitometric measurements. Our results indicate that PrP^C molecules from uninfected and infected brains are predominantly glycosylated, whereas PrP^{Sc} molecules are more often non-glycosylated.

EXPERIMENTAL

Brains

The brains of healthy mice (strain STU) were derived from the breeding facility of the German Primate Centre (DPZ), Germany. Brains of mice (strain C57BL/6) infected with scrapie (strain Chandler) were obtained from M. Groschup (Institute of Novel and Emerging Infectious Diseases, Federal Research Centre for Virus Diseases on Animals, Insel Riems, Germany). A bovine stem brain sample of a healthy animal was provided by W. Schulz-Schaeffer (Institute of Neuropathology, Georg-August-University, Göttingen, Germany). A stem brain sample of a cow that had clinical evidence of BSE infection (case number 97/00091) was kindly provided by the Veterinary Laboratories Agency Weybridge (Addlestone, Surrey, U.K.).

Preparation of protein extracts (homogenization and subcellular fractionation)

To hinder denaturation and inactivation processes, all steps were performed at 4°C. After thawing, aliquots of brain material were homogenized using a glass Potter–Elvehjem homogenizer in Tris buffer (10 mM Tris/HCl, 100 mM KCl, 320 mM sucrose, pH 7.5) to a final concentration of 10%. To pellet nuclei, cytoskeletal and cytoplasmic membrane fractions, the homogenates were centrifuged at 2000 g for 10 min. The pellet was removed and saved, and the resulting supernatant was sedimented at 10 000 g for 15 min yielding a pellet containing the mitochondria. Subsequently, the remaining supernatant was layered on to a 20% (w/v) sucrose cushion and clarified by ultracentrifugation at 160 000 g for 4 h. Three fractions were recovered, a synaptosomal/microsomal fraction at the bottom, the sucrose fraction and the supernatant. The pellets of the three centrifugation steps, the sucrose layer and the remaining supernatant were stored at –80°C. The total protein content of all fractions was determined using a protein assay kit (Bio-Rad).

Detergent solubilization and IMAC

PrP was enriched and partially purified by a modification of the IMAC protocol, as described by Pan et al. [23]. To solubilize membranes containing PrP, samples with a total protein content of 1–4 mg/ml were incubated with 2.5% (w/v) of the mild non-ionic detergent octylglucoside for 90 min at 4°C in the presence of the protease inhibitors leupeptin, aprotinin and pepstatin A

(0.1 µg/ml each). Octylglucoside is known to maintain the native conformation and enzymatic activity of many proteins under a variety of conditions [24]. The samples were adjusted to 1% (w/v) octylglucoside by adding Tris buffer (pH 7.5) and were agitated at 4°C overnight. Residual cell debris were removed by a short centrifugation step (11 000 g, 5 min, 4°C) and the supernatants were collected and applied to the IMAC column. The total protein content of the detergent-containing supernatants was determined with the RC DC Assay Kit (Bio-Rad).

A 1-ml IMAC column packed with iminodiacetic acid coupled to chelating fast-flow Sepharose (Amersham Pharmacia) was charged with three column volumes of 100 mM CuSO₄, washed with five column volumes of washing buffer (20 mM Tris/HCl, 500 mM KCl, 1 mM imidazole, pH 8.0), and equilibrated with ten column volumes of washing buffer containing 1% (w/v) octylglucoside. Then the solubilized sample containing a total of 1–5 mg of protein with a mean concentration of 0.5 mg/ml was applied on to the column and the flow-through was collected in 1-ml fractions. After washing with five column volumes of washing buffer, the bound proteins were eluted with five column volumes of 100 mM EDTA, pH 8.0, by releasing the copper ions from the IMAC resin. All steps were performed at a flow rate of 0.5 ml/min. Subsequently, remaining salts, EDTA and Cu²⁺ ions were removed by dialysis or centrifugation using YM-10 Centriprep centrifugal filter devices (Amicon). Aliquots of flow-through, wash fraction and eluate corresponding to 15–90 µg of total protein were concentrated by ethanol precipitation and analysed by SDS/PAGE and immunoblotting. Before analysis some samples were digested with PK. All experiments were done at least in triplicate.

Denaturation

To further characterize the binding affinities of PrP^C and PrP^{Sc} to the Cu²⁺-loaded resin the PrP-containing samples were denatured by boiling in 4% SDS for 10 min before applying to the IMAC column.

PK digestion

To determine the PrP^{res} content of the solubilized fractions and the IMAC fractions, protein samples corresponding to 15–40 µg of total protein were precipitated with three volumes of 96% (w/v) ethanol. The resulting pellets were dissolved in digestion buffer [10 mM Tris/HCl, 1 mM CaCl₂, 0.5% (w/v) SDS, pH 7.8] and incubated with PK (Boehringer) concentrations ranging from 0 µg/ml to 5 µg/ml for 30 min at 37°C. Digestion was terminated by addition of an equal volume of SDS/PAGE loading buffer [40 mM Tris/HCl, 250 mM dithiothreitol, 7.5 M urea, 2.3% (w/v) SDS, 0.05% (w/v) Bromophenol Blue, pH 6.8] and immediately heated to 95°C for 5 min.

Discontinuous SDS/PAGE analysis and immunoblotting

The proteins were separated using 9–16% (w/v) polyacrylamide gradient gels containing 1% (w/v) SDS according to the protocol of Laemmli [25], and electroblotted on to 0.45 µm NC (nitrocellulose) membranes (Schleicher and Schuell) using the method described by Towbin et al. [26]. The NC sheets were incubated for 90 min at 37°C in blocking buffer [PBS with 5% non-fat dry milk, 0.1% (v/v) Tween 20]. PrP was visualized by staining with a mixture of the mAbs (monoclonal antibodies) 3B5, 4F2, 8G8, 11C6, 12F10 and 14D3 (1:3000 for each). The mAbs specifically react with human, murine and bovine PrP epitopes [27], but are unable to discriminate between PrP^C and PrP^{Sc}. NC sheets were incubated for 1 h at 37°C. Subsequently, the sheets were

Figure 1 Subcellular localization of PrP

PrP^C enriched in cell fractions of uninfected bovine stem brain (**A**), and PrP^C/PrP^{Sc} enriched in cell fractions of BSE-infected bovine stem brain (**B**). Brain samples were homogenized, subcellular fractionated and immunoblotted as described. PrP contents of cytoplasmic membrane fraction (lane 1), mitochondrial fraction (lane 2), synaptosomal/microsomal fraction (lane 3), sucrose phase (lane 4), supernatant of the ultracentrifugation (lane 5) and a positive control comprising 10 ng of recbPrP (lane 6) were determined using a mix of the anti-PrP mabs 3B5, 4F2, 8G8, 11C6, 12F10 and 14D3. The diglycosylated (a), monoglycosylated (b) and unglycosylated (c) PrP forms are indicated. Molecular masses are indicated in kDa.

incubated for 1 h at 37 °C with a peroxidase-conjugated anti-mouse IgG (1:4000; Dianova). Western blots were developed with an ECL detection system (Amersham Pharmacia) according to manufacturer's instructions and exposed on X-ray HyperFilms (Amersham Pharmacia). Signals were quantified by comparing with known quantities of recbPrP (recombinant bovine PrP; Prionics), and the relative PrP content was determined with the ScionImage analysis software (Scion Corporation). The limit of detection was approx. 1 ng of recbPrP. The general distribution of proteins was determined by staining the NC sheets with 2 % (w/v) Ponceau S in 3 % (v/v) acetic acid.

RESULTS

PrP localization in subcellular fractions of infected and uninfected bovine stem brain and whole mouse brains

To localize PrP^C and PrP^{Sc} within different cellular fractions, brain tissue was homogenized and adjusted with buffer to a final concentration of 10 %. Subsequently, the samples were differentially centrifuged to pellet nuclei, cytoplasmic membranes and cytoskeleton in the first step, mitochondria in the second and synaptosomal/microsomal membranes in the final step. After subcellular fractionation of normal bovine stem brain (Figure 1A) and normal murine brain, PrP^C was enriched in all membrane-containing fractions. Of the three centrifugation pellets, the cytoplasmic membrane fraction contained not only the highest PrP content (Table 1), but also the highest PrP total protein ratio (results not shown). In the sucrose fraction only a small amount of PrP^C was observed, whereas in the remaining supernatant containing all soluble proteins, PrP^C-specific signals were absent.

From infected bovine stem brain (Figure 1B) and infected murine brain, PrP^C and PrP^{Sc} were enriched in all three centrifugation pellets, as well as in the sucrose fraction. In the remaining supernatant, PrP was absent. Similarly to uninfected specimens the cytoplasmic membrane fraction of infected brain specimens contained the highest PrP content (Table 1) and PrP total protein ratio (results not shown). The distribution of PrP in the subcellular fractions of normal bovine stem brain and BSE-infected bovine stem brain differed. In uninfected stem brain 89 % of PrP^C were enriched in the cytoplasmic membrane fraction. After infection with the BSE agent, the total PrP content of the cytoplasmic membrane fraction was found to be diminished by about 15 % in favour of the mitochondrial fraction.

IMAC

PrP^C and PrP^{Sc} were enriched in all three subcellular pellet fractions, namely in the cytoplasmic membrane fraction and the mitochondrial fraction, as well as in the synaptosomal/microsomal fraction. To take variation in the conformational appearance or modification status of different PrP^C and PrP^{Sc} species in different subcellular fractions into consideration, we decided to subject a pool of the three centrifugation pellets to IMAC treatment. However, single IMAC experiments with individual fractions suggested no differences regarding the IMAC elution profiles of single fractions or the fraction pool (results not shown). To release PrP from the membranes, the pooled pellet fractions were incubated in 2.5 % octylglucoside. Whereas PrP^C is solubilized by this treatment, PrP^{Sc} remains to be incorporated into aggregated structures [21]. After sedimentation of cell debris the supernatant containing the whole PrP was applied to the Cu²⁺-loaded column. The elution profiles of the PrP isoforms of both infected and

Table 1 Mean content of PrP^C and PrP^C/PrP^{Sc} within fractions after subcellular fractionation of murine and bovine specimens

The mean contribution (%) of the PrP content of each fraction compared with the combined signals (set as 100 %) are shown. The relative PrP contents were determined with image analysis software.

Specimen	Fraction ...	Contribution (%)				
		Cytoplasmic membrane	Mitochondrial	Synaptosomal/microsomal	Sucrose	Supernatant
Bovine	PrP ^C	89	9	2	0	0
	PrP ^C /Sc	73	22	3	2	0
Murine	PrP ^C	53	33	14	0	0
	PrP ^C /Sc	60	32	8	2	0

Figure 2 Partial purification of PrP^C by IMAC

Octylglucoside extracts from normal mouse brain (**A**) and normal bovine stem brain (**B**) were applied to and eluted from a Cu²⁺-loaded IMAC column. Flow-through samples (**A**, lanes 1–8, total protein in each lane, 90 µg; **B**, lanes 1–2, total protein in each lane, 35 µg), a positive control comprising recbPrP (**A**, lane 9; **B**, lane 6; 10 ng of each), washes (**A**, lanes 10–12, total protein in each lane, 90 µg; **B**, lanes 3–5, total protein in each lane, 35 µg) and eluted samples (**A**, lanes 13–15, total protein in each lane, 15 µg; **B**, lanes 7–10, total protein in each lane, 55 µg) were immunoblotted using a mixture of the anti-PrP mabs 3B5, 4F2, 8G8, 11C6, 12F10 and 14D3. PrP^C was detected in the eluted samples. Molecular masses are indicated in kDa.

uninfected mouse brain and bovine stem brain were recorded by ECL immunoblotting.

IMAC of normal mouse brains and normal bovine stem brains

PrP^C was found only in the eluate of the IMAC column, indicating that PrP^C binds the Cu²⁺-loaded resin. Immunoblotted fractions of an uninfected murine brain specimen (Figure 2A) and uninfected bovine stem brain specimen (Figure 2B) after IMAC are shown. PrP-specific signals were not detected in the flow-through or in the wash fractions. A decreased protein/detergent ratio during solubilization turned out to be crucial for recovery of PrP^C from the suspended membranes and its affinity to Cu²⁺ ions. A 4-fold PBS-diluted specimen resulted not only in a higher content of solubilized membrane-released proteins, but also led to a more extensive binding of PrP^C to the IMAC column. In the case of undiluted bovine stem brain, 22 % of the total PrP^C amount was bound on average (results not shown). However, a 4-fold PBS dilution of the same specimen resulted in an almost 5-fold higher affinity of PrP^C to the Cu²⁺-loaded column, leading to a complete binding (within our detection limit). Furthermore, we observed that repeated usage of the columns yielded a better recovery of PrP^C. IMAC with an unused column led to an incomplete binding of PrP^C. Thus columns had to be used repeatedly to obtain reproducible, quantitative results.

Normal bovine stem brain and normal mouse brain were characterized by stronger signals for the fully glycosylated form of PrP^C. Accordingly, after IMAC involving loss of substantial amounts of protein no unglycosylated PrP^C was detectable. The relative intensities of the PrP-specific signals before and after IMAC did not differ. In Figure 3 a glycotyping pattern is shown for six uninfected murine whole brain specimens.

IMAC of scrapie-infected mice brains and a BSE-infected bovine stem brain

In case of infected material PrP was found both in the flow-through and in a lower content in the eluate. Figure 4 shows fractions after

Figure 3 PrP^C and PrP^{Sc} glycotyping patterns of murine specimens after IMAC

Western blot signals after IMAC were quantified with image analysis software. For this to be carried out, all PrP-specific signals in the flow-through were regarded as being PrP^{Sc}, and all PrP-specific signals in the eluate were regarded as PrP^C. Mean contributions (%) of di-, mono- and un-glycosylated PrP bands to the combined signals (set as 100 %) are shown. The numbers (n) of specimens examined are given.

Figure 4 Partial purification and separation of PrP^C and PrP^{Sc} by IMAC

Octylglucoside extracts from mouse brain infected with the scrapie strain Chandler (**A**) and BSE-infected bovine stem brain (**B**) were applied to and eluted from a Cu²⁺-loaded IMAC column. Flow-through samples (lanes 1–4, total protein in each lane, 15 µg), a positive control comprising recbPrP (lane 5, 10 ng), washes (lanes 6–8, total protein in each lane, 15 µg) and eluted samples (lanes 9–12, total protein in each lane, 50 µg) were immunoblotted as described in Figure 1. The diglycosylated (a), monoglycosylated (b) and unglycosylated (c) PrP forms in the eluted samples are indicated. Molecular masses are indicated in kDa.

IMAC of a mouse brain specimen infected with the Chandler scrapie strain (Figure 4A) and of a BSE-infected bovine stem brain specimen (Figure 4B). In contrast with uninfected samples, PrP

Figure 5 PK digestion of IMAC fractions of scrapie-infected murine brain and BSE-infected bovine stem brain

Octylglucoside extracts from murine brain infected with the scrapie strain Chandler (**A**) and BSE-infected bovine stem brain (**B**) were applied to and eluted from a Cu^{2+} -loaded IMAC column. Pooled flow-through samples (500 $\mu\text{g/ml}$ total protein), pooled eluted samples (1000 $\mu\text{g/ml}$ total protein) and solubilized samples immediately prior to application to the Cu^{2+} -loaded column (750 $\mu\text{g/ml}$ total protein) were digested for 30 min at 37 °C in the presence of the PK concentrations indicated. In case of the flow-through sample in (**A**), digested with 0.5 $\mu\text{g/ml}$ PK, 750 $\mu\text{g/ml}$ total protein was used. After SDS/PAGE (total protein in each lane, 30–50 μg) immunoblotting was carried as described in Figure 1. The positive controls (Pos) comprised 15 ng of recbPrP. Molecular masses are indicated in kDa.

was found not only in the retained fraction, but also in the flow-through of the IMAC column. Thus we hypothesized that the bands in the flow-through (Figure 4A, lanes 2–3, and Figure 4B, lanes 1–4) comprised PrP^{Sc} .

The diglycosylated, monoglycosylated and unglycosylated forms of PrP were observed in the IMAC flow-through, whereas in the eluate only weak signals of the unglycosylated form were found. The latter corresponds to the results obtained with uninfected specimens before and after IMAC. In Figure 3 a glycotyping pattern is shown for three infected murine whole brain specimens.

PK digestion of IMAC fractions of infected mouse brains and an infected bovine stem brain

Additional PrP-specific signals appearing in the IMAC flow-through of infected specimen (Figure 4A, lanes 2–3, and Figure 4B, lanes 1–4) may be explained as follows. The TSE-associated isoform was actually enriched in the flow-through and consequently separated from the cellular isoform in the retained fraction. However, the total PrP content is significantly higher in infected specimens. Thus it cannot be excluded that the PrP-specific signals in the flow-through appeared as a consequence of an insufficient capacity of the column. Since PrP^{C} and PrP^{Sc} are undistinguishable by specific antibodies, both isoforms can only be monitored by their differing PK resistance.

The resistance of all IMAC fractions with respect to digestion with PK was analysed. Each fraction was digested with a range of PK concentrations from 0 to 5 $\mu\text{g/ml}$. The results shown in Figure 5 indicate a substantial difference in the PK resistance of PrP in different IMAC fractions. In the positive control, comprising the solubilized material before application to the IMAC column, PrP-specific signals were observed up to PK concentrations of 5 $\mu\text{g/ml}$. A band displacement appeared at 0.25 $\mu\text{g/ml}$ PK for the scrapie-infected murine specimen. The BSE-infected

Figure 6 Partial purification of denatured PrP isoforms by IMAC

Octylglucoside extracts from murine brain infected with the scrapie strain Chandler were denatured by boiling in 4 % SDS for 10 min and applied to and eluted from a Cu^{2+} -loaded IMAC resin (**A**). PrP-containing eluate fractions were pooled and digested for 30 min at 37 °C in the presence of PK at the concentrations indicated (**B**). Flow-through samples (**A**, lanes 1–4, total protein in each lane, 25 μg), a positive control comprising recbPrP (**A**, lane 5; **B**, Pos; 10 ng of each sample), washes (**A**, lanes 6–8, 25 μg total protein each), eluted samples (**A**, lanes 9–12, total protein in each lane, 25 μg) and PK-digested pooled eluate fractions (**B**, 500 $\mu\text{g/ml}$ each) were immunoblotted as described in Figure 1. The diglycosylated (a), monoglycosylated (b) and unglycosylated (c) PrP forms in the eluted samples are indicated. Molecular masses are indicated in kDa.

bovine specimen was resistant up to 5 $\mu\text{g/ml}$ PK. Thus, under the conditions used here, bovine PrP was found to be more resistant to proteolysis than murine PrP. In the flow-through PrP-specific signals were still undisplaced at PK concentrations of 0.25 $\mu\text{g/ml}$ (scrapie-infected murine specimen) or 5 $\mu\text{g/ml}$ PK (BSE-infected bovine specimen) respectively. Thus the specimens showed an even greater PK resistance after applying to the Cu^{2+} -loaded resin. In contrast with the flow-through, in the eluate no PrP-specific signals were detected after proteolytic treatment with 0.01 $\mu\text{g/ml}$ PK (scrapie-infected murine specimen) or 5 $\mu\text{g/ml}$ PK (BSE-infected bovine specimen) respectively. These observations confirmed our hypothesis that the flow-through contains PrP^{Sc} -molecules, whereas only PrP^{C} binds to the Cu^{2+} -loaded resin and can be eluted with EDTA.

IMAC of denatured scrapie-infected mouse brains

To further analyse the inability of PrP^{Sc} to bind to an IMAC column, scrapie-infected murine brain tissue was homogenized and suspended to a final concentration of 10 %. The homogenate was denatured by boiling in 4 % SDS for 10 min and applied to Cu^{2+} -loaded columns. In all IMACs of denatured specimens, all PrP-specific signals were observed in the eluate. Furthermore, all three PrP-specific bands were always detected (Figure 6A). Thus after SDS treatment both PrP isoforms were retained by the column, as demonstrated with scrapie-infected mouse brain. To test the resistance of the denatured and IMAC-subjected PrP molecules to proteolytic treatment, the eluate was digested with different PK concentrations (Figure 6B). Digestion by 0.5 $\mu\text{g/ml}$ PK completely degraded all PrP molecules without producing an intermediate band displacement.

IMAC of PK-digested and denatured scrapie-infected mouse brains

The observation that denatured PrP^{Sc} binds to the IMAC resin prompted us to determine whether this ability is actually based on the

Figure 7 Partial purification of denatured and PK digested PrP isoforms by IMAC

Octylglucoside extracts from murine brain infected with the scrapie strain Chandler (lane 1, 15 μ g total protein) were digested for 30 min at 37°C in the presence of 1 μ g/ml PK (lane 2, total protein, 15 μ g) and denatured by boiling in 10% SDS for 10 min. Subsequently the sample was applied to and eluted from a Cu^{2+} -loaded IMAC column and flow-through samples (lanes 3–4, total protein in each lane, 10 μ g), washes (lanes 5–7, total protein in each lane, 19 μ g) and eluted samples (lanes 8–11, total protein in each lane, 30 μ g) were immunoblotted as described in Figure 1. Molecular masses are indicated in kDa.

existence of the octarepeats containing the proposed Cu^{2+} -binding function. Therefore, a 10% homogenate of scrapie-infected mouse brain tissue was digested with 1 μ g/ml PK for 30 min, denatured by boiling in 4% SDS, and applied to and eluted from an IMAC column (Figure 7). After proteolytic treatment the N-terminal amino acids containing the Cu^{2+} -binding site are lost, yielding the PK-resistant core fragment of scrapie-associated PrP (PrP^{res}). Consequently, a band displacement appeared. After applying the specimen on to the IMAC column, all PrP-specific signals were detected in the flow-through. Surprisingly, the PrP bands always appeared not only at a higher molecular mass than the PK-digested PrP, but also at a higher mass than the untreated PrP prior to PK digestion and IMAC. As expected no bands were observed in the eluate.

IMAC-mediated enrichment of PrP isoforms is influenced by the washing conditions

The positive co-operative Cu^{2+} -binding site on PrP comprises the PHGGGWGQ segment within each of its four highly conserved octarepeats spanning residues 60 to 91 in the highly flexible N-terminal half of the PrP sequence. Cu^{2+} binding is pH dependent. At pH 7.4, four Cu^{2+} ions are chelated by the octarepeats, at acidic conditions only two Cu^{2+} ions are bound. In any case, the histidine residues are responsible for the binding to the metal ions [28]. To chelate metal ions not only are different amino acid residues suitable, but also molecules of a low molecular mass containing an amino function, such as ammonium chloride and imidazole. In agreement with this fact, before elution, a washing buffer containing the displacing agent imidazole was used to compete for binding to Cu^{2+} and consequently to remove non-specifically bound proteins. By changing the imidazole concentration the distribution of other cellular proteins with respect to flow-through/wash fraction or eluate was influenced (results not shown). Using a washing buffer containing 1 mM imidazole, other cellular proteins showed an increased non-specific binding to the IMAC column and were found in the retained fraction. Increased the imidazole concentration before releasing the Cu^{2+} ions by addition of the chelating agent EDTA resulted in a like-wise increased content of cellular proteins, which were enriched in the wash fraction. In contrast, the imidazole concentration did not affect the binding behaviour of PrP^{Sc} . Consequently, the imidazole concentration allows an

optimization of the enrichment of PrP^{C} and denatured PrP^{Sc} in the eluate or native PrP^{Sc} or PrP^{res} in the flow-through.

DISCUSSION

After infection with prions the cellular PrP is converted into its pathology-associated isoform and consequently the amount of PrP^{Sc} increases. Thus in the infected material analysed the PrP^{Sc} content was considerably higher than the PrP^{C} content, reflecting the clinical stage of the specimens examined. Our observations are comparable with quantitative analyses reported previously [29]. As expected the subcellular fractionation of uninfected murine brain homogenate and uninfected bovine stem brain homogenate indicated that PrP^{C} is mainly localized in the cytoplasmic membrane fraction. In summary, these experiments showed that PrP predominantly occurs in a membrane-associated form. In infected bovine stem brain an increased amount of PrP was found in the mitochondrial fraction, whereas in whole mouse brains the distribution of PrP was similar for uninfected and infected brains. This may reflect an increased PrP concentration in bovine stem brain material.

To separate both PrP isoforms and to partially purify them from other proteins we conducted an IMAC study using uninfected and infected brain specimens. In each IMAC experiment a pool of membrane fractions was solubilized in 2.5% octylglucoside and the IMAC elution profile was recorded by immunoblotting and total protein staining. IMAC experiments analysing uninfected murine brains and uninfected bovine stem brains indicated that under optimized conditions (see the Experimental section) PrP^{C} was found only in the eluate of the column. Since the eluted PrP was PK sensitive under the experimental conditions used in our study, a Cu^{2+} -induced PK-resistant PrP^{C} species [30] was excluded. However, the effect of copper on PrP detection appears to be subtle [31–33] and has to be investigated in further studies.

In contrast with uninfected specimens, the elution profiles of scrapie-infected murine brains and BSE-infected bovine stem brains exhibited PrP-specific bands in the IMAC flow-through. These PrP molecules proved to be more resistant to PK digestion than the PrP molecules in the eluted fractions. To assess the validity of the remaining PrP bands after PK treatment (PrP^{res}), a concurrent complete digestion of PrP^{C} is of importance. In this context, not only the PK concentration and incubation time, but also the PK total protein ratio and the extraction detergents, leading to distinct solubilities of the PrP isoforms, are of importance to avoid misinterpretation of the remaining PrP^{C} bands as PrP^{Sc} specific. Thus we concluded that the signals in the flow-through were PrP^{Sc} specific and that PrP^{Sc} actually does not adhere to a Cu^{2+} -loaded IMAC column. Since PrP^{Sc} does not bind to the IMAC column, PrP^{C} conversion presumably results in an isoform that does not bind Cu^{2+} . Consequently, the IMAC technique not only allowed us to concentrate and to partially purify, but also to separate both isoforms.

To further analyse the deviation in Cu^{2+} -binding behaviour, additional IMAC experiments were conducted. Infected murine brains were denatured before IMAC by treatment with SDS and also treated with SDS following PK digestion. Altogether, in contrast with native PrP^{C} and denatured PrP^{Sc} , native PrP^{Sc} and PrP^{res} can be distinguished by their inability to be retained by a Cu^{2+} -loaded resin. The results observed with denatured PrP^{Sc} also support previous findings [34] that denatured PrP^{Sc} does not refold into the original PrP^{Sc} conformation under the conditions of the experimental protocol used in the present study. Surprisingly, after IMAC of PK-digested and denatured murine scrapie brain specimens the PrP-specific signals appeared not only at a higher

molecular mass than the PrP bands after PK treatment only, but were also higher than the PrP bands prior to PK digestion and IMAC. Probably, aggregation occurred and seems to be responsible for the observed effect. Although after denaturation with loading buffer containing SDS no aggregates should remain, and chemical reactions yielding covalent modifications during PK treatment, SDS denaturation or IMAC are not known, but cannot be excluded.

In agreement with the IMAC treatments of PK-digested and SDS-denatured specimens, the octarepeats proved to be responsible for the Cu²⁺-binding function of both PrP^C and denatured PrP^{Sc}. However, we could not confirm the proposed presence of a second Cu²⁺-binding site [23] downstream from the N-terminal repeats. The N-terminal region of PrP containing the Cu²⁺-binding site is described as highly flexible, metastable and devoid of a well-defined structure. Thus one can assume that the different affinities of PrP^C and PrP^{Sc} to Cu²⁺ in the experiments described here are a consequence of the deviations in the N-terminal secondary and tertiary conformation of both isoforms. Of course, it cannot be excluded that the inability of native PrP^{Sc} to bind to the Cu²⁺-loaded columns results from PrP^{Sc} aggregation which buries the octarepeats. In addition, an incomplete solubilization of PrP^{Sc} could have a similar effect, as octylglucoside solubilizes PrP^C whereas some PrP^{Sc} could remain to be incorporated into rafts.

It has been proposed that glycan side chains can alter the PrP^{Sc} structure (conformation theory) or bind to neuronal receptors which then reproduce homologously glycosylated PrP^{Sc} (target-cell theory) [35]. The relative amounts of PK-digested and denatured PrP glycoforms of infected brains have been used to discriminate between TSE strains [15]. However, only little attention was dedicated to the glycosylation pattern of native PrP^C from uninfected individuals or cells [36] in comparison with the glycosylation pattern of native PrP^{Sc}. The results of such glycotyping studies should be carefully interpreted, because small variations during homogenization [37] and PK digestion, i.e. autolysis of cells, pH value [15], redox potential etc., may have unpredictable effects on PrP structure and immunogenicity. Since the IMAC technique enabled us to separate both PrP isoforms in native-like conformations, we used the Western blot signals of uninfected and infected murine IMAC specimens for glycotyping. We observed that PrP^C isolated from uninfected and infected murine brain appeared predominantly to be diglycosylated. This is not surprising, since a recent study suggests that the majority of PrP^C in pig brain appears in a fully-glycosylated form [38]. If PrP^C fulfils its biological function at the plasma membrane this seems to be evident. For the PrP^{Sc} isoform we observed significantly higher portions of especially the non-glycosylated state. One may speculate that this incomplete glycosylation of PrP^{Sc} could be specific for TSE diseases and may even favour their transmissibility or development. This hypothesis is consistent with studies in a cell-free conversion assay [39] and in neuroblastoma cells [40,41].

We thank W. Luke and J. Derer for preliminary experiments and D. Motzkus for carefully correcting the manuscript. We also thank W. Schulz-Schaeffer. This work was supported in part by a European Community BIOMED grant (BMH4-CT98-6040).

REFERENCES

- Prusiner, S. B. (1998) Prions. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13363–13383
- McKinley, M. P., Taraboulos, A., Kenaga, L., Serban, D., Stieber, A., DeArmond, S. J., Prusiner, S. B. and Gonas, N. (1991) Ultrastructural localization of scrapie prion proteins in cytoplasmic vesicles of infected cultured cells. *Lab. Invest.* **65**, 622–630
- Wadsworth, J. D., Jackson, G. S., Hill, A. F. and Collinge, J. (1999a) Molecular biology of prion propagation. *Curr. Opin. Genet. Dev.* **9**, 338–345
- MacGregor, I. (2001) Prion protein and developments in its detection. *Transfus. Med.* **11**, 3–14
- Martins, V. R., Linden, R., Prado, M. A., Walz, R., Sakamoto, A. C., Izquierdo, I. and Brentani, R. R. (2002) Cellular prion protein: on the road for functions. *FEBS Lett.* **512**, 25–28
- Ermonval, M., Mouillet-Richard, S., Codogno, P., Kellermann, O. and Botti, J. (2003) Evolving views in prion glycosylation: functional and pathological implications. *Biochimie* **85**, 33–45
- Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S. J., Smart, E. J., Anderson, R. G., Taraboulos, A. and Prusiner, S. B. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14945–14949
- Naslavsky, N., Stein, R., Yanai, A., Friedlander, G. and Taraboulos, A. (1997) Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. *J. Biol. Chem.* **272**, 6324–6331
- Shyng, S. L., Huber, M. T. and Harris, D. A. (1993) A prion protein cycles between the cell surface and an endocytic compartment in cultured neuroblastoma cells. *J. Biol. Chem.* **268**, 15922–15928
- Rudd, P. M., Endo, T., Colominas, C., Groth, D., Wheeler, S. F., Harvey, D. J., Wormald, M. R., Serban, H., Prusiner, S. B., Kobata, A. and Dwek, R. A. (1999) Glycosylation differences between the normal and pathogenic prion protein isoforms. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13044–13049
- Rudd, P. M., Wormald, M. R., Wing, D. R., Prusiner, S. B. and Dwek, R. A. (2001) Prion glycoprotein: structure, dynamics, and roles for the sugars. *Biochemistry* **40**, 3759–3766
- Nielsen, D., Gyllberg, H., Ostlund, P., Bergman, T. and Bedecs, K. (2004) Increased levels of insulin and insulin-like growth factor-1 hybrid receptors and decreased glycosylation of the insulin receptor alpha- and beta-subunits in scrapie-infected neuroblastoma N2a cells. *Biochem. J.* **380**, 571–579
- Parchi, P., Capellari, S., Chen, S. G., Petersen, R. B., Gambetti, P., Kopp, N., Brown, P., Kitamoto, T., Tateishi, J., Giese, A. and Kretzschmar, H. (1997) Typing prion isoforms. *Nature (London)* **386**, 232–234
- Wadsworth, J. D., Hill, A. F., Joiner, S., Jackson, G. S., Clarke, A. R. and Collinge, J. (1999) Strain-specific prion-protein conformation determined by metal ions. *Nat. Cell Biol.* **1**, 55–59
- Vorberg, I. and Priola, S. A. (2002) Molecular basis of scrapie strain glycoform variation. *J. Biol. Chem.* **277**, 36775–36781
- Notari, S., Capellari, A., Giese, I., Westner, A., Baruzzi, B., Ghetti, B., Gambetti, P., Kretzschmar, H. A. and Parchi, P. (2004) Effects of different experimental conditions on the PrP^{Sc} core generated by protease digestion: implications for strain typing and molecular classification of CJD. *J. Biol. Chem.* **279**, 16797–16804
- Kramer, M. L., Kratzin, H. D., Schmidt, B., Romer, A., Windl, O., Liemann, S., Hornemann, S. and Kretzschmar, H. (2001) Prion protein binds copper within the physiological concentration range. *J. Biol. Chem.* **276**, 16711–16719
- Garnett, A. P. and Viles, J. H. (2003) Copper binding to the octarepeats of the prion protein. Affinity, specificity, folding, and cooperativity: insights from circular dichroism. *J. Biol. Chem.* **278**, 6795–6802
- Pergami, P., Jaffe, H. and Safar, J. (1996) Semipreparative chromatographic method to purify the normal cellular isoform of the prion protein in nondenatured form. *Anal. Biochem.* **236**, 63–73
- Yin, S. M., Zheng, Y. and Tien, P. (2003) On-column purification and refolding of recombinant bovine prion protein: using its octarepeat sequences as a natural affinity tag. *Protein Expression Purif.* **32**, 104–109
- Shaked, Y., Rosenmann, H., Hijazi, N., Halimi, M. and Gabizon, R. (2001) Copper binding to the PrP isoforms: a putative marker of their conformation and function. *J. Virol.* **75**, 7872–7874
- Pergami, P., Bramanti, E. and Ascoli, G. A. (1999) Structural dependence of the cellular isoform of prion protein on solvent: spectroscopic characterization of an intermediate conformation. *Biochem. Biophys. Res. Commun.* **264**, 972–978
- Pan, K. M., Stahl, N. and Prusiner, S. B. (1992) Purification and properties of the cellular prion protein from Syrian hamster brain. *Protein Sci.* **1**, 1343–1352
- Jacobs, E., Fuchte, K. and Bredt, W. (1988) Isolation of the adherence protein of *Mycoplasma pneumoniae* by fractionated solubilization and size exclusion chromatography. *Biol. Chem. Hoppe Seyler* **369**, 1295–1299
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Krasemann, S., Groschup, M., Hunsmann, G. and Bodemer, W. (1996) Induction of antibodies against human prion proteins (PrP) by DNA-mediated immunization of PrP0/0 mice. *J. Immunol. Methods* **199**, 109–118

- 28 Brown, D. R. (2001) Copper and prion disease. *Brain Res. Bull.* **55**, 165–173
- 29 Lee, D. C., Stenland, C. J., Hartwell, R. C., Ford, E. K., Cai, K., Miller, J. L., Gilligan, K. J., Rubenstein, R., Fournel, M. and Petteway, Jr, S. R. (2000) Monitoring plasma processing steps with a sensitive Western blot assay for the detection of the prion protein. *J. Virol. Methods* **84**, 77–89
- 30 Quaglio, E., Chiesa, R. and Harris, D. A. (2001) Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform. *J. Biol. Chem.* **276**, 11432–11438
- 31 Thackray, A. M., Madec, J. Y., Wong, E., Morgan-Warren, R., Brown, D. R., Baron, T. and Bujdoso, R. (2003) Detection of bovine spongiform encephalopathy, ovine scrapie prion-related protein (PrP^{Sc}) and normal PrP^C by monoclonal antibodies raised to copper-refolded prion protein. *Biochem. J.* **370**, 81–90
- 32 Wong, B. S., Li, R., Sassoon, J., Kang, S. C., Liu, T., Pan, T., Greenspan, N. S., Wisniewski, T., Brown, D. R. and Sy, M. S. (2003) Mapping the antigenicity of copper-treated cellular prion protein with the scrapie isoform. *Cell. Mol. Life Sci.* **60**, 1224–1234
- 33 Kuczius, T., Buschmann, A., Zhang, W., Karch, H., Becker, K., Peters, G. and Groschup, M. H. (2004) Cellular prion protein acquires resistance to proteolytic degradation following copper ion binding. *Biol. Chem.* **385**, 739–747
- 34 Callahan, M. A., Xiong, L. and Caughey, B. (2001) Reversibility of scrapie-associated prion protein aggregation. *J. Biol. Chem.* **276**, 28022–28028
- 35 Neuendorf, E., Weber, A., Saalmüller, A., Schatzl, H., Reifenberg, K., Pfaff, E. and Groschup, M. H. (2004) Glycosylation deficiency at either one of the two glycan attachment sites of cellular prion protein preserves susceptibility to bovine spongiform encephalopathy and scrapie infections. *J. Biol. Chem.* **279**, 53306–53316
- 36 Monnet, C., Marthiens, V., Enslin, H., Frobert, Y., Sobel, A. and Mege, R. M. (2003) Heterogeneity and regulation of cellular prion protein glycoforms in neuronal cell lines. *Eur. J. Neurosci.* **18**, 542–548
- 37 Grathwohl, K. U., Horiuchi, M., Ishiguro, N. and Shinagawa, M. (1996) Improvement of PrP^{Sc}-detection in mouse spleen early at the preclinical stage of scrapie with collagenase-completed tissue homogenization and Sarkosyl-NaCl extraction of PrP^{Sc}. *Arch. Virol.* **141**, 1863–1874
- 38 Nieznanski, K., Rutkowski, M., Dominik, M. and Stepkowski, D. (2005) Proteolytic processing and glycosylation influence formation of pig prion protein complexes. *Biochem. J.* **387**, 93–100
- 39 Priola, S. A. and Lawson, V. A. (2001) Glycosylation influences cross-species formation of protease-resistant prion protein. *EMBO J.* **20**, 6692–6699
- 40 Korth, C., Kaneko, K. and Prusiner, S. B. (2000) Expression of unglycosylated mutated prion protein facilitates PrP^{Sc} formation in neuroblastoma cells infected with different prion strains. *J. Gen. Virol.* **81**, 2555–2563
- 41 Winklhofer, K. F., Heller, U., Reintjes, A. and Tatzelt, J. (2003) Inhibition of complex glycosylation increases the formation of PrP^{Sc}. *Traffic* **4**, 313–322

Received 30 July 2004/19 January 2005; accepted 19 January 2005

Published as BJ Immediate Publication 19 January 2005, DOI 10.1042/BJ20041291