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Journal of Chromatography B, 818 (2005) 75–82
JOURNAL OF CHROMATOGRAPHY B
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Copper binding to prion octarepeat peptides, a combined metal chelate affinity and immunochemical approaches

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Received 14 June 2004; accepted 1 October 2004
Available online 13 November 2004

Abstract

Based on the hypothetical proposal of Sulkowski [E. Sulkowski, FEBS Lett. 307 (2) (1992) 129] for the implication of transition metal ions in the structural changes/oligomerisation of normal cellular prion protein (PrPc) resulting in the pathological isoform (PrPsc), we focused our study on the octarepat domain of this protein which has been supposed to be the metal binding site. We have studied the copper binding to synthetic prion octarepeat peptides (PHGGGWGQ)*n* (*n* = 1, 3, 6) using metal chelate and size-exclusion modes of chromatographies. This copper binding induces oligomerisation resulting in multiple aggregates. Moreover, heterogeneity of metal bound octarepeat oligomers by ESI-MS has been demonstrated. In addition, anti prion antibodies specific to the octarepeat region were used to discriminate between metal free and copper, nickel and zinc bound hexamer octarepeat peptide. Differential recognition of Cu(II) and Zn(II) bound complexes has been observed which signify differences in exposed epitopes of aggregated peptides.

Keywords: Prion; Octarepeat peptides; Copper; Metal chelate affinity; Mass spectrometry; Anti-prion peptide antibody

1. Introduction

According to the Prusiner's theory, the term "Prions" is used to describe the causative agent responsible of Transmissible Spongiform Encephalopathies (TSEs) including Creutzfeldt-Jakob disease in human as well as scrapie and bovine spongiform encephalopathy (BSE) in animals. It is composed essentially, if not exclusively, of a protein abnormally folded (PrPsc). PrPsc derives from the normal form (PrPc) through post-translational modifications which induce a conformational change and confer on the PrPsc a partial resistance to degradation by proteases as well as a marked insolubility in the presence of detergents. Conformational change in the host cellular prion protein (PrPc) has been reported to be the key molecular event associated with the illnesses [2]. The pathological "scrapie" PrP conformation (PrPsc) is characterized by increased β-sheet, decreased α-helical contents, higher tendency to self-aggregation, insolubility and protease resistance [3,4].

The primary sequence of normal PrPc contains four (or five in bovine PrP) tandem octarepeats (PHGGGWGQ) in the N-terminal region (residues 61–91). These octarepeats were found to be the major Cu(II) binding site, as investigated by chromatography methods, Raman and adsorption spectroscopy [5,6].

Moreover, in some situations, it has been observed that there is a positive correlation between the pres-

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doi:10.1016/j.jchromb.2004.10.011

ence of additional copies (5–10) of these octarepeats and the occurrence of Creutzfeld-Jacob disease [7].

As early as 1992, Sulkowski [1] proposed the hypothesis of metal-induced oligomerisation of octarepeat peptides's implication in the formation of PrPsc. Recent reports have shown that metal ions, such as Cu(II) could be implicated in the conversion of PrPc to PrPsc. Stockel et al. [8] showed by means of circular dichroism (CD) and tryptophan (Trp) fluorescence spectroscopies that Cu(II)-induced changes to the tertiary structure of recombinant Syrian hamster prion protein (ShaPrP (29–231)) and also promoted the conformational shift from α-helical to a β-sheet structure. More recently, Lehmann [9] reviewed the relationship between the prion protein and metal ions, which seemed to play a critical role in the physiopathology of prion diseases.

Other very sensitive methods for studying conformational changes are the immunological methods, which have been already used for the diagnosis of prion pathologies [10]. Williamson et al. [11] used 19 PrP-specific recombinant monoclonal antibodies from phage display libraries and they could distinguish the epitopes present on PrPc but absent or non-accessible on PrPsc. Moreover, monoclonal antibodies were reported to be used to determine the sensitivity of abnormal prion protein (PrPres) to proteases, and thus, they could be new tools to facilitate the progress in qualitative and quantitative studies of prion proteins [12]. Recently, Leclerck et al. [13] have shown using a panel of recombinant antibodies reactive with different parts of normal ShaPrP (23–231) that structural rearrangements occur spontaneously within 6 days of immobilization of PrP on a sensor chip. Using surface plasmon resonance (SPR) they showed the significant modifications in the central position (residues 90–120) and also loss of antibody binding to N-terminus of alpha helix and to the octarepeat region of the immobilized PrP. Thus, the antibodies have high potential for studying conformational changes of PrP, which is a promising approach for understanding the prion pathology.

In our study, based on the hypothesis of Sulkowski [1] that metal binding to PrP could induce conformational changes/oligomerisation by the coordination of transition metal ions to the octarepeats, we investigated the copper binding to monomer, trimer and hexamer octarepeat peptides by size-exclusion and metal chelate affinity chromatographies and correlated with the binding of anti-octo-repeat antibodies. Appearance of oligomeric forms and heterogeneity of the mixture containing copper were detected by size-exclusion chromatography and ESI-MS analysis. Anti prion antibodies specific to the octarepeat region were applied to study metal free and metal bound hexamer octarepeat peptide. Differential binding of the free and the Cu(II) bound octarepeat hexamers to the antibodies suggested conformational changes resulting in modified epitope exposure.

2. Materials and methods

2.1. Materials

Octarepeat trimer and hexamer peptides were custom-synthesized by Genosphere Biotechnology company, Paris, France.

Monoclonal anti-PrP antibodies were raised in KO mice ($PrP^{0/0}$) immunized either with human recombinant protein (4F2 and 3B5) [14–16] or denatured Scrapie Associated Fibrils (SAFs) from scrapie infected hamster brain (SAF-X series) [12]. They all bind the octarepeat region of PrP as demonstrated by their capacity to bind a synthetic peptide of the following sequence (GGWGQPHGGGWGQG, Grasi et al., unpublished results).

Prepacked HPLC gel-filtration column Superdex 75, Sephadex G75 and HiTrap chelating column were purchased from Amersham Biosciences, Sweden. All other reagents were of analytical grade and ultrapure water was used throughout.

2.2. Chromatography procedures

2.2.1. Immobilized metal-ion affinity chromatography (IMAC)

IMAC was performed on HiTrap chelating column with bed volume of 5 ml (Amersham Biosciences, Sweden) at a flow rate of 1 ml/min. The column was charged with 0.05 M $CuSO_2$ in sodium acetate buffer 20 mM pH 4, NaCl 1 M. Unbound metal ions were removed by H_2O and sodium acetate buffer 20 mM pH 4, NaCl 1 M washes. The column was then equilibrated with HEPES 20 mM, pH 7, NaCl 1 M. 0.1 mg of peptide was injected and a pH decreasing step gradient from pH 7 to 4 was applied. After each run, the gel was regenerated by flushing with five column volumes of 0.05 M EDTA and thoroughly washed with water before a new metal load was applied.

2.2.2. HPLC and low pressure liquid size-exclusion chromatography

Chromatographies were performed at flow of 1 ml/min on Superdex 75 (HPLC) and 0.1 ml/min on Sephadex G 75. Amount of 0.1 mg or 1 mg of synthetic octarepeats with and without metal ion preincubation, were applied on HPLC or low pressure gel-filtration columns, respectively, in Tris 0.05 M, pH 7, NaCl 1 M. Elution pattern was followed by 270 nm absorbance.

2.2.3. Electrospray ionisation mass spectrometry (ESI-MS)

Octarepeat hexamer peptide and metal (Cu(II), Ni(II) and Zn(II))–hexamer complexes were analysed by a single quadropole mass spectrophotometer (Finnigan SSQ 710, San Jose, CA, USA) with 110 °C drying gas electrospray ionisation source (Analytica source of Brandford, CT, USA) under 2.5–3.5 kV. One volume of octarepeat hexamer sample was diluted in one volume of methanol–acetic acid (100:1, v/v).

In the case of the non-covalent metal–hexamer complexes, a sheath liquid of methanol was used when aqueous solution (1.5 mM Tris, pH 7) were injected in the mass spectrometer. Drying gas temperature was 70 °C.

The flow rate was set at 1 μl/min by a syringe pump (Harvard Apparatus, South Natick, USA). Multiply charged ions from horse myoglobin were used to calibrate the *m*/*z* scale of the mass spectrometer.

2.3. ELISA

Microtiter plates coated with the eight different anti-octarepeat antibodies (4F2, 3B5, SAF-15, SAF-32, SAF-33, SAF-34, SAF-35, SAF-37) as well as the corresponding biotin-labelled conjugates were prepared as previously described [17]. Final detection was obtained using acetylcholinesterase streptavidine conjugates [17].

Hundred microliters (10 ng/ml) of hexamer with and without metal ion preincubation in 2.5 mM Tris, pH 7.4, BSA 1 mg/ml, NaN_3 0.01% were reacted for 1 h 30 at room temperature (RT) on the antibody coated solid phase. After a first washing biotin-labelled antibodies (SAF32, SAF 34 or 3B5) were incubated for 4 h 30 at RT. After second washing, streptavidine acetylcholinesterase conjugate was added and reacted for 2 h RT and solid-phase bound enzyme activity was measured as previously described [17].

3. Results and discussion

3.1. Affinity of prion octarepeat peptides for chelated Cu(II)

Each PrP octarepeat peptides contains one histidine residue and also one tryptophan which are amino acids known to be involved in the coordination of transition metal ions, e.g. Cu(II), Ni(II), Zn(II), Co(II). Metal ions, and especially Cu(II) were supposed to play an important role in prion pathologies. Many reports have shown that the cellular prion protein binds Cu(II) in vivo and that the main binding site is located in the N-terminal region where four octarepeat peptides $(PHGGGWGQ)_4$ were identified [6,9,18]. Metal binding could induce conformational changes/oligomerisation of octarepeat region and it could be, as postulated by Sulkowski [1], the nucleation site for spontaneous conversion of PrPc to PrPsc. Thus, to explore the binding of prion peptides to Cu(II) we first used the immobilized metal-ion affinity chromatography (IMAC) [19]. IMAC is not only one of the most popular purification method, but it is widely used for protein structure studies [20,21] and it may give an information on the possible conformational changes of prion peptides containing different copies of octarepeats bound to Cu(II).

Prion octarepeat peptide $(PHGGGWGQ)_1$ has already been shown to bind chelated Cu(II) [5]. Based on the finding that in many cases of CJD genetic forms [22] additional copies of prion octarepeats were accumulated when the prion pathologies occurred we were interested in the interaction between chelated Cu(II) and synthetic peptides with increasing number: one, three and six octarepeats, named monomer, trimer and hexamer. One prompted hypothesis could be the differential recognition of Cu(II) ions by different peptides and detection of structural changes/oligomerisation due to the reorganization of peptide structure.

Table 1
IMAC (IDA-Cu(II)) retention of different octarepeat oligomers

Peptide	Migration time (min)
Acetylated monomer	14.65
Monomer	14.72
Acetylated trimer	14.96
Trimer	15.13
Acetylated hexamer	15.56

Chromatography conditions: matrix, HiTrap chelating column (5 ml) charged with Cu(II); flow rate of 1 ml/min; injection sample of 0.1 mg. Elution with decreasing pH gradient (see Section 2 for details).

We demonstrated that the strength of binding to Cu(II) chelate was monomer $(PHGGGWGQ)_1$ < trimer $(PHGGGWGQ)_3$ < hexamer $(PHGGGWGQ)_6$ (Table 1). Consequently, His are accessible for Cu(II) chelate and when their number increases the affinity is stronger which is in accordance with the ground rules in IMAC for protein recognition established by Sulkowski [23]. Thus, IMAC could be a useful method for identifying the presence of extra copies of the octarepeat sequence possibly involved in the development of some forms of CJD. Moreover, we also studied the chromatographic behaviour on IDA-Cu(II) of acetylated peptides in order to prevent the contribution to the binding of the amino group [24]. Our results show that monomer and trimer without acetylation have higher affinity for chelated Cu(II) compared to the acetylated forms meaning that the amino group contributed to the binding. Consequently, acetylated peptides were used for all further studies in order to ensure the metal binding due only to the histidine residues.

However, we also explored if IMAC/metal-ion transfert (MIT) events took place, and if the octarepeat peptides scavenged metal ions when passed through the Cu(II) chelated column which can exert conformational change/oligomerisation. Mixed, IMAC/MIT events have been already observed in the case of proteins, like apocarboxypeptidase A and albumin, having higher affinity for metal ions [23,25,26]. To find out if MIT phenomenon occurred, ESI-MS analysis of fractions retained on IDA-Cu(II) were carried out (data not shown). Important heterogeneity was detected in the fractions and mass-spectra were not conclusive. This heterogeneity could be due to the binding of Cu(II) to the peptide in the fractions after retention on IDA-Cu(II) and formation of different conformation peptides/oligomers. Consequently, we further investigated prion peptide binding to free Cu(II) by size-exclusion chromatography which could allow the detection of oligomeric forms.

3.2. Binding of free Cu(II) to prion peptides

Size-exclusion chromatography, in both low pressure and HPLC modes, were carried out in order to investigate the behaviour of trimer and hexamer synthetic prion peptides with and without preincubation with Cu(II). In both modes of sieving separation at pH 7, oligomeric structures of octarepeats–Cu(II) complexes, four to five times bigger than the octarepeat peptides alone, were observed (Fig. 1). These oligomers could be attributed to Cu(II) coordination to the His residue(s) which could prompt a hypothesis that Cu(II) ions could trigger oligomerisation of PrPc and this may be implied in the spontaneous conversion of PrPc to PrPsc. Previously, it has been postulated that (His-X)n repeats may give rise to protein dimers via transition metal bridges [27]. ESI-MS analysis showed important heterogeneity in both trimer and hexamer synthetic prion peptides with and without preincubation with Cu(II) (data not shown). The heterogeneity of the trimer and the hexamer in solution at pH 7 could be due to the structural rearrangements of prion

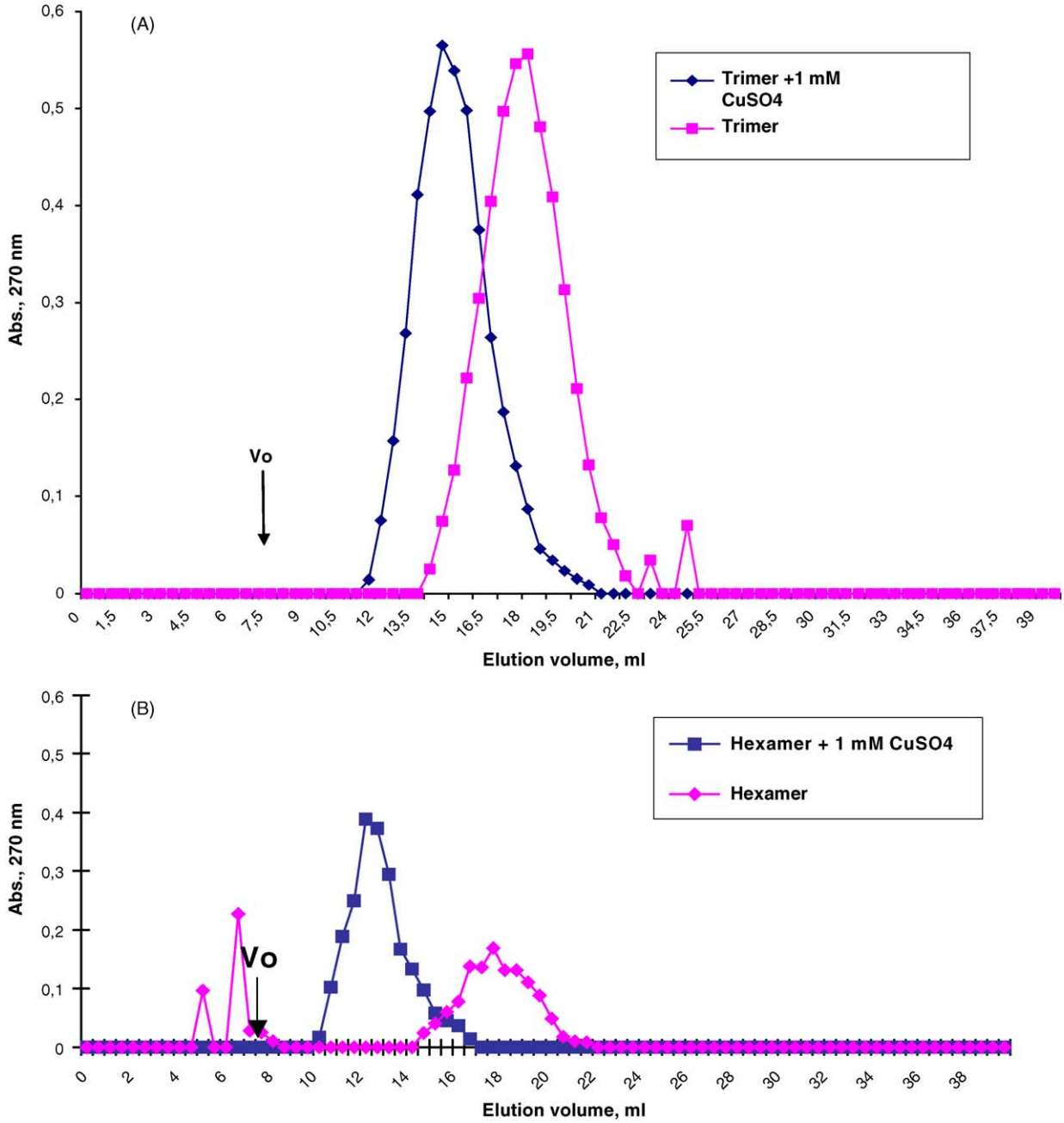

Fig. 1. Size-exclusion separation using Sephadex G 75: (A)trimer and trimer–Cu(II) complex; (B) hexamer and hexamer–Cu(II) complex. *Chromatography conditions*: matrix, Sephadex G 75; flow rate, 0.1 ml/min; injection sample, 1 mg of synthetic octarepeats with and without metal ion preincubation; buffer, Tris 0.05M, pH 7, NaCl 1 M. Vo: blue dextran (2103000 relative molecular mass).

peptides. This observation is supported by the findings of Leclerck et al. [13] that under physiological solvent conditions, monomeric PrPc is an intrinsically unstable molecule prone to conformational rearrangement. Thus, the propensity of PrPc regions to spontaneously undergo conformational changes may have important implications for investigation of prion pathology and the implication of metal ions could induce/accelerate the formation of different conformational structures.

Since the presence of extra copies of the octarepeat sequence is associated with CJD genetic forms, we further focused our study on the hexa octarepeat (hexamer) prion peptide and its metal complexes.

3.3. Study of hexamer–metal complexes by electrospray ionisation mass spectrometry (ESI-MS)

ESI-MS has been shown to be a suitable method for monitoring metal complex formation [28]. Recently, ESI-MS has been employed in order to measure the binding of Cu(II) ions to synthetic peptides, especially the octarepeat domain conserved be-

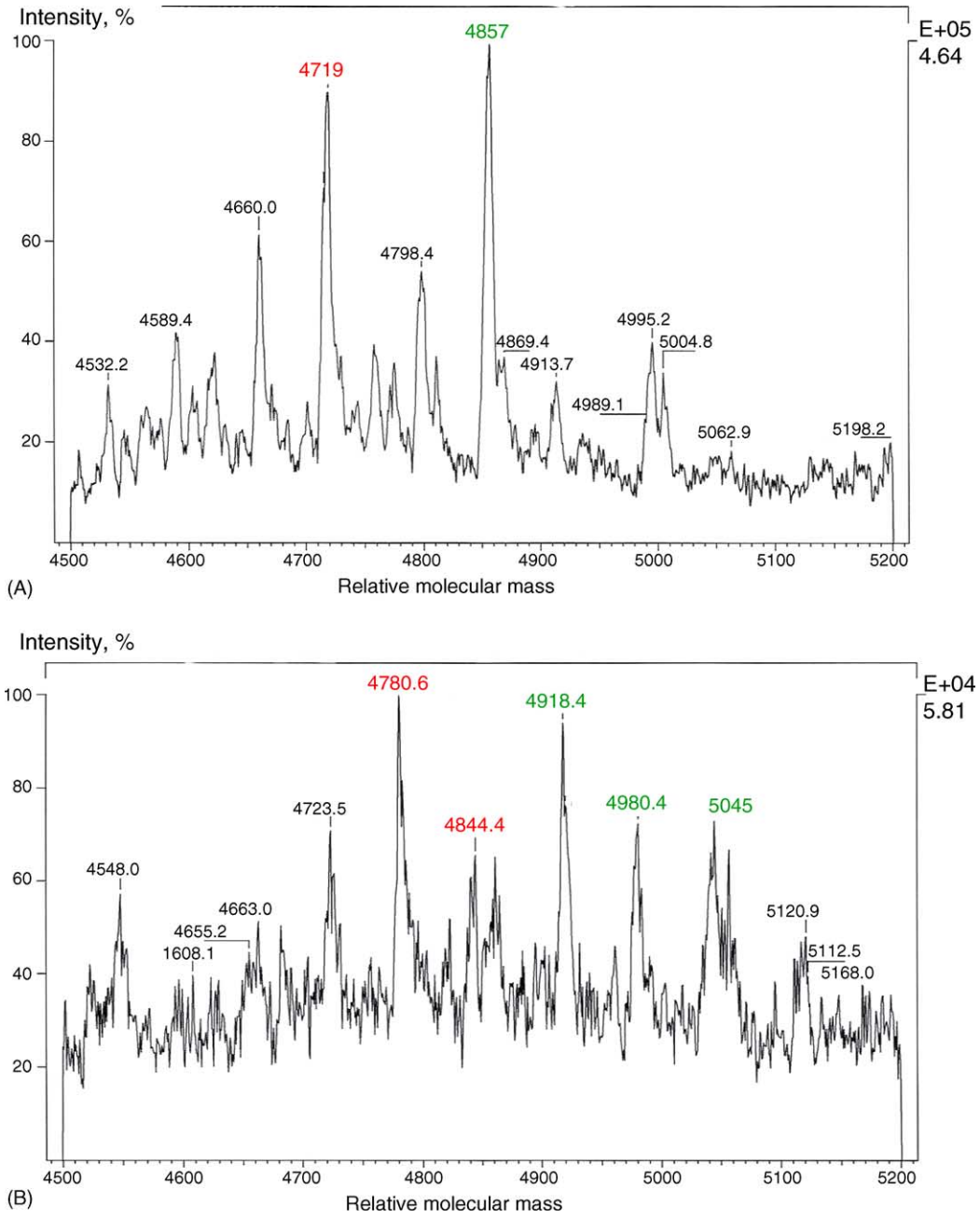

Fig. 2. ESI-MS spectra of (A) hexamer; (B) hexamer–Cu(II) complex. The absence of Cu(II) ions in the sample of free hexamer was confirmed by ESI-MS analysis (A).

tween various species, of the mature prion protein PrPc [29].

In our experiments, two populations one of 4719 relative molecular mass, corresponding to the theoretical mass and another species of 4857 relative molecular mass were detected in the commercial preparation of hexamer (Fig. 2A). ESI-MS spectra of Cu(II)–hexamer complex clearly show the binding of one, two and three Cu(II) ions at pH 7 (Fig. 2B and Table 2). Whittal et al. [29] studied the stoichiometries of prion peptides metal complexes and they found that the binding is pH dependent: a peptide containing four octarepeats chelates two Cu(II) ions at pH 6 but four Cu(II) ions at pH 7.4. Recently, Burns et al. [30] studied the molecular features of the Cu(II) binding sites in the octarepeat domain of PrPc and they established the first atomic resolution view of the Cu(II) binding site (HGGGW) within an octarepeat.

However, the ESI-MS spectra of Ni(II) and Zn(II)–hexamer complexes showed the binding of one metal ion to the peptide (Table 2). The weaker affinity of hexamer octarepeat prion peptide for Ni(II) and Zn(II) in solution at pH 7 compared to Cu(II) coincides with the requirements for specific structure conformation of His residues; at least two His should be fully exposed for binding to Ni(II) and a cluster of His (His-$X_{2,3}$-His) is required for binding to Zn(II). Our ESI-MS spectra confirmed that at pH 7 these three His residues are exposed to the Cu(II) but that no His cluster structure exist. Moreover, the ESI-MS analysis was carried out in the appropriate conditions for monitoring non-covalent complexes. However, the ESI-MS spectra gave us only information on the stochiometry of the binding of Cu(II) to the hexamer. Although we could clearly identify the appearance of oligomeric forms by size-exclusion chromatography, the detection of these structures by ESI-MS was not possible probably due to their important heterogeneity.

These findings showed that the Cu(II) is the major transition metal involved in the oligomerisation of prion octarepeat peptides. In order to check for any conformational modifications induced by Cu(II) binding, we undertook immunological approaches which are highly sensitive methods for studying conformational changes of molecules.

Table 2
Metal binding to prion hexa octarepeat peptide determined by ESI-MS

Metal	Molecular mass of Me–hexamer complex, relative molecular mass		Number of bound Me ions
Cu	I	4780.6	1
		4844.4	2
	II	4918.4	1
		4980.4	2
		5045	3
Ni	I	4776.6	1
	II	4916	1
Zn	I	4782.6	1 NS
	II	4920.1	1 NS

NS, not significant; I, Me(II) binding to the first species of hexamer of 4719 relative molecular mass; II, Me(II) binding to the second species of hexamer of 4857 relative molecular mass.

3.4. Study of hexamer–metal complexes by immunological methods

Anti-octarepeat antibodies binding to metals (Cu(II), Ni(II) and Zn(II))–hexamer complexes with the reference to free hexamer was investigated. Eight antibodies which recognise the octarepeat region of PrP were used in a conventional two-site immunometric assay (sandwich immunoassay) based on the use of two anti-octarepeat monoclonal antibodies (see methods). The results showed that in some cases of Cu(II) bound hexamer, when SAF15 and SAF37 solid phase antibodies and SAF 32, SAF 34 and 3B5 secondary antibodies were used, an increase of immunological response was observed (Fig. 3, Table 3). Statistically, in the 58% of immunoassays, the increase in the immunological response in the case of hexamer–Cu(II) metal complex was observed, whereas, in almost all the cases (80% of immunoassays) of Zn(II) bound hexamer complexes, a decrease of immunological response was detected.

In contrast, Ni(II) bound to hexamer did not exert a significant effect on the antibody binding—58% of immunoassays gave binding response similar to those of the hexamer without preincubation with metals (Table 3).

These variations in the immunological responses in the case of different metal (Cu(II), Ni(II) and Zn(II))–hexamer complexes may suggest differential epitope exposure and probably conformational changes of metal complexes. Moreover, Cu(II) binding site is probably different from those for Ni(II) and for Zn(II) resulting in different conformation, and thus, in differential recognition by anti-octarepeat antibodies. These findings show that metal ions may induce conformational changes of prion peptides, which in turn may result in the pathology of prions. Moreover, the epitopes of hexamer–metal complexes recognized by the anti-octarepeat monoclonal antibodies seem to be differently exposed depending on the bound metal. This is the first report on the differential recognition of prion peptides–metal (Cu(II), Ni(II) and Zn(II)) complexes by monoclonal antibodies and this could be of help in understanding of the prion diseases.

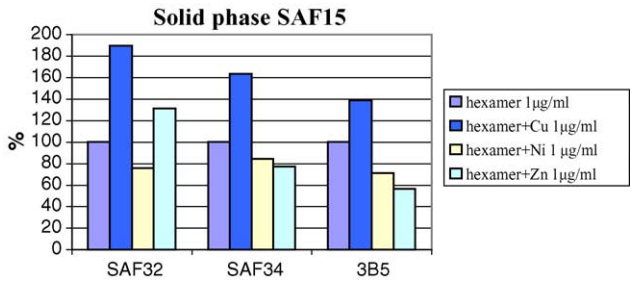

Fig. 3. Metal binding to hexamer as investigated by ELISA two site immunometric assay.

Table 3
Binding response of metal hexa octarepeat peptide complexes

Solid phase antibody	Secondary antibody	Binding response (%)			
		Hexamer	Hexamer–Cu(II)	Hexamer–Ni(II)	Hexamer–Zn(II)
SAF15	SAF32	100	189.5	75.7	131.5
	SAF34	100	163.4	84.7	77.5
	3B5	100	139	71.5	56.5
SAF37	SAF32	100	116.2	116	65.2
	SAF34	100	145.9	120	83.5
	3B5	100	144.9	135.4	96.6
3B5	SAF32	100	119.4	102.6	67.4
	SAF34	100	121.3	97.9	82.5
	3B5	100	92.5	85.3	101.6
SAF35	SAF32	100	78.3	88.1	50.6
	SAF34	100	106.5	101.3	65.8
	3B5	100	110.6	111.3	60.3
4F2	SAF32	100	94.2	103	53.8
	SAF34	100	109.1	98	65.9
	3B5	100	105.8	86	59.6
SAF34	SAF32	100	101	97.8	96.2
	SAF34	100	104.8	97.6	92.8
	3B5	100	91.9	96.6	65.8
SAF32	SAF32	100	90.4	100.2	62.8
	SAF34	100	102.1	101.2	69.3
	3B5	100	97.7	105.1	48.7
SAF33	SAF32	100	88	90.7	63.5
	SAF34	100	94.1	93.3	70.3
	3B5	100	89.5	102	55.2

Acknowledgements

The authors thank Dr. E. Sulkowski from Roswell Park Cancer Institute, Buffalo, NY, USA for his suggestions, comments and fruitful discussions. The authors also thank Mr. H. Adenier from Université de Technologie de Compiègne (UTC), France for his technical assistance in mass spectrometry studies.

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