Diss ETH Nr. 14053

Copper binding and thermodynamic stability of the recombinant murine prion protein PrP(23-231) and its variants

A dissertation submitted to the

Swiss Federal Institute of Technology Zürich

for the degree of

Doctor of Natural Sciences

Zürich, 2. 10. 2001

Prof. Dr. R. Glockshuber
Institut für Molekularbiologie
und Biophysik
ETH Hönggerberg HPK
CH-8093 Zürich

presented by

Grazia Maria Cereghetti
Dipl. Natw. ETHZ
born on Juny 17, 1974
Switzerland

accepted on the recommandation of:
Prof. Dr. Rudi Glockshuber, examiner
Prof. Dr. Arthur Schweiger, co-examiner
2001

Index

Abstract

Transmissible spongiform encephalopathies (TSEs) in mammals and humans are progressive neurodegenerative diseases supposed to be caused by an infectious agent, PrP^{Sc}, the prion, consisting exclusively of a cellular host protein, PrP^{C}, in its changed conformation. The innovative view of infectivity transmitted by alteration of the overall fold of a protein, without nucleic acid components, is known as "protein-only" hypothesis.

PrP^{C} is a cell-surface, GPI anchored protein which is strongly expressed in cells of the central nervous system; it consists of 209 amino acids and is covalently modified by the addition of several high mannose glycans. The structures of PrP^{C}s from mouse, hamster, human and bovine are known and show a similar fold, characterised by an N-terminal, disordered tail, residues 23-120, and a C-terminal domain, residues 121-231, consisting of three α-helices and a short antiparallel β-sheet. The single disulfide bridge lies in the buried core of the structured domain and is solvent-inaccessible. The structure of PrP^{Sc}, the abnormal, oligomeric form of PrP is not known, but Fourier transform infrared spectroscopy shows an increased β-sheet content at the expenses of α-helical regions compared to PrP^{C}. Moreover, proteinase K digestion of PrP^{Sc} generates protease resistant subunits denominated PrP27-30 which comprise residues 90-231 of the full-length protein, suggesting that the region 90-120 is no longer unstructured in PrP^{Sc}. The mechanism underlying the conformational change from PrP^{C} to PrP^{Sc} is until now unknown.

In this work, the recent question about copper(II) binding to the prion protein is approached using electron paramagnetic resonance (EPR) spectroscopy on recombinant PrP(23-231) and PrP(121-231) samples. It was found that also the C-terminal, structured domain of the protein, and not only the histidine rich N-terminal region, is able to bind up to four copper(II) ions. Six additional Cu(II) ions are found to bind to the N-terminal tail, in accordance with previous results. Copper(II) binding was shown to be pH dependent. The finding that the structured region of PrP binds Cu(II) opens new questions about a possible involvement of copper(II) ions in PrP^{Sc} formation and infectivity.

To investigate the role of Cu(II) binding on the structure of the N-terminal region of PrP(23-231), limited proteolysis of the protein was performed with several proteases, in the absence and presence of copper(II), and the digestion patterns compared to identify the possible formation of structures in the disordered domain that could impede the proteolytic attack. The experiments show that copper(II) binding does not induce increased proteolytic resistance due to tertiary structure formation within the segment 23-120.

Furthermore, the conditions favouring the formation of the previously identified, scrapie-like folding intermediate found at acidic pH in urea-induced unfolding transitions for PrP(121-231) were investigated. It was shown that intermediate formation at pH 4.0 also occurs at the level of full-length PrP(23-231). In addition, formation of the intermediate is strongly dependent on protein concentration and is only observed at concentrations above 15 μM, This suggests an oligomeric, most likely dimeric state of the folding intermediate. Ionic strength was found to be another critical factor. The intermediate was not formed at ionic strengths below 50 mM, but well populated at physiological ionic strength (~150 mM). The finding that formation of this PrP^{Sc}-like intermediate requires oligomerization is a good argument supporting the nucleation-condensation model proposed for PrP^{Sc} formation.

Finally, the thermodynamic stability of PrP(23-231) and PrP(121-231) wild-type and some variants thereof was analysed at pH 4.0 and 7.0 under the conditions that favour formation of the scrapie-like unfolding intermediate described above. The selected variants bear point mutations that cause spontaneous formation of infectivity in inherited human TSEs. It should be clarified whether these variants are thermodynamically less stable than the wild-type, or perhaps more prone to the formation of the folding intermediate at acidic pH. We found that no general pathway of destabilisation can be found for the variants investigated with respect to the wild-type, nor a particular propensity to the formation of the scrapie-like intermediate during folding. The increased tendency of the variants to form PrP^{Sc} spontaneously *in vivo* therefore must have another origin, perhaps the better interaction with other factors involved in the conformational change leading to the infectious isoform, faster kinetics of PrP^{Sc} formation or higher stability of PrP^{Sc}.

Sommario

Le encefalopatie spongiformi trasmissibili (TSE) nei mammiferi e negli uomini sono malattie neurodegenerative progressive e fatali causate presumibilmente da un agente infettivo, PrP^{Sc}, il prione, consistente esclusivamente di una proteina cellulare, PrP^{C}, in una conformazione modificata. La visione innovativa della possibilità di trasmissione di una malattia attraverso la conformazione alterata di una singola proteina, senza la collaborazione di altre entità quali gli acidi nucleici, è conosciuta come "protein-only hypothesis" (ipotesi di solo proteina).

PrP^{C} è una proteina espressa principalmente in cellule del sistema nervoso centrale e situata sulla superficie cellulare, a cui è ancorata per mezzo di una unità di GPI; è composta di 209 aminoacidi ed è modificata covalentemente dall'aggiunta di alcuni residui zuccherini. Sono finora note le strutture delle PrP^{C} del topo, del criceto, dell'uomo e della mucca, e tutte presentano una conformazione simile, caratterizzata da una coda amoniterminale disordinata, comprendente gli aminoacidi 23-120, e un dominio carbossiterminale (residui 121-231) consistente di tre alfa-eliche e un corto foglietto beta antiparallelo. L'unico ponte disulfurico giace in una tasca nascosta del dominio strutturato ed è inaccessibile al solvente. La struttura della PrP^{Sc}, la forma oligomerica anormale della PrP^{C}, non è nota, ma dati di spettroscopia infrarossa con trasformata di Fourier evidenziano un accresciuto contenuto di foglietti beta alle spese di parti alfa-elicali rispetto alla struttura della PrP^{C}. Perdipiù, la digestione con la proteinase K della PrP^{Sc} genera subunità resistenti all'attacco della protease denominate PrP27-30: queste comprendono i residui 90-231 della proteina intera, suggerendo che la regione 90-120 nella PrP^{Sc} non è priva di struttura come nella PrP^{C}. Il meccanismo alla base della conversione da PrP^{C} a PrP^{Sc} non è finora noto.

In questo lavoro, abbiamo avvicinato il recente dibattito a proposito del legame dello ione di rame(II) alla proteina del prione applicando la spettroscopia paramagnetica elettronica (EPR) a campioni di PrP(23-231) e PrP(121-231) ricombinante. Abbiamo trovato che anche la parte carbossiterminale, dominio strutturato della proteina, e non solo la regione aminoterminale ricca di istidine, è in grado di legare fino a quattro ioni di rame(II). Sei ioni Cu(II) aggiuntivi si legano poi alla parte aminoterminale, in accordo con

i risultati precedenti. Abbiamo inoltre mostrato che il modo di legame del rame(II) è dipende dal pH. La scoperta che la parte strutturata della PrP lega Cu(II) apre un nuovo dibattito a proposito di un possibile coinvolgimento degli ioni di rame(II) nella formazione della PrP^{Sc} e nell'infettività.

Per indagare il ruolo del legame di Cu(II) nella struttura della parte aminoterminale di PrP(23-231), abbiamo approntato digestioni limitate della proteina con diverse proteasi in assenza e presenza di ioni di rame e abbiamo confrontato i modi di digestione per identificare un'eventuale formazione di strutture all'interno del dominio disordinato, che potrebbero ostacolare l'attacco proteolitico. Gli esperimenti mostrano che il rame(II) non induce un aumento della resistenza alla proteolisi a causa di formazione di struttura terziaria nel segmento 23-120.

In seguito sono state indagate le condizioni che favoriscono la formazione dello stato intermedio, con proprietà caratteristiche di PrP^{Sc}, trovato precedentemente a pH acido durante l'unfolding di PrP(121-231) indotto da crescenti concentrazioni di urea. Abbiamo innazitutto mostrato che lo stato intermedio viene formato anche durante l'unfolding dell'intera proteina, PrP(23-231). Inoltre, abbiamo trovato che la formazione dello stato intermedio è strettamente dipendente dalla concentrazione della proteina ed è possibile solo a concentrazioni superiori a 15 μM. Questo suggerisce che lo stato intermedio è oligomerico, molto probabilmente un dimero. La forza ionica è pure un fattore determinate in questo ambito: lo stato intermedio non è osservato a forze ioniche inferiori a ~50 mM, ma è molto popolato a una forza ionica fisiologica (~150 mM). La scoperta che la formazione di questo stato intermedio con caratteristiche della forma infettiva del prione richiede oligomerizzazione della proteina è un buon argomento a favore del modello di nuclezione-condensazione proposto per la formazione della PrP^{Sc}.

Infine, abbiamo analizzato la stabilità termodinamica della forma selvatica e di alcune forme mutanti di PrP(23-231) e PrP(121-231) a pH 4 e pH 7, nelle condizioni che favoriscono durante l'unfolding la formazione dello stato intermedio descritto sopra. Le mutanti utilizzate contengono mutazioni puntiformi che causano spontaneamente infettività nelle forme ereditarie di TSE. Lo scopo era di chiarire se queste mutanti sono da un punto di vista termodinamico meno stabili della forma selvatica, o eventualmente più predisposte alla formazione dello stato intermedio a pH acido. Abiamo trovato che

non esiste una regola universale di destabilizzazione termodinamica delle mutanti rispetto alla forma selvatica della proteina, né un'accresciuta tendenza generale alla formazione dello stato intermedio. In conclusione, dobbiamo assumere che l'osservata predisposizione delle mutanti alla formazione della PrP^{Sc} *in vivo* deve essere attribuita ad altri fattori, come per esempio a una migliore interazione con altri elementi coinvolti nel processo di cambiamento conformazionale della PrP^{C}, a un'accelerata cinetica di formazione della PrP^{Sc}, o a una accresciuta stabilità termodinamica della PrP^{Sc} stessa nelle forme mutanti.

1. INTRODUCTION

1.1. Prions

In 1982 Stanley B. Prusiner introduced the name "prion" to christen a particle that, on the basis of clinical and biochemical observations, appeared to be a totally new infectious agent, seeming to consist only of an endogenous cell surface protein (Prusiner, 1982). The word "prion" was just a free splicing from the adjectives *pro*teinaceous and *in*fectious to characterise this new pathogen. The cellular prion protein (PrP^C) was hypothesised to be able to induce transmissible spongiform encephalopathies (TSEs) in mammals by changing its conformation through an as yet not understood pathway, ending up in the formation of a "diseased" isoform, the so called "scrapie form" of the protein (PrP^{Sc}) (Griffith, 1967; Pan et al., 1993; Prusiner, 1991). PrP^{Sc}, in contrast to the soluble, monomeric PrP^C, has been found to have a high tendency to associate into insoluble oligomers shaped as amorphous aggregates inducing a fatal degeneration of the central nervous system where the aggregates accumulate (Caughey et al., 1991; Safar et al., 1993; Zlotnik and Stamp, 1961). Still before 1982 it was recognised that apparently the infectious agent was composed only of host PrP in its changed conformation, without any nucleic acid component, as claimed first by Alper in 1967 (Alper et al., 1967); the concept was subsequently reformulated by Prusiner in 1982 and presented as "protein-only" hypothesis (Prusiner, 1982). This hypothesis was at the beginning widely contested, but meanwhile is generally accepted, as underlined by the Nobel prize awarded to Prusiner in 1998 although it cannot be considered as proven (Manuelidis and Fritch, 1996; Lasmezas et al., 1997; Chesebro, 1998; Chesebro et al., 1993).

1.2. Prion diseases

The diseases found to be related to the prion protein are known as transmissible spongiform encephalopathies (TSEs) (s. *Table 1*), e.g. scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, or chronic wasting disease (CWD) in mule deer and elk. In humans, many forms of prion disease are known; they include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome,

fatal familial insomnia (FFI), prion protein cerebral amyloid angiopathy (PrP-CAA) and kuru (for review s. Prusiner, 1995; Young et al., 1999). These diseases may present as inherited, sporadic or infectious disorders; the inherited forms account for 10-15% of cases observed, whereas the most cases correspond to sporadic CJD, and only some cases appear to be the result of transmission of the pathogenic agent to humans from contaminated surgical instruments, or by the therapeutic administration of infected human growth hormone. Very recently, however, a new variant of CJD (nvCJD) has been found in Great Britain which is almost certainly caused by transmission of BSE to man. This disease has up to now caused 90 deaths in Great Britain in 1996-2000 and may become epidemic.

Already in the eighteenth century scrapie was a well-known disease of sheep in Great Britain as well as in continental Europe (Parry, 1983). Successively, the other prion diseases were found in other animals as well as in human.

Disease	**Host**	**Mechanism of pathogenesis**
Kuru	Fore people	Infection through ritualistic cannibalism
CJD	Humans	Germ-line mutation in PrP gene
GSS	Humans	Germ-line mutation in PrP gene
FFI	Humans	Germ-line mutation in PrP gene
nvCJD	Humans	Infection through bovine prions
FSI	Humans	Somatic mutation?
CAA-PrP	Humans	Somatic mutation?
Scrapie	Sheep and goat	Infection in genetically susceptible sheep or goat
BSE	Cattle	Infection with prion-contaminated MBM
TME	Mink	Infection with prions from sheep or cattle
CWD	Mule deer, elk	Unknown
FSE	Cats	Infection with prion-contam. bovine tissues or MBM

Table 1. Mammalian prion diseases

The first scientific study on the transmissibility of prion diseases from infected animals to healthy animals came 1936 with the finding by Cuille and Chelle that scrapie can be induced in healthy sheep by intraocular injection of spinal cord material from diseased animals (Cuille and Chelle, 1936). The agent responsible for transmission was

further found to be particularly resistant to inactivation by heat or formaldehyde treatment, methods usually employed to inactivate bacteria and viruses (Gordon, 1946; Pattison, 1965). So a first hint to the extraordinary nature of the scrapie agent was evident.

In 1986, several cases of BSE were diagnosed in cattle in Great Britain (Wells et al., 1987); since then the number of cases increased dramatically until 1992 with more than 3000 cases reported each month. The epidemic then decreased progressively, but indeed more than 177 000 cattle in the UK had died between 1986 and April 1998. The explosive spread of disease seems to have been caused by the feeding of cattle with meat and bone meal (MBM) infected by the BSE agent. In fact, in the late seventies and early eighties the manufacturing of MBM was changed, and the new procedure was unable to inactivate the BSE agent (Dormont, 1999). The banning of MBM reduced the BSE cases observed, further supporting the idea of disease transmission to cattle from contaminated foodstuffs.

In humans, approximately 1 person per million world-wide succumb to spontaneous CJD each year. So, the new prion pathogen remained almost unknown until 1996: at that time the appearance of a new variant of Creutzfeldt-Jakob disease (vCJD or nvCJD) brought prions to the centre of public attention, because of the suspect that nvCJD was caused by transmission of the disease to humans from BSE contaminated meat (Collinge et al., 1996).

Moreover, since usually an incubation time of several years is interposed between the infection event and appearance of the first disease symptoms in the brain, the urgency in understanding the mechanisms underlying disease formation becomes clear in view to develop as soon as possible therapeutics against a potential epidemic explosion of transmissible spongiform encephalopathy cases in humans after the consumption of BSE contaminated meat (Raymond et al., 1997; Bruce et al., 1997; Scott et al., 1999).

1.3 The phenotype of prion diseases

The pathologic findings in prion diseases are confined to the central nervous system (CNS), although infectious material is also found in the lymphoreticular system (Bertrand

et al., 1937; Brownlee, 1940; Holman and Pattison, 1943). Interestingly, no cellular infiltrates, no inflammation and no evidence of an immune response are found, in contrast to other infectious diseases of the central nervous system. The prion diseases manifest as progressive degeneration of the CNS, characterised by spongiform degeneration and reactive astrocytic glyosis as shown by electron microscopy examination of tissues from CNS of diseased humans or animals (Zlotnik and Stamp, 1961). The "spongy" aspect of neurons is a consequence of abnormal proliferation of small vacuoles that coalesce and enlarge in large vacuoles leading to neuron loss; the degree of spongiform degeneration is variable from case to case. The extent of astrocytic proliferation is on the other hand correlated with the degree of neuron loss (Masters and Richardson, 1978). The distribution of affected areas within the CNS is dependent on both the genotype of the acceptor and on the strain of scrapie agent.

Depending on the type of infectious prion, the clinical course in humans is characterised by rapidly progressing dementia followed by loss of coordination as in CJD (Bell and Ironside, 1993), or by slowly progressing disease in which ataxia is the predominant sign, followed by the onset of dementia, as found in GSS (Ghetti et al., 1995); FFI presents mainly with sleep disturbances recrudescing in insomnia paralleled by dementia (Goldfarb et al., 1992). Disease onset in inherited human TSEs is in general after the age of fourty and seems to depend on many factors; death occurs within a few years or sometimes within a few months after appearance of first symptoms. Interestingly, the cases of new variant CJD observed recently in Europe are characterised by an earlier onset, often still in the second decade of life, and lead to death within several months to one year (Weissmann and Aguzzi, 1997).

1.4 Spread of infectivity

After intracerebral PrP^{Sc} inoculation in mice, infectivity appears first within days in the lymphoreticular system, including the spleen and lymph nodes; the level remains almost constant until death occurs (Bueler et al., 1993). In the spleen, PrP is present on the surface of both B and T cells, although at very low levels (Cashman et al., 1990); after infection PrP^{Sc} is mainly found in follicular dendritic cells (Kitamoto et al., 1991). Only after a few weeks infectivity begins to become detectable in the CNS, but there

reaches rapidly much higher titers than in the spleen. On the other hand, no infectivity is found in the serum, blood clot, heart, lung or skeletal muscle; low levels of infectivity are found in the blood.

Very intriguingly, PrP knock-out mice are resistant to infection with prions (Bueler et al., 1993). This means that PrP expressing cells are necessary for the disease to be propagated. This evidence is further supported by the fact that PrP knock-out mice with a PrP overexpressing graft, surgically inserted in their brain, only show the typical signs of prion diseases within the engrafted tissue, i.e. no pathological changes are observable in other brain regions (for review s. Aguzzi, 1998). In addition, since susceptibility to PrP^{Sc} is observed in PrP knock-out mice expressing PrP under the control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter, while no expression of PrP is detectable in the neurons of these animals, it seems likely that astrocytes are involved in prion replication in the brain.

B lymphocytes were shortly claimed to be essential for PrP^{Sc} neuroinvasion, because mice devoid of mature B lymphocytes do not show spread of prions from the periphery to the central nervous system (Klein et al., 1997), but further investigations allowed to attribute them just an indirect role (Klein et al., 1998), namely as essential for the maturation of follicular dendritic cells (FDCs), which are found to be the real carrier responsible for the transport of PrP^{Sc} from the periphery to the CNS (Brown et al., 1999b): indeed, treatment of mice with an agent that prevents FDC maturation also avoid scrapie propagation after inoculation with prions (Montrasio et al., 2000). In accordance with this finding, temporary inactivation of FDCs is able to delay to process of neuroinvasion (Mabbott et al., 2000).

Still unclear is how the prions ingested by eating get to the CNS.

1.5 The "protein X" hypothesis

As mentioned before, several years of incubation time are interposed between the event of transmission of infectious prions and appearance of the first symptoms of disease. This incubation time still gets longer if the infecting and the infected individuals belong to different species: this phenomenon is referred to as "species barrier". The stronger species barrier effect is observed during the first passage of prions from the

donor to the host. During the second passage to an homologous uninfected host, the incubation time shortens, and it remains constant through further passages (Kimberlin and Walker, 1979).

In the case of hamsters and mice, the incubation time after the first passage of hamster prions to mice is too long for the mice to get sick before they die of other motifs. A more detailed analysis of these mice infected with hamster prions however showed that their brains presents the disease hallmarks albeit the mice apparently do not get sick for at least two years after inoculation (Hill et al., 2000). Inoculation of brain extracts from this apparently healthy mice into other mice not yet infected with scrapie agents leads to an early manifestation of illness with the usual signs of disease, abrogating the previous view of absolute resistance of some species to prion infection from other species (Hill et al., 2000). This new evidence raises further alarming questions about the eventual transmission of prions between species from an apparently healthy animal to another (Balter, 2000).

In contrast to wild type mice, transgenic mice constitutively expressing the hamster prion protein are sensitive to inoculation with prions from diseased Syrian hamster brains (Prusiner et al., 1990; Scott et al., 1989; Scott et al., 1993). This evidence confirms that the difference in PrP sequence between the prion donor and recipient is essential in determining the species barrier effect.

The pattern of interaction between PrP^{C} and inoculated PrP^{Sc} was investigated creating mice that express a chimeric protein where the region between amino acids 96-167 of the mouse prion protein was exchanged with the hamster PrP sequence (Kocisko et al., 1995). Inoculation of these chimeric mice with hamster prions was followed by onset of disease. Furthermore, it was shown that inoculation of chimeric mice with infectious material from brains of the same chimeric mice but death of prion disease, even shortened the disease incubation time, as expected from the species barrier hypothesis.

Another example of species barrier is observed if transgenic mice expressing PrP with a disease related point mutation are injected with prion preparations from brains of mice suffering of the disease caused by this same point mutation: those mice have

earlier disease onset with respect to the homologous wild type mice not carrying the point mutation (Telling et al., 1995).

Most of these experiments were done also with transgenic mice expressing the human prion protein either together with the mouse prion protein or on a mouse prion protein knock-out background. Interestingly, in the case of simultaneous expression of mouse and human PrP, the mice didn't show signs of disease after infection with human prions. On the contrary, inoculation with human prions on a mouse PrP knock-out background was followed by disease onset. These results caused Telling et al. to postulate a third component essential in the PrP^{Sc} formation process, something like a host encoded wrong folding chaperone, able to bind to both PrP^{Sc} and the converting PrP^{C}. This component was denominated "protein X", because of its likelihood to be a protein; protein X is supposed to interact better with endogenous prions than with prions from a foreign species, allowing further explanations of the species barrier phenomenon. The possible region of interaction between protein X and PrP was assumed to be located between residues 215 and 230 (Telling et al., 1994; Telling et al., 1995).

In summary, studies on mice expressing chimeric PrP allowed Prusiner and colleagues to propose two regions within the PrP sequence essential for the conversion of PrP^{C} to PrP^{Sc} and the species barrier: the region between residues 96 to 167 may be the site of binding of PrP^{Sc} to PrP^{C}, and the region between residues 215 to 230 could be responsible for the binding of protein X. Further investigations will hopefully lead to further insights into the existence and the nature of protein X.

1.6 PrP^{C} biosynthesis

Both PrP^{C} and PrP^{Sc} are found to be encoded by the same chromosomal gene (Basler et al., 1986); the entire open reading frame of the PrP gene of mammalian and avian PrP is located within a single exon (Hsiao et al., 1989), so no diversity due to different splicing exists.

The cellular PrP gene or protein is found in all studied vertebrates, including chicken (Gabriel et al., 1992), salmon (Gibbs and Bolis, 1997) and turtle (Simonic et al., 2000). PrP is a cell surface protein present at high levels in brain tissues, mainly in neurons (Kretzschmar et al., 1986), but also detected in atrocytes and oligodendrocytes (Moser

et al., 1995). Albeit at lower levels, expression is found also in other tissues, such as heart, lung and spleen (Oesch et al., 1985). The prion protein, consisting of about 254 amino acids, is well conserved between species. During processing in the endoplasmic reticulum (ER), the first 22 residues are removed (Basler et al., 1986) and a glycosylphosphatidylinositol (GPI) anchor is added to residue 231 and segment 232-254 is cleaved off (Stahl et al., 1990; Stahl et al., 1987); two high mannose sugars are non-obligatory linked to asparagine residues 181 and 197 (Endo et al., 1989); furthermore, a disulfide bridge between cysteine residues 179 and 214 is formed (Turk et al., 1988). The high mannose sugars are modified in the Golgi and the protein is finally transferred to the external cell surface where it remains anchored via the GPI group.

The prion protein is found to constitutively cycle in clathrin-coated pits between the cell surface and an endocytic compartment during its lifetime (Shyng et al., 1993). The importance of this pathway is unknown. Since the prion protein is GPI anchored and thus never exposed to the cytoplasm, a PrP^C receptor was supposed to clarify how an interaction between PrP and the intracellular components of coated pits such as clathrin and adaptor proteins can occur (Harris et al., 1996).

Because both PrP^C and PrP^{Sc} are found to have a GPI anchor, it is assumed that the conversion from PrP^C to PrP^{Sc} is a post-translational process taking place after PrP^C has reached the cell surface (Borchelt et al., 1990; Taraboulos et al., 1995). Interestingly, while GPI anchored PrP^C is sensitive to digestion with phosphatidylinositol phospholipase C (PIPLC), a bacterial phospholipase that specifically cleaves the GPI anchor, PrP^{Sc} and some disease related PrP^C mutants are resistant to digestion. A possible consequence is that isoforms of the prion protein are differently attached to the cell surface (Lehmann and Harris, 1995).

1.7 Structure of PrP

The NMR solution structure of the recombinant prion protein revealed an N-terminal, disordered fragment up to residue 120, and a well structured, C-terminal domain consisting of residues 121-231, forming three α-helices spanning amino acids 144-154, 179-193, 200-217, and a short two-stranded antiparallel β-sheet involving residues 128-131 and 161-164 (see *Figure 1.7.1*) (Donne et al., 1997; Riek et al., 1996).

The folded C-terminal domain is stabilised by a hydrophobic core comprising side chains of the second helix, the third helix and tyrosine 150 from the mostly hydrophilic first helix, as well as residues belonging to the three loop regions. Comparison of the PrP amino acid sequences of several mammals shows that the invariant residues form an important part of this hydrophobic core and involve residues not in the regions of regular secondary structure. In contrast, most of the amino acid residues linked with the point mutations associated with inherited forms of human prion diseases are located in regular secondary structure elements or immediately adjacent to them.

The single disulphide bond between cysteine residues 179 and 214 is found in the buried, not solvent accessible core of the protein. It links the first turn of the second helix and the last turn of the third helix.

The surface of the globular C-terminal domain of PrP, PrP(121-231), is

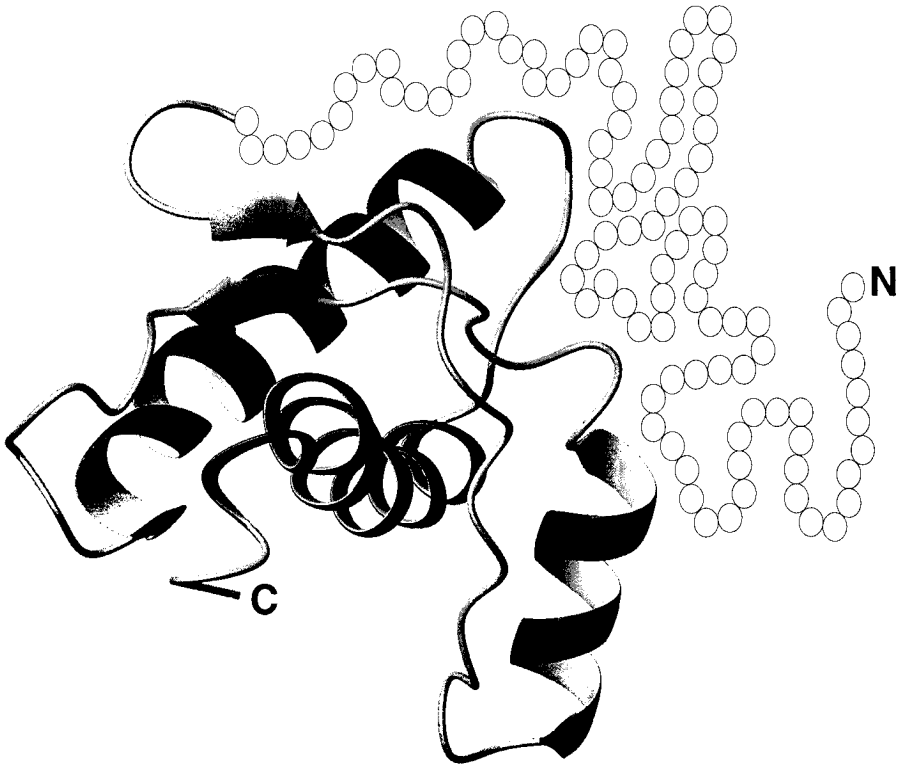

Figure 1.7.1. Structure of the mouse prion protein PrP(23-231) as solved by NMR; the unstructured, N terminal region and the folded, C-terminal domain are shown.

characterised by an uneven distribution of positively and negatively charged residues. The two glycosylation sites at asparagine 181 and 197 are located in the negatively charged surface of the protein.

The recent NMR solution structures of the bovine (Lopez Garcia et al., 2000) and human (Zahn et al., 2000) prion proteins have shown that the three dimensional structure of these prion proteins is almost identical to that from mouse and hamster. However, characteristic difference patterns in the structures of the prion proteins invesitigated interestingly agree with the species barrier hypothesis, showing that high structural homology well correlates with a lower species barrier and vice-versa.

Also the NMR solution structures of human PrP variants with three point mutations, where the amino acid present in the human sequence is exchanged with the corresponding amino acid in the mouse sequence in the region of binding of the postulated "protein X", caused a structural change of human towards mouse PrP. Whether structural similarities and differences between recombinant mammalian PrPs indeed always reproduces the observed species barrier still has to be established (Calzolai et al., 2000).

The infectious isoform, or "scrapie form" PrP^{Sc}, is an insoluble oligomer which tends to accumulate in the brain as amorphous aggregates, that are often resistant to proteinase K digestion. Treatment with proteinase K results in a fragment spanning the region in the vicinity of residue 90 up to residue 231. This fragment is called PrP27-30 referring to its apparent mass on SDS gels varying between 27 and 30 kDa depending on the exact site of digestion by proteinase K and extent of glycosylation.

Albeit the structure of the insoluble PrP^{Sc} is not known Fourier transform infrared (FTIR) spectroscopy allowed to attribute to PrP^{Sc} a higher β-sheet content compared to its normal, non-infectious isoform PrP^{C}. The conformational change leading to the formation of regions rich in β-sheet, possibly at the expense of the α-helical regions is supposed to be the modification triggering aggregation of PrP^{Sc} and disease manifestation.

1.8 PrP^{C} to PrP^{Sc} conversion

Although, as described in the previous paragraph, a basic structural diversity between PrP^{C} and PrP^{Sc} is found, which could give some indications about the conformational changes the normal protein should undergo to reach the diseased conformation, the real process of conversion still remains one of the most important and unsolved questions in the context of prion disease pathogenesis.

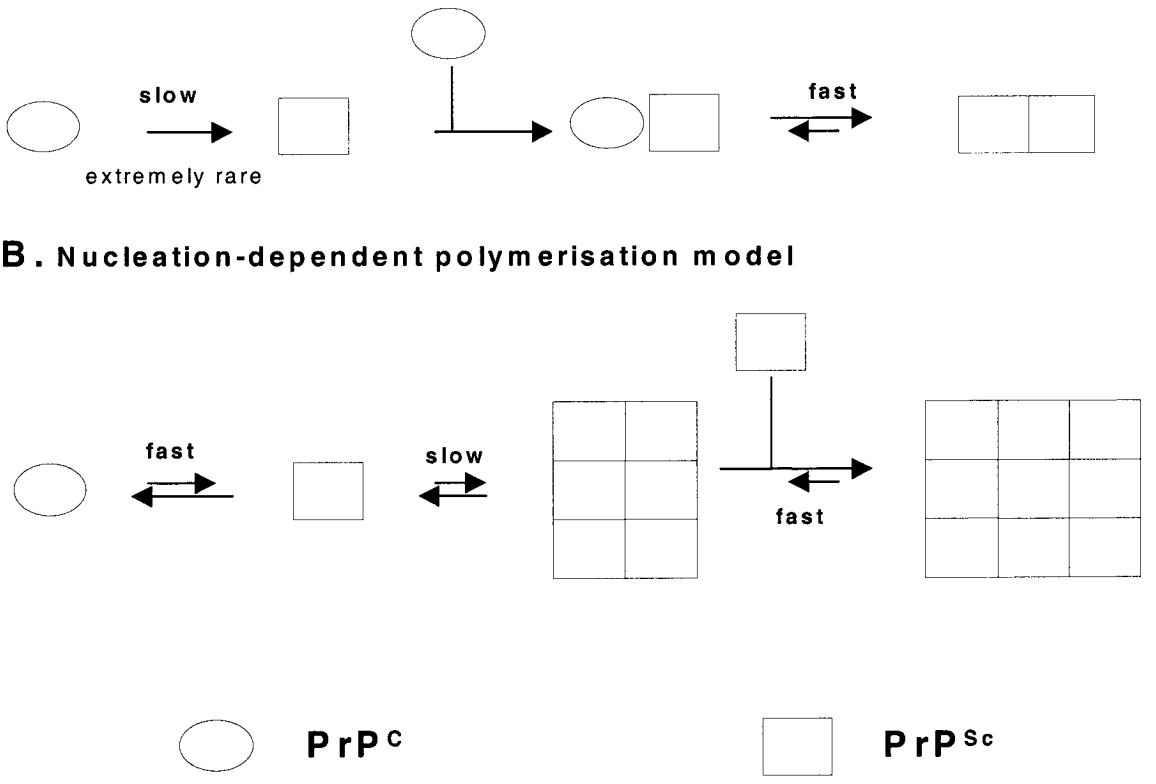

Figure 1.8.1. Proposed models for the self-replication of the prion agent. (A) conformational model; (B) nucleation-dependent polymerisation model

Two main models for the conversion and propagation of prions have been proposed: the nucleation-polymerization model (Jarrett and Lansbury, 1993) and the template-assistance or conformational model (Prusiner, 1991) (see *Figure 1.8.1*).

The nucleation-dependent polymerization model states that after the slow formation of a nucleus of a few scrapie prions from pre-existing PrP^{Sc} monomers in thermodynamic equilibrium with the corresponding normal conformation, a fast transmission of the bad conformation to other 'normal' shaped proteins occurs leading to the very quick formation of large prion aggregates. A possible argument against this view is the supposed high energy barrier for the formation of the scrapie isoform from PrP^{C}. However the model best explains the "strain" phenomenon observed in TSEs, namely in terms of slightly different quarternary structures of different prion strains that are retained during prion propagation.

The template-assistance model seems to better allow for a clarification of the findings related to prion pathogenesis, such as the different incubation time for different species pairs; it claims that scrapie propagation is a very fast process once the irreversible formation of a PrP^{Sc} monomer has occured which then further catalyses misfolding of normal shaped proteins through PrP^{C}-PrP^{Sc} conctacts.

Many attempts to reproduce *in vitro* the conformational transition of PrP^{C} to PrP^{Sc} have been made, but nobody has until now been successful in inducing infectious PrP^{Sc} from PrP^{C} or recombinant PrP *in vitro* without adding PrP^{Sc} purified from brain extracts from diseased animals under defined conditions (Kocisko et al., 1994; Caughey et al., 1995; Morillas et al., 1999). In this context, Kocisko et al. proposed a so called *in vitro*-conversion, by which mixing of radioactively labelled recombinant PrP^{C} from cultured eukaryotic cells with brain isolates from scrapie infected animals, PrP^{C} is converted into a protease resistant PrP isoform, denominated PrP^{res}, which is reminescent of PrP^{Sc} (Kocisko et al., 1994). Caughey et al. showed that these cell-free conversion studies indicate that PrP^{Sc} aggregates are necessary for the reaction, in accordance with the seeding-model hypothesis for prion conversion (Caughey et al., 1997). Anyway, it has to be pointed out that since the PrP^{Sc} purification process does not yield entirely pure PrP^{Sc}, the suspect that additional undetectable components play a crucial role in the conversion is in principle still acceptable. Another point is the possible need of cellular components, such as the proposed 'protein X' for the formation of the leader PrP^{Sc} molecules that are then able to propagate the conformational change by the one or the

other of the models proposed (DebBurman et al., 1997; Kocisko et al., 1995; Saborio et al., 1999).

Many conditions have been found to produce fibrils similar to PrP^{Sc} fibrils obtained after proteinase K treatment. All of the fibrils checked to now for infectivity in mice are found to be non infectious. In addition, prion rods may be an artifact of detergent extraction followed by proteinase K treatment of the amorphous aggregates of PrP^{Sc} found in diseased brains (McKinley et al., 1991). So, prion rod formation appears to be restricted to the proteinase K digestion product PrP27-30 of PrP^{Sc}. Proteinase K resistance of PrP is also found not to correlate necessarily with PrP^{Sc} infectivity (Hill et al., 1999).

The success in producing *in vitro* infectious PrP^{Sc} without the addition of pre-existing PrP^{Sc} would be the best demonstration of the protein-only hypothesis. On the other site, the apparent difficulty to achieve this task is in the meanwhile a good argument to raise dissent about the hypothesis.

1.9 A prion-like small prion: the 'prion-doppel'

As mentioned in paragraph 1.4, prion protein knock-out mice are resistant to scrapie infection and develop more or less normally, indicating that the prion protein is not essential for the normal life of a laboratory mouse. However, some strains of $PrP^{o/o}$ mice were found to develop late-onset ataxia, the origin of which was not clear (Sakaguchi et al., 1996).

These observations prompted Moore et al. to perform a thorough investigation of the PrP gene locus and they found a novel locus, denominated *Prnd*, 16 kb downstream of the mouse prion protein gene *Prnp*. The protein encoded by *Prnd* was designated doppel, abbreviated Dpl. Dpl is composed of 179 amino acids and albeit the overall degree of sequence homology between PrP and Dpl is only 25%, because Dpl lacks the N-terminal octapeptide repeat containing region as well as a region analogous to the fragment 106-126, supposed to be neurotoxic, Dpl has a higher sequence homology to the C-terminal, structured domain of PrP, PrP(121-231). Interestingly, Dpl is expressed minimally in the central nervous system, in contrast to PrP, but is found to be upregulated in those lines of PrP knock-out mice that developed ataxia. So, Dpl is the

first PrP-like protein found in mammals and it may play, in concert with PrP, a role in the pathogenesis of prion diseases (Moore et al., 1999).

2. COPPER(II) BINDING TO THE PRION PROTEIN

2.1. Copper(II) binding to PrPs: what was known

The function of the prion protein remains up to now unknown, and only hypotheses have been made about the physiological role of this protein, which is most likely not indispensable for normal life as shown by the normal behaviour and development of PrP knock-out mice ($PrP^{0/0}$ mice) (Bueler et al., 1992). Examples for the potential roles attributed to PrP are a possible interaction with a ligand, which would result in a signal essential for proper neuronal function (Shmerling et al., 1998), or a role in targeting the neuronal nitric oxide synthase (nNOS) to its normal subcellular localisation in cholesterol-rich membrane microdomains (Keshet et al., 1999).

The finding of Brown et al. in 1997 that the prion protein is able to bind Cu^{2+} ions *in vivo* (Brown et al., 1997a) opened new questions about the biological relevance of copper(II) binding and the possible involvement of PrP in the copper(II) metabolism in neuronal cells.

A first hint to a Cu^{2+} binding ability of PrP^{C} *in vitro* came from the observation that hamster PrP^{C} can be enriched by Cu^{2+} chelate affinity chromatography (Pan et al., 1993). The hypothesis that PrP is also able to bind copper(II) *in vivo* was raised after the observation that the Cu^{2+} content in membrane preparations of brains from $PrP^{o/o}$ mice was only 20% of that in wild-type mice (Brown et al., 1997a); Waggoner et al. recently claimed that these data could not be reproduced (Waggoner et al., 2000), but anyway the reported non-reproducibility is however also not entirely convincing because of the large statistical errors in the data. The possibility that the copper(II)-content in the brains of knock-out mice is in fact lower than in the brains of normal mice remains still open.

However, a great deal of other information indeed support the idea of an essential role of copper(II) in the biological function of PrP as well as in the pathogenesis of prion diseases. Cu^{2+} was shown to stimulate endocytosis of the prion protein in cultured neuroblastoma cells, suggesting that PrP^{C} could serve as a receptor for the uptake of Cu^{2+} ions in neuronal cells from the extracellular milieu; in fact, no other protein performing this task in this type of cell is until now known (Pauly and Harris, 1998). A possible localisation of PrP^{C} in the presynaptic membrane is also discussed, where PrP^{C}

may modulate synaptic transmission by recapturing Cu^{2+} ions released into the synaptic cleft (Herms et al., 1999).

In vivo studies report that $PrP^{o/o}$ mice show an higher sensitivity to copper(II) toxicity and oxidative stress conditions compared to the their wild-type counterpart (Brown et al., 1997b; Brown et al., 1998; White et al., 1999), albeit they develop normally and are not sensitive to infection with prions from diseased animals (Bueler et al., 1993; Bueler et al., 1992). This finding suggests that the prion protein plays a role in preventing oxidative damages and may prevent the toxic effects of too high copper(II) concentrations in neuronal cells by trapping excess copper(II); in addition, the fact that a 32-amino acids peptide corresponding to residues 59-91 of the human PrP, supposed to be the region of copper binding in PrP(23-231) (see below), is able to promote survival of $PrP^{0/0}$ cerebellar cells in the presence of toxic amounts of Cu^{2+} was used as another argument that PrP is involved in the copper(II) metabolism of neuronal cells (Brown et al., 1998).

On the other side, the evidence that mice fed with cuprizone, a copper(II) chelating agent, do not propagate prion disease after infection (Pattison and Jebbett, 1973a; Pattison and Jebbett, 1973b) confirms the crucial role played by copper(II) in the process of disease spread. According to this, Cu^{2+} ions have been shown to apparently enhance recovery of proteinase K resistance after moderate treatment of PrP^{Sc} with guanidine hydrochloride (GdmCl), followed by dilution of the protein solution to lower GdmCl concentrations (McKenzie et al., 1998).

Later, the observation that brain extracts from $PrP^{0/0}$ mice show a decreased activity of the cuproenzyme superoxide dismutase (SOD) led Brown et al. to the hypothesis that PrP^{C} acts as a copper(II) storage protein guaranteeing sufficient biological acitivity of SOD (Brown and Besinger, 1998). Thereafter, the same authors suggested from *in vitro* experiments that the cellular prion protein itself has a SOD-like activity, but only after refolding of the recombinant protein in the presence of excess Cu^{2+} ions (Brown et al., 1999a; Wong et al., 2000d); this view has until now not been addressed by other investigators in the prion research field, and it was neither disproven nor confirmed by others; nevertheless, Brown et al. reported in the meanwhile a lot of experimental data supporting this hypothesis. In this context, PrP^{C} was found to be also able to bind

manganese(II) ions and to acquire some SOD-like activity also after binding of ions of manganese(II) (Brown et al., 2000); in addition, SOD activity measurements on cell cultures appeared to confirm the antioxidant function of PrP^C in a more physiological milieu (Wong et al., 2000b; Wong et al., 2000c).

In summary, at the moment the findings that suggest that the prion protein is involved in the copper(II) metabolism of neuronal cells have raised much more questions on the biological activity of PrP^C in relation to its binding ability than have yielded clear-cut answers.

Anyway, albeit a clear link between copper(II) binding and PrP function has not yet been established, more detailed knowledge is available on the mode of binding. Unfortunately, not so many studies have been done on the copper(II) binding behaviour of the full-length protein, since most experiments have been performed on fragments of PrP corresponding to synthetic peptides of different length from the unstructured, N-terminal domain of the protein, which was the most obvious candidate for copper binding due to a fourfold repeat of eight amino acids with the consensus sequence (PHGGGWGQ) containing one histidine residue per repeated unit. The high avidity of this type of residue for copper(II) ions is well known, and indeed the most usual pattern known for copper(II) binding in proteins involves up to three histidine residues per bound metal ion. Other fragments candidates for copper(II) binding are the amino acids cysteine and methionine. Water or oxygen molecules can fill up the coordination sphere of the metal ion. According to the type of amino acids responsible for binding, three classes of copper-centres in copper(II) binding proteins are distinguished, called type I to type III copper-centres (s. *Table 2.1.1*).

The most likely type of copper(II) centre in the prion protein is type II; type I is indeed excluded because the only two cysteine residues in the protein are located in the C-terminal, folded domain and are connected by a disulfide bridge. Copper(II) binding as in the type III complexes is also excluded because the typical absorption at 350 and at 600 nm cannot be observed for the copper(II)-bound prion protein.

Type and coordinating ligands	Function and spectroscopic properties
Type I: two histidines, one cysteine, one methionine	'Blue' copper(II) centres Function: reversible electron-transfer: $Cu^{II} + e^- \rightarrow Cu^{I}$ Absorption of the Cu(II) form at approximately 600 nm (ε>2000 $M^{-1}cm^{-1}$)
Type II: three histidines and one water molecule	Normal, 'not-blue' copper centres Function: activation of O_2, in conjunction with organic coenzymes from the Cu^{I}-state Typical Cu(II) absorptions with ε>1000 $M^{-1}cm^{-1}$
Type III: connection via an oxygen molecule of two copper(II) centres coordinated each by three histidines	Copper-dimers Function: O_2-uptake from the Cu^{I}-Cu^{I} state After O_2-uptake intensive absorption at 350 and 600 nm ($\varepsilon \approx$20000 $M^{-1}cm^{-1}$ and 1000 $M^{-1}cm^{-1}$)

Table 2.1.1. Classical copper(II) centres in proteins.

Regarding the stoichiometry of Cu^{2+} binding to PrP, 2-6 copper(II) ions have been proposed to bind per PrP^{C} molecule (Hornshaw et al., 1995a; Hornshaw et al., 1995b; Stockel et al., 1998; Viles et al., 1999; Miura et al., 1999; Brown et al., 1997a; Whittal et al., 2000). The differences in the stoichiometry may be caused by different, pH-dependent protonation states of the histidine residues and by buffer artifacts.

Stöckel and co-worker found by equilibrium dialysis experiments that the Syrian hamster prion protein, SHaPrP(29-231) binds 2 Cu^{2+} ions at pH 6. The model of binding proposed by this group suggests that two octapeptide repeat units are involved in the coordination of one metal ion with one histidine and perhaps one glycine carbonyl as chelating agents (*Figure 2.1.1*). In addition, tryptophan fluorescence and circular dichroism spectroscopy experiments were interpreted such that binding of copper(II)

induces a more defined structure to the otherwise disordered, N-terminal region (Stockel et al., 1998).

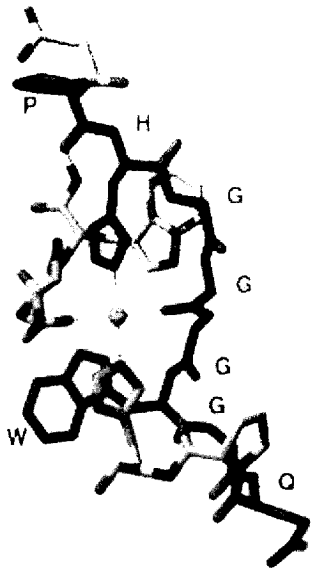

Figure 2.1.1. Possible conformation of Cu^{2+} binding to two PrP octapeptide repeats. Copper is coordinated by two His residues and two Gly carbonyl oxygens and is bound in a square planar geometry. Modeling was performed using the Sybyl software package (Tripos Inc.), and the structure was validated with PROCHECK (*34*). [from Stockel et al., 1998].

The more recent copper binding model from Viles et al. proposes cooperative binding of 4 copper(II) ions at pH 7.5 to a PrP(58-91) peptide containing all 4 octarepeats. Viles and co-workers also analysed separately copper(II) binding on peptides corresponding to one up to four octapeptide repeat units. In their model, the coordination sphere in the four octapeptide region is supposed to be practically identical for all of the four copper(II) ions bound, consisting of a square-planar geometry with three nitrogen ligands and one oxygen ligand (*Figure 2.1.2*). Copper(II) binding was analysed by absorption and circular dichroism spectroscopy in the visible wavelength region, as well as with electron paramagnetic resonance and nuclear magnetic resonance spectroscopy (Viles et al., 1999).

Very recently, Whittal et al. investigated fragments from the unstructured, N-terminal domain of Syrian hamster PrP and found that the binding stoichiometry of Cu^{2+} to PrP^{C} is pH dependent, with 2 copper(II) bound to the octapeptide repeat region at pH 6 and four copper(II) bound at pH 7.4 (Whittal et al., 2000).

In comparison, the previous *in vivo* analysis by Brown et al. (1997a) showed binding of 5.6 ± 0.4 Cu^{2+} ions at neutral pH per peptide corresponding to the N-terminal region of PrP, PrP(23-98). The copper(II) ions were supplied as a copper(II)-glycine$_2$ complex to enhance the copper(II) solubility at neutral pH. The amount of copper was determined by atomic absorption spectroscopy on samples from equilibrium dialysis experiments (Brown et al., 1997a).

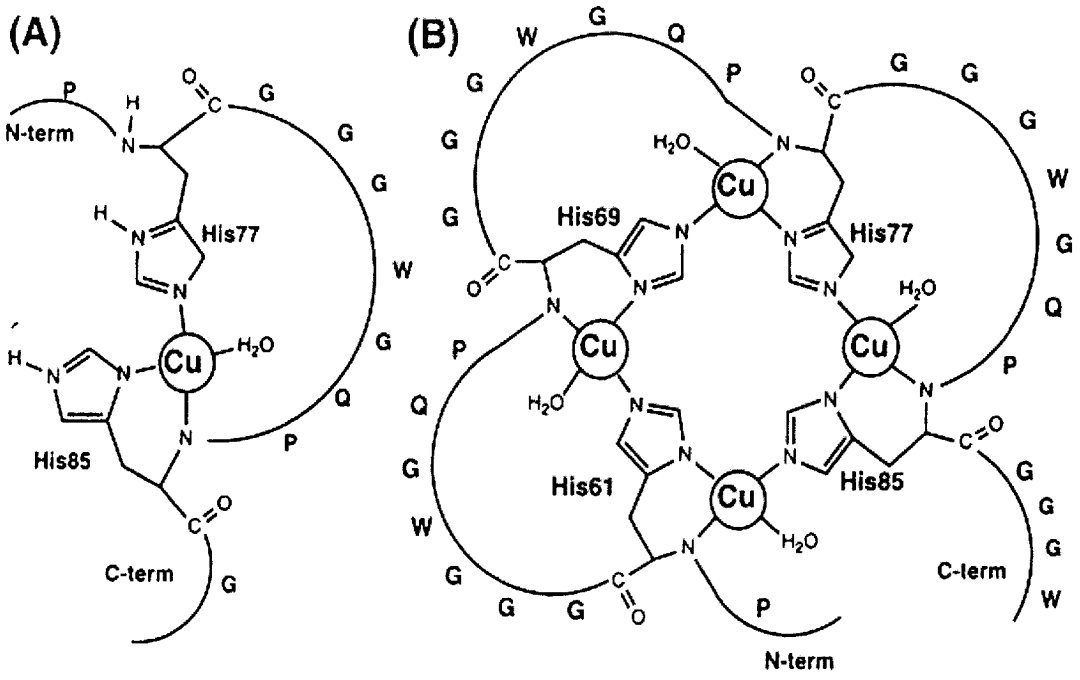

Figure 2.1.2. (A) A plausible structure for the complex of Cu(II) with PrP(76-86). (B) A plausible structure for the bridged complex of four Cu(II) ions with PrP(58-91) [from Viles et al., 1999].

2.2. Contributions of this work to the investigation on copper(II) binding

In this work, we analysed copper(II) binding to the recombinant mouse prion protein, PrP(23-231), as well as to its isolated C-terminal domain PrP(121-231), through the pH range 3-8 with electron paramagnetic resonance (EPR) spectroscopy. We also analysed the binding behaviour of some of the PrP(23-231) point mutants which are related to inherited forms of prion disease in human.

By titration experiments we found that up to ten Cu^{2+} ions bind to full-length PrP(23-231) at pH 4.0 and 6.0; very interestingly, analogous experiments with PrP(121-231) revealed binding of up to four copper(II) ions also to the structured, C-terminal domain of PrP. Furthermore, the pattern of binding to PrP(23-231) is the same as that found for PrP(121-231) at a copper(II) concentration of up to 4 molar equivalents. In summary, of the ten molar equivalents of copper(II) found to bind overall to PrP(23-231), four are supposed to be trapped by the C-terminal domain, and the remaining six likely bind to the N-terminal, unstructured region, in accordance with the *in vivo* results of Brown et al. (Brown et al., 1997a). Moreover, since in the case of PrP(23-231) the complexes formed first during titration with copper(II) are the same as those found in PrP(121-231), the C-terminal domain may have a higher avidity for Cu^{2+} than the N-terminal part, in contrast to the previous view that the octapeptide repeat region is most prone for copper(II) binding.

Thereafter, we analysed in more detail the copper(II) centres found within the C-terminal domain; we could distinguish three different modes of binding; the relative distribution of the three complexes was found to be pH-dependent. In all of the three complexes observed, the EPR parameters of copper(II) were typical of type II copper centres as expected. One of the copper(II) complexes in PrP(121-231), and correspondingly in PrP(23-231), was shown by its pH dependence as well as by pulse EPR and electron nuclear double resonance (ENDOR) spectroscopy to involve a histidine residue. Simultaneous involvement in the coordination sphere of more than one histidine residue from the C-terminal domain is excluded because they are located too far apart in the three dimensional structure.

In order to determine which of the three histidine residues contained in the C-terminal domain is responsible for binding, we cloned three point mutants of PrP(121-

231) in which one single histidine residue was exchanged by a serine residue. Unfortunately, these mutants were extremely unstable and had a high tendency to aggregate; this made solid EPR measurements impossible.

Furthermore, we analysed copper(II) binding to three of the mutant proteins related to inherited TSEs in humans. We found that the mutant proteins E200K and F198S of PrP(23-231), reported to be responsible in human for the Gerstmann-Straeussler-Scheinker syndrome (Unverzagt et al., 1997) and the inherited form of Creutzfeldt-Jakob disease (Spudich et al., 1995), respectively, had both a pattern of binding different to that found in the wild-type protein and also differed to each other in their binding behaviour; the D178N/M129 mutation of PrP(23-231), inducing fatal familial insomnia (Gambetti et al., 1995), did instead not have a modified pattern of copper(II) binding to the structured, C-terminal part.

Overall we want to stress that the unprecedented finding that the C-terminal domain PrP(121-231) of the mouse prion protein is also able to bind copper(II) ions supplies a new exciting argument in the discussion about the role of copper(II) ions in the process of PrP^{Sc} formation. In fact, since the octapeptide repeats are not required for prion propagation after infection (Shmerling et al., 1998; Flechsig et al., 2000), copper(II) binding to the remaining fragment of the protein may give an explanation to the previously found involvement of copper(II) in prion toxicity.

2.3. Electron Paramagnetic Resonance (EPR) spectroscopy

Electron paramagnetic resonance (EPR), also called electron spin resonance (ESR), is a spectroscopic technique that allows to specifically detect unpaired electrons in a sample by their absorption of energy from microwave irradiation (ca. 10^{10} Hz) when the sample is placed in a strong magnetic field.

Since only molecules with unpaired electrons can be investigated by this technique, the use of EPR in biology is restricted to the analysis of protein-bound transition metal ions with unpaired electrons such as Cu(II), Fe(III), Mn(II) and Co(II), the detection of free radicals, such as the intermediate states in the photosynthetic process, and the study of molecules modified with so called spin labels. Spin labels are free radicals that

are stable to about 80°C and over the pH range 3-10. The most commonly used labels contain the nitroxide moiety N^+-O^-; two examples of them are shown in *Figure 2.3.1*:

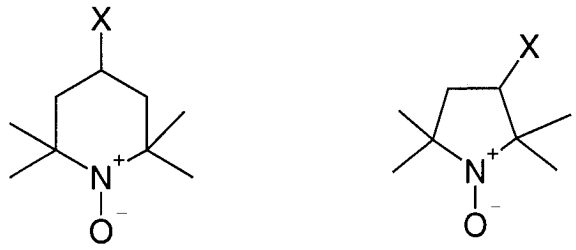

Figure 2.3.1. Examples of spin labels with nitroxide moiety.

Spin labels may be either attached (via the X-group) to specific functional groups on the protein being investigated, or intercalated into regions of interest. In addition, it is possible to make spin-labeled substrates or ligands. The major advantage of the spin label method in biology is the possibility to study the motion of proteins, e.g. the lateral diffusion of proteins in membranes.

The possibility of detecting unpaired electrons is based on the fact that, due to their spin 1/2, the electrons possess a magnetic moment, so applying an external field B_0, the magnetic moment of the electron is induced to position itself in one of the only two allowed orientations with respect to this external field: the parallel and the antiparallel orientation, which have different energies and correspond to the two spin states of the electron ($m_s=\pm 1/2$). Transitions between the two spin states can be induced if electromagnetic radiation of the appropriate frequency ν is applied perpendicularly to the external magnetic field B_0. The basic resonance condition in EPR results then to be:

$$h \nu = g \beta B_0$$

h: Planck constant (6.63×10^{-34} Js)

ν: frequency of the electromagnetic radiation

g: electron g-factor

β: Bohr magneton ($0.92\ 10^{-23}$ JT)

B_0: external field

Due to practical aspects, the frequency ν is maintained constant and the external field B_0 is varied in the EPR experiment.

An EPR spectrum is characterised by four main parameters:

1. the **signal intensity**, proportional to the concentration of the unpaired spin giving raise to the spectrum;
2. the **line width** of the signal, which gives information about any dynamic processes;
3. the **g-value**, indicative of the immediate environment of the unpaired electron; this value characterises the position of a resonance and it is a characteristic quantity;
4. the multiplet structure, represented by the **A-value**, that allows to analyse the interaction of the unpaired electron with the nuclear spin.

In addition, many of the EPR parameters are anisotropic, i.e. they have different values in different directions, because the orbitals containing the unpaired electrons are in general not spherical and so not spatially symmetric (orbitals of the p-, d- or f-type). However, if a fast rate of motion in all directions is allowed, all orientations can be averaged out, and the anisotropy resolves in a isotropic-like situation. This is the case e.g. in solutions measured at room temperature; if the measurement is carried on at liquid nitrogen temperature (120 K), the motion is no more free in space and the EPR parameters g and A show anisotropy.

The anisotropy of the g-value is characterised by three principal g-parameters: g_{xx}, g_{yy} and g_{zz}, positioned along the principal axes. In the case of axial symmetry, g_{zz} is by convention also designated $g_{//}$; g_{xx} and g_{yy} are defined $g_{\perp}$ (*Figure 2.3.2*). At any intermediate orientation, the g-values depend on the angle θ between the magnetic field and the principal axes.

The anisotropy of the hyperfine interaction A, which arises from the dipolar interaction of the magnetic field of the electron with the magnetic field of the nucleus is also visible at lower temperatures. The hyperfine interaction A is also characterised by 3 principal values: A_{xx}, A_{yy} and A_{zz}. As for the g-value, in the case of axial symmetry A_{zz} is also defined $A_{//}$ and A_{xx} and A_{yy} are also represented by $A_{\perp}$.

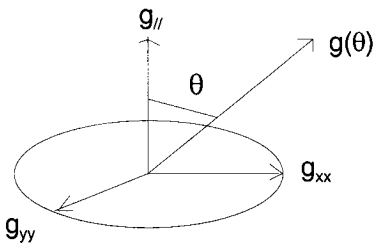

Figure 2.3.2. Variation of g-value with the magnetic field orientation for an axially symmetric system.

In the study of transition metal ions in biological systems, the unpaired electrons are primarily localized to d-orbitals, and the number of electron-spin energy levels is given by 2S+1, where S represents the total spin of the unpaired electron, so the EPR spectrum depends on the number of unpaired electrons and on the arrangement (symmetry) and nature of the coordinated ligands. The g-values can vary over a wide range (values of 1.4 to 10 have been observed). Anyway, g-values for copper(II) centres in analogous environments are similar, therefore knowledge of the coordination sphere of metals in proteins of which the structure in the presence of the metal is solved is very helpful to have some insight into the nature of the coordination sphere in other proteins,

for which only the EPR parameters are known, and no structure with bound metal ions is available.

EPR has become a standard tool in the study of copper(II) binding proteins; every type of copper(II) site in a protein has its characteristic continuous wave (CW) EPR spectrum for type II copper centres in proteins (paragraph 2.1). The EPR spectrum taken at about 9.4 GHz (X-band) is almost axial and the copper hyperfine splitting is resolved in the $g_{//}$ region; however, in most cases the interaction of the unpaired electron with other nuclei in the copper site remains hidden under the EPR line. By means of pulse EPR and electron nuclear double resonance (ENDOR) spectroscopy it is possible to resolve such hyperfine interactions and to get some insight into the structure of the copper(II) complexes.

In the ENDOR method, the EPR absorption is first saturated (reduced) by strong microwave irradiation; thereafter nuclear transitions are induced by means of a radio frequency field, that changes the populations of the energy states of the electrons and therefore induces increased EPR absorption.

A disadvantage of the EPR technique is that the protein concentration needed to observe a spectrum with a sufficiently good signal-to-noise ratio is rather high, in the range of 100 μM; this often renders a direct comparison with the physiological protein concentrations difficult. In addition, the low temperature needed to obtain a high resolution of the spectra (sometimes the temperature has to be lowered down to 4 K) is also not necessarily comparable with the physiological case.

2.4. EPR measurements on PrP(23-231) and PrP(121-231) wild-type

2.4.1. Titration of copper(II) binding to PrP(23-231) and PrP(121-231) at pH 4.0 and 6.0

In order to determine the number of copper(II) ions bound per PrP(23-231) or PrP(121-231) molecule, we measured the EPR spectra of samples of these proteins, at a concentration of 0.1 mM, in the presence of 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15 and 20 molar equivalents $CuCl_2$ at pH 4.0 in 10 mM formic acid/NaOH buffer or at pH

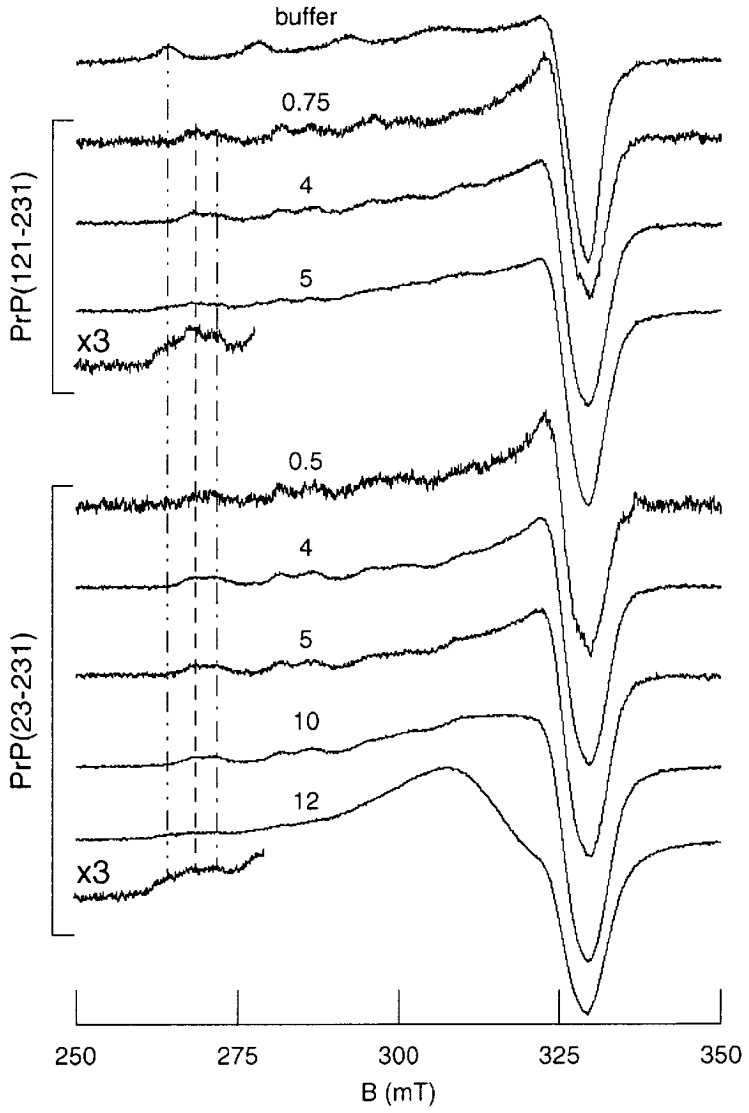

Figure 2.4.1.1. Titration of PrP(23-231) and PrP(121-231) with 0-12 molar equivalents of $CuCl_2$ at pH 4.0 at a protein concentration of 100 μM. Over each EPR spectrum the number of $CuCl_2$ molar equivalents present in the sample is indicated.

6.0 in 10 mM sodium cacodylate/HCl buffer. These pH values were chosen because copper(II) salts are almost insoluble at neutral and basic pH, what would render the interpretation of the titration experiment difficult due to copper(II) precipitation, and also because the pH range 4.0-6.0 is typical for the endosomal compartments, which are actually supposed to be the subcellular site where the scrapie isoform of the prion protein is produced (Shyng et al., 1993; Hornemann and Glockshuber, 1998).

In all cases analysed, the signal intensity was found to increase linearly with the number of molar equivalents $CuCl_2$ added; this represents a good proof of the suitability of use EPR to perform titration in the system we studied.

Addition of 0.25 to 4 molar equivalents of $CuCl_2$ to PrP(121-231) yielded EPR spectra with a mixture of two complexes (*Figure 2.4.1.1* and *Figure 2.4.2.1*); there seemed to be no preference for one of the two because they were both already visible by addition of less than 1 molar equivalent of Cu^{2+}. We called these complexes complex **1** and complex **2** to distinguish them (the characterisation of these complexes is presented in the following paragraph). From 5 molar equivalents onwards, a third component appeared at pH 4. Comparison with the spectrum of the formate buffer in the presence of copper(II) ions and in absence of protein allowed to attribute this additional signal to a formate-copper(II) complex, i.e. to the buffer also starting bind Cu^{2+} ions. While the signal intensity of the copper(II)-formate complex increased by further Cu^{2+} addition, the signals of the protein-copper(II) complexes remained constant, indicating that PrP(121-231) at pH 4.0 binds a maximum of 4 copper(II) ions. The same behaviour

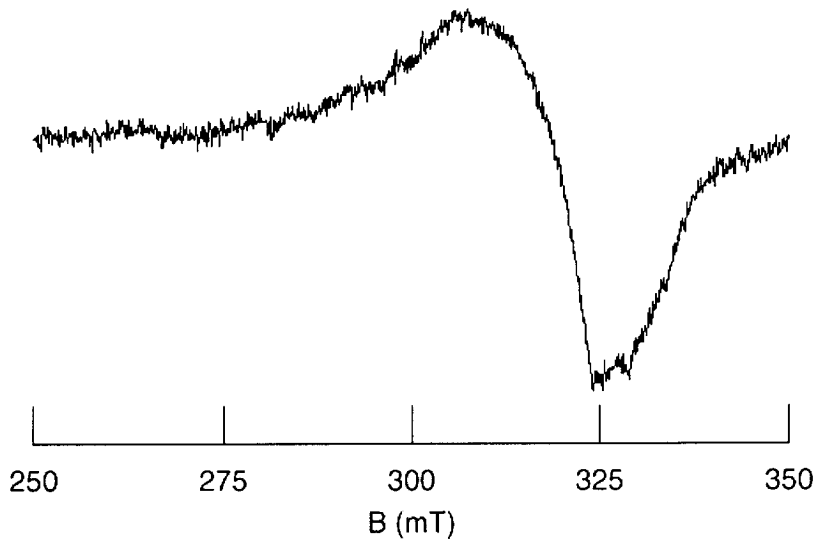

Figure 2.4.1.2. Difference spectrum at pH 4.0 of PrP(23-231) in presence of 10 molar equivalents $CuCl_2$ and 4 molar equivalents $CuCl_2$. The spectrum should correspond to the spectrum of copper(II) bound to the N-terminal region.

as for PrP(121-231) was observed for PrP(23-231) by addition of up to 4 molar equivalents Cu^{2+}, but interestingly further addition of Cu^{2+} did not result in the appearance of the signal of the copper(II) bound buffer; only from 12 molar equivalents Cu^{2+} added onwards, a contribution from the copper(II)-formate complex was visible in the PrP(23-231) samples. Albeit the sample with 11 molar equivalents Cu^{2+} was not analysed, the intensity of the buffer signal at 12 molar equivalents Cu^{2+} allows to argue that a buffer signal is visible after addition of 11 molar equivalents, i.e. the full-length PrP(23-231) is able to bind up to 10 molar equivalents of copper(II) ions at pH 4.0 (*Figure 2.4.1.1*). Analysis of the shape of the EPR spectra from 5 to 10 molar equivalents Cu^{2+} suggests that besides the two complexes observed for both PrP(23-231) and PrP(121-231) a third component is formed, which, because of partial overlapping of the signals from different copper(II) centres, only becomes visible if the spectrum of the protein in the presence of more than 4 molar equivalents Cu^{2+} is subtracted from the spectrum observed with less than 4 molar equivalents Cu^{2+} (*Figure 2.4.1.2*). The low resolution of this spectrum allows to conclude that it arises from copper(II) centres that are in close vicinity to each other, because high local concentrations of Cu(II) complexes are shown to cause inevitable broadening of the EPR lines. This picture matches well with the hypothesis that from 5 molar equivalents Cu^{2+} added onwards, the N-terminal, unstructured portion of PrP(23-231) starts binding copper(II); as expected from the models previously proposed for binding to the octapeptide repeat region, the metal ions have to be positioned close to each other in order to allow simultaneous binding (*Figure 2.1.1 and 2.1.2*).

Titration at pH 6.0 instead of pH 4.0 gave analogous results; the only difference was that upon addition of 4 or more molar equivalents Cu^{2+}, the C-terminal domain PrP(121-231) started to aggregate, allowing no further investigation by EPR. In the presence of 0-3 molar equivalents $CuCl_2$ the signal intensity was found to increase linearly with the Cu^{2+} concentration; this indicates that, at pH 6.0, PrP(121-231) can bind at least 3 molar equivalents copper(II). On the contrary, the full-length protein PrP(23-231) did not aggregate even after addition of 20 molar equivalents $CuCl_2$. For 0-4 molar equivalents the typical spectrum shown in *Figure 2.4.1.1* was observed. Addition of more molar equivalents Cu^{2+} resulted in alteration of the spectrum, albeit the changes were not as

clearly interpretable as at pH 4.0. At copper(II) concentrations of 12 molar equivalents onwards, the signal of the cacodylate-copper(II) complex appeared. Thus, also at pH 6.0 up to 10-11 copper(II) ions are bound per PrP(23-231) molecule, and at least 3 of them are bound to the C-terminal region of the protein.

The evidence that also in the case of PrP(23-231) first the same complexes as for PrP(121-231) are found, and only later the broadening due to the copper(II) centres in the N-terminal region appears, allows to argue that PrP(23-231) starts binding copper to its C-terminal, structured domain, and only thereafter Cu^{2+} binding to the unstructured region with the four octapeptide repeats takes place. As we mentioned, this result is unexpected from previous observation on prion proteins and fragments thereof, where the histidine residues in the octapeptide repeat region were claimed to be the most likely candidates for copper(II) coordination.

2.4.2. pH-dependence of the PrP-copper(II) interaction

After having determined that PrP(121-231) is able to bind up to 4 Cu(II) ions at pH 4.0 and at least 3 Cu(II) ions at pH 6.0, while the full-length protein PrP(23-231) binds up to 10 Cu(II) ions at both pHs, we investigated samples of both PrP proteins in the presence of copper(II) at different pH values in order to analyse if the formation of protein-copper(II) complexes was dependent on the pH of the solution.

We found that the shape of the spectra and thus the relative populations of the different copper(II) complexes is in fact pH dependent (*Figure 2.4.2.1 A and B*).

For the pH range 3.0-6.0, 0.1 mM protein samples supplied with 3 molar equivalents of $CuCl_2$ were analysed (*Figure 2.4.2.1 A*). Due to the lack of titration data for higher pH values and due to the low solubility of $CuCl_2$, the samples at pH 7.0, 7.4 and 8.0 were prepared by dialysing the protein at a concentration of 0.1 mM against buffer with 50 µM $CuCl_2$ concentration, followed by extensive dialysis of the copper(II)-bound protein against buffer without $CuCl_2$ to remove unbound Cu^{2+} (*Figure 2.4.2.1 B*). $CuCl_2$ containing buffer solutions without protein were also analysed as control for all conditions used. The buffers used were 10 mM formic acid/NaOH for pH 3.0 and 4.0, 10

mM sodium acetate/HCl for pH 5.0, 10 mM sodium cacodylate/HCl for pH 6.0 and 10 mM MOPS/NaOH for pH 7.0, 7.4 and 8.0.

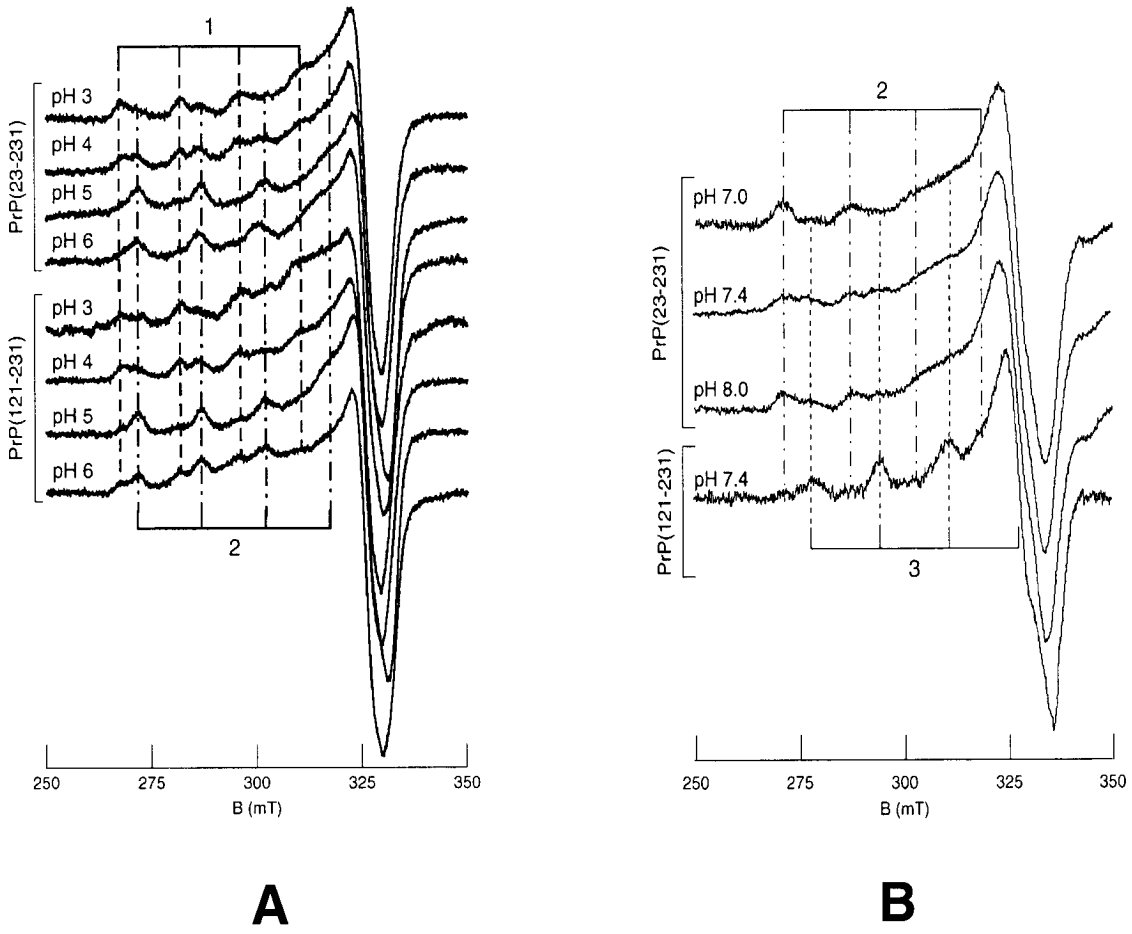

Figure 2.4.2.1. A. pH dependent formation of complex **1** and complex **2** of PrP(121-231) and PrP(23-231) with 3 molar equivalents $CuCl_2$. B. pH dependent formation of complex **2** and **3** of PrP(23-231) and PrP(121-231) after copper(II) binding.

Through the pH range 3.0 to 6.0, the two complexes denominated complex **1** and complex **2** were observed; the ratio of both binding modes varies by varying the pH. The $g_{//}$ and $A_{//}$ values of complex **1** are in agreement with three possible types of copper(II) ligation: ligation to two nitrogens and two oxygens (2N2O), ligation to one nitrogen and three oxygens (1N3O) or ligation to four oxygens (4O). The shift to lower $g_{//}$ and higher $A_{//}$ values observed for complex **2** compared with complex **1** is an indication for a larger number of nitrogen ligands and less oxygen ligands; in fact for complex **2** the EPR

parameters are in agreement with the three combinations: 3N1O, 2N2O or 1N3O, although the $g_{//}$ and $A_{//}$ values observed are also similar to those observed for type-2 azurin mutants where Cu(II) is ligated to one nitrogen, two oxygens and one sulphur; involvement of two sulfur atoms in the ligation can be ruled out for both complexes.

The same complex **1** and complex **2** are found both in the case of PrP(23-231) as well as PrP(121-231) at all pH values investigated, once more giving evidence to the fact that in PrP(23-231) the structured, C-terminal part first binds copper(II) ions.

To test the stability of the two complexes formed, samples prepared at pH 4.0 were extensively dialysed against distilled water at 4°C to a final pH of 5.6. The same contributions of complex **1** and **2** were still present, as for the undialysed samples at pH 5.0 and 6.0.

At pH 7.0 to 8.0 the EPR spectra of the dialysed samples of PrP(23-231) lack complex **1**, but exhibit an additional contribution. As a control, the measurement of a sample of 50 μM $CuCl_2$ in MOPS/NaOH buffer (pH 7-8) did not show any signal, so it could be excluded that this signal came from a buffer-copper(II) complex. Instead, an additional copper(II) binding mode is present at neutral and basic pH. This third complex was referred to as complex **3**. The $g_{//}$ and $A_{//}$ parameters of complex **3** (*Table 2.4.2.1*) are in agreement with the ligation modes 4N, 3N1O, and 2N2O, although they are also similar to those observed for type-2 azurin mutants with Cu(II) ligated to two nitrogens, one sulfur and one oxygen (den Blaauwen and Canter, 1993).

Complex **2** is still observed at pH 7-8 and has the same EPR parameters as those observed at acidic pHs.

In summary, complex **1** is only observed at acidic pH (range 3-6), while complex **3** is only formed at neutral and basic pH values. Complex **2** is visible throughout the whole pH range analysed (3 to 8), albeit its contribution to the spectrum varies, with a maximum population around pH 6.0.

Since PrP(121-231) shows a strong aggregation tendency upon addition of $CuCl_2$ at pH 7.0-8.0, the concentration of soluble protein after dialysis was too low to give interpretable EPR spectra. However, a spectrum of PrP(121-231) in the presence of less than 1 molar equivalent $CuCl_2$ at pH 7.4 with an acceptable signal-to-noise ratio was obtained. The components of complex **2** and complex **3** contributing to this spectrum

were also in this case the same as those found for the full-length protein, suggesting that complex **3** is also localised to the C-terminal domain of the protein.

After having characterised the complexes present in both the C-terminal domain and the full-length protein, the remaining complex due to binding to the N-terminal region was denominated complex **4**. As mentioned in the previous paragraph, the bad resolution of the spectrum of complex **4** did not allow for a detailed characterisation of the g and A parameters (*Figure 2.4.1.2*).

A summary of the physical EPR parameters of the complexes found is shown in *Table 2.4.2.1.*

complex	$g_\perp$ (±0.003),	$g_\parallel$ (±0.002)	$A_\perp$ (± 10) [MHz]	$A_\parallel$ (± 10) [MHz]	Location	pH
1	2.068	2.332	12	452	mPrP(121-231)	3-6
2	2.068	2.295	20	457	mPrP(121-231)	3-8
3	2.055	2.230	50	495	mPrP(121-231)	7-8
4	2.114		not resolved		mPrP(23-120)	4+6

Table 2.4.2.1. EPR parameters of the type-2 Cu(II) complexes observed in copper containing full-length mPrP(23-231). The hyperfine data are given for ^{63}Cu. The location of the Cu(II) complexes in the protein and the pH values at which they are observable are also given.

2.4.3. Characterisation of the copper(II) complexes in the C-terminal domain

After the surprising result that the C-terminal domain of the full-length prion protein PrP(23-231) binds copper(II) and shows an even higher avidity for the metal ion than the N-terminal tail, we tried to better characterise the three complexes found to be formed within the structured part of the protein using pulsed EPR and electron nuclear double resonance spectroscopy (ENDOR). To better profit from the advantages of these techniques, the pH of the sample were chosen so as to maximize the fraction of the complex of interest. Since at pH 5.6 the contribution of complex **2** to the spectra is maximum for both PrP(23-231) and PrP(121-231), this complex was investigated at this pH. For complex **3** the most suitable pH was 7.4, while for complex **1** a pH of about 3 would be the best. We first started with the

characterisation of complex **2**, and then, with the knowledge of the different spectra observed for this complex, proceeded to the study of complexes **1** and **3**.

Complex **2**. The pH dependence found for the population of complex **2**, with a decreasing intensity at pH values lower than 6, still suggested that a histidine residue is involved in this complex since histidine residues have a pK_a of about 6 and therefore at lower pH values the histidine nitrogen responsible for copper(II) coordination should become protonated and no longer be able to coordinate Cu(II).

Pulse EPR and ENDOR investigations allowed us to ascertain the involvement of the amino acid histidine in the coordination sphere, because if a histidine-copper(II) complex is formed both the directly coupled and the remote nitrogen gives an unequivocal shape to the spectra. In fact, using these techniques, we were able to observe a weak interaction with a nitrogen nucleus as well as a strong interaction with at least one other nitrogen ligand. The weak interaction is due to a nitrogen that is not directly bound to the copper(II) centre; this nitrogen is supposed not to be a backbone nitrogen because the parameters found for this atom do not agree with parameters typical for the interaction of copper(II) with backbone nitrogens found in the literature. The interaction is much more likely due to the remote nitrogen from a histidine residue. The strong interaction reveals parameters that are in the range of the ones observed for directly bound histidine nitrogens in different copper(II) containing proteins. In summary, both the strong and the weak interaction observed for complex **2** strongly suggest that a histidine residue is one of the ligands in the coordination sphere of this complex.

Only three histidine residues are contained in the C-terminal domain of PrP(23-231): His140, His177 and His187. As shown in *Figure 2.4.3.1* these three residues are too far apart in the three dimensional structure to allow simultaneous binding of two or three histidines to a single metal ion, therefore the most likely picture is that complex **2** only involves one of these three residues. In order to find out which of them acts as copper(II) ligand we produced corresponding PrP(121-231) mutants where one of each histidine was exchanged by a serine; the results of these experiments are presented in the next paragraph.

In addition, strong proton interactions are found in the coordination sphere of complex **2**; measurement of a sample where H_2O was exchanged by D_2O showed that these interactions did not disappear; so apparently the presence of water molecules as ligands can be ruled out. This interaction can instead be attributed to a non-

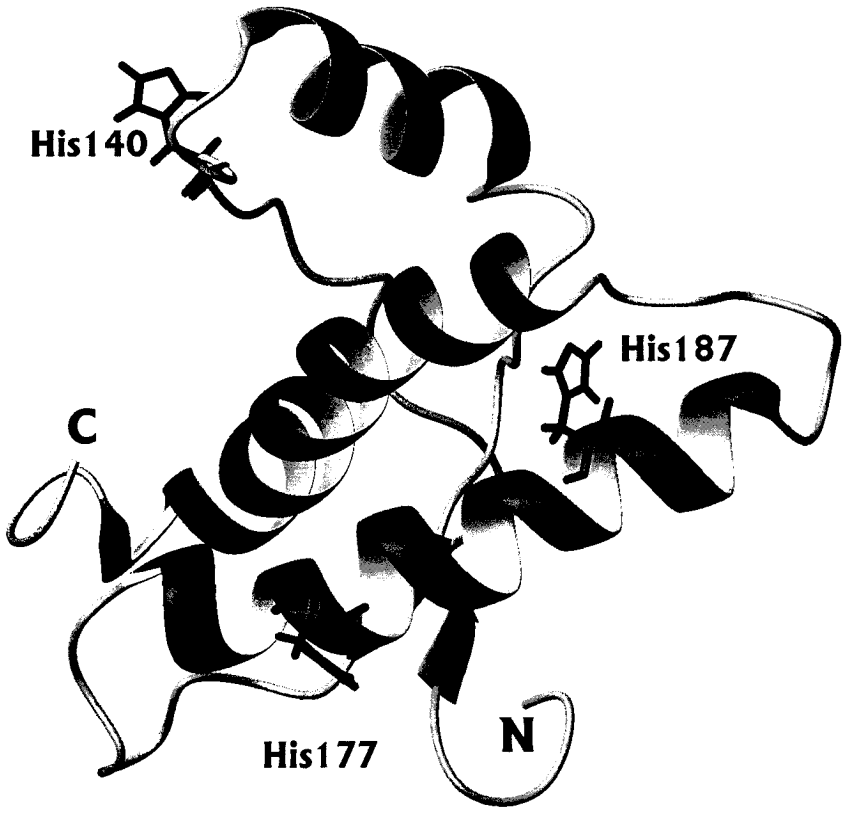

Figure 2.4.3.1. View of the three histidine residues in the three dimensional structure of murine PrP(121-231).

exchangeable proton of the protein, being either a C-bound or a non-accessible proton.

Complex **1**. Only preliminary results were obtainend by the ENDOR analysis of this complex; the EPR parameters indicate an involvement of aspartic acid or glutamic acid, possibly with a decreased pK_a due to copper(II) binding. Pulse EPR and ENDOR also indicate that no nitrogen atom is involved as ligand in this complex.

Complex **3**. According to the ligation modes expected from the $g_{//}$ and $A_{//}$ values (4N, 3N1O, 2N2O or 2N1S1O), the ENDOR measurements also argue that more than one nitrogen atom is present as ligand in this complex that is only visible at pH ≥ 7; the EPR parameters argue for the involvement of a backbone nitrogen.

To ascertain that the identified complexes **1-3** are located within the C-terminal

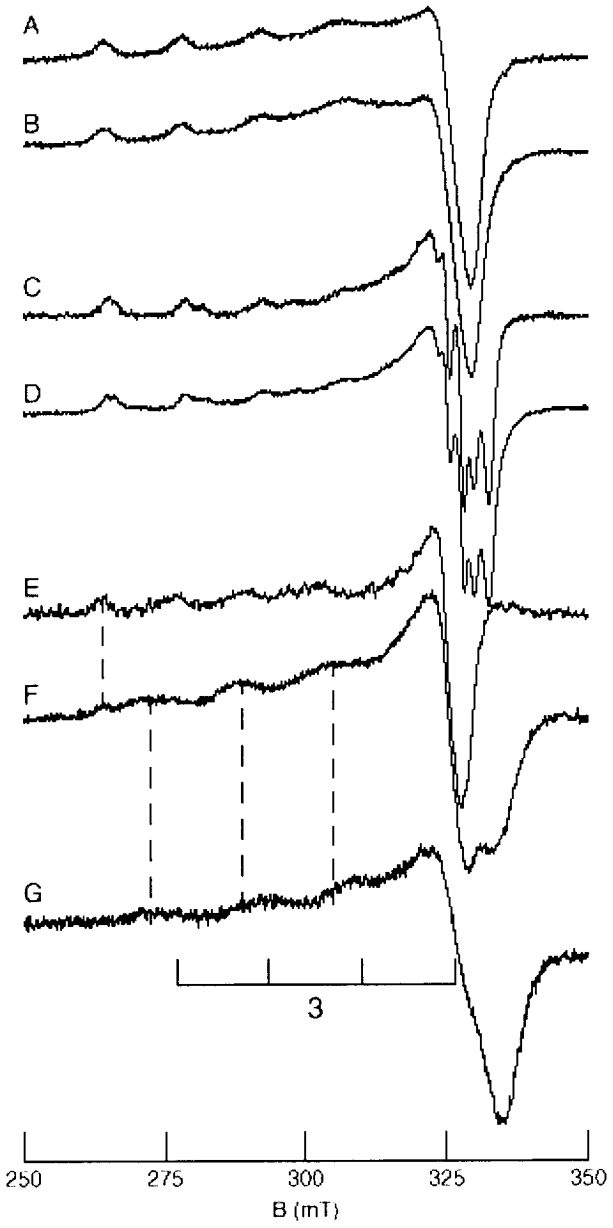

Figure 2.4.3.2. EPR-spectra of the peptide 58-91 between pH 3 and 7.4 in the presence of Cu^{2+}. Spectra of buffer at pH 4 with 0.3 mM $CuCl_2$ (A), mPrP(58-91) with 4 molar equivalents of Cu^{2+} at pH 4 (B), buffer at pH 5 with 0.3 mM $CuCl_2$ (C), mPrP(58-91) with 4 molar equivalents of Cu^{2+} at pH 5 (D), buffer at pH 6 with 0.3 mM $CuCl_2$ (E), mPrP(58-91) with 4 molar equivalents of Cu^{2+} at pH 6 (F), mPrP(58-91) with 4 molar equivalents of Cu^{2+} at pH 7.4 (G) are shown.

domain, control measurements were done on a synthetic peptide with a sequence corresponding to the region containing the four octapeptide repeat units supposed to bind copper in PrP, i.e. PrP(58-91) (sequence: GQPHGGGWGQPHGGSWGQPHGGSWGQPHGGGWGQ). The PrP(58-91) peptide was acetylated at the N-terminus and amidated at the C-terminus to avoid artifacts due to artificial charges. For the measurements of Cu(II) binding of PrP(58-91), 2 or 4 molar equivalents of $CuCl_2$ were added to a stock solution of the peptide to reach a final 0.1 mM peptide concentration at pH 3, 4, 5, 6 and 7.4. The buffers used were the same as that used for pH dependent measurements on PrP(23-231) and PrP(121-231). We found that PrP(58-91) only starts binding Cu(II) at pH values above 5, confirming that the signals of complex **1** and **2** found at lower pH values arise from binding to the C-terminal region (*Figure 2.4.3.2.* B and D, and corresponding protein-free buffer-Cu(II) spectra, *Figure 2.4.3.2.* A and C). At pH 6, the EPR spectrum of a sample of PrP(58-91) in the presence of four molar equivalents Cu(II) consists of two components (*Figure 2.4.3.2.* F), one being the copper(II) bound cacodylate signal (compare with the buffer-Cu(II) spectrum, *Figure 2.4.3.2.* E), the other the peptide bound Cu(II). This second component presents EPR parameters corresponding to those of complex **4** found in PrP(23-231). These data thus demonstrate that the N-terminal region, in accordance with the results of Viles et al. (1999), starts binding copper at pH≥6, and that at lower pH values the C-terminal domain is the preferred copper(II) ligand.

At pH 7.4, the EPR spectrum of the Cu(II)-PrP(58-91) complex consists of two components (*Figure 2.4.3.2.* G): one corresponds to complex **4** still found at pH 6, the other is a complex **3**-like coordination type. A recent detailed analysis of copper(II) binding to the N-terminal region of PrP also showed these same modes of binding at pH 6 and 7.45 (Aronoff-Spencer et al., 2000). Since we also find a complex **3** coordination type in PrP(121-231) at pH between 7 and 8, it is very likely that two different complexes are formed with similar EPR parameters: both complexes present a large involvement of nitrogen atoms in the Cu(II) ligation (2O2N, 1O3N, 4N or 2N1S1O). So at pH≥7 both PrP(121-231) and PrP(58-91) have very similar complex 3-type Cu(II) binding sites that give rise to quasi the same EPR spectrum. Further investigation should elucidate the nature of these distinct complex **3**-type coordination modes.

2.4.4. Measurements on histidine mutants of the C-terminal domain PrP(121-231)

In order to determine which of the three histidine residues of the C-terminal domain (*Figure 2.4.3.1*) is involved in copper(II) binding we prepared the three corresponding point mutations His140Ser, His177Ser and His187Ser and analysed them by EPR after purification from periplasmic extracts of *Escherichia coli* bacteria.

2.4.4.A. Cloning

For construction and expression of the mPrP(121-231) histidine to serin mutants the previously described phagemid pPrP-CRR was used (Hornemann and Glockshuber, 1996). Site-directed mutagenesis was performed according to (Kunkel, 1985; Kunkel et al., 1991) using uridinylated single stranded pPrP-CRR from M13K07 phage infection of the E.coli strain CJ236 with oligonucleotide primers (5'-CCAATCATTGCCAAAAGAGATCATGGGTCGACT-3') for His140Ser, (5'-AATATTGACGCAGTCGCTCACGAAGTTGTTCTG-3') for His177Ser and (5'-CGTCGTGGTGACCGTCGACTGCTTAATGGT-3') for His187Ser.

Successful mutagenesis was verified by dideoxynucleotide sequencing of the entire PrP(121-231) genes.

2.4.4.B. Purification

The purification of the histidine variants was performed following the purification protocol established for the wild-type PrP(121-231), as previously described (Liemann and Glockshuber, 1999). The molecular mass of the purified mutants was confirmed by MALDI mass spectrometry.

Circular dichroism (CD) spectra of the mutants in distilled water (pH 5.6) confirmed that they were folded correctly and had a tertiary structure similar to that of the wild-type protein.

The purification yields were very high for the His140Ser and the His177Ser mutants, in the range of 10-13 mg pure protein per litre of bacterial culture, while the yield of the His187Ser mutant was only 0.2 mg/L bacterial culture. The purification of the His187Ser from periplasmic inclusion bodies was unsuccessful. Very likely, the introduction of a serine residue in the environment of the residue 187 results in a large destabilisation of the protein as was previously found for the Phe198Ser

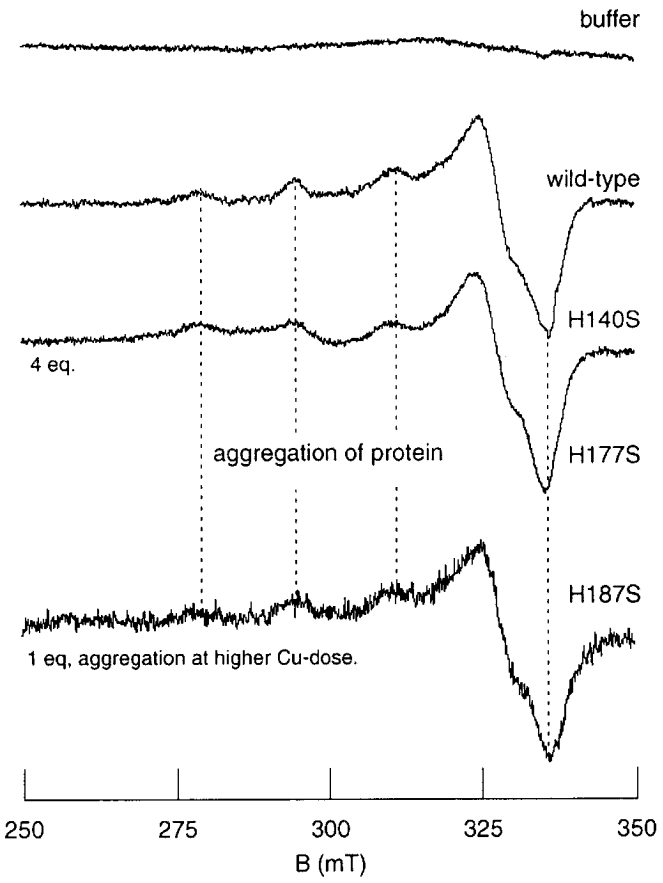

Figure 2.4.4.1. EPR spectra of PrP(121-231) at pH 7.4 in the presence of the Cu(II) molar equivalents indicated.

mutation of the C-terminal domain, that is located in the same region in the three dimensional structure (Liemann and Glockshuber, 1999).

2.4.4.C. EPR measurements

To determine which of the three histidine residues in the C-terminal domain is involved in complex **2**, measurements at pH 6 were planed since at this pH complex **2** is maximally populated. Unfortunately, strong aggregation of the histidine variants H177S and H187S at this pH already in the presence of one molar equivalent $CuCl_2$ impeded EPR measurements. The H140S variant was partially soluble in the presence of one molar equivalent $CuCl_2$, but the resolution of the spectrum obtained was too low to allow interpretation of the signal.

Measurements at pH 4 were not favourable because comparing the CD spectra of the PrP(121-231) histidine mutants in 10 mM formic acid/NaOH buffer with the wild-type spectrum under the same conditions indicated that not all of the variants were properly folded at this pH. Therefore, measurement at pH 4 was not appropriate.

Measurements at pH 7.4 in 10 mM MOPS/NaOH were done on 0.1 mM H140S and H187S samples. The H140S variant was soluble even upon addition of 4 molar equivalents $CuCl_2$; the H187S variant in the presence of one molar equivalent Cu(II) aggregated slowly, so that a spectrum with a reliable signal-to-noise ratio was obtained (*Figure 2.4.4.1*). Since also the spectrum of the wild-type protein in the presence of four molar equivalents Cu(II) does not show a discernible complex **2** component at pH 7.4, and only the dominant complex **3** signal is visible, measurements on the variants at neutral pH allowed exclusively to establish that complex **3** is observed also in the H140S and H187S variants, but anything could be established about the formation of complex **2**. Therefore, no information about the possibility of formation of this complex could be obtained by the preparation of these proteins.

2.4.5. EPR measurements on PrP(23-231) mutants D178N, F198S and E200K

In order to determine a possible link between copper(II) binding and prion diseases, we analysed the behaviour of three point mutations of full-length mPrP(23-231) corresponding to three mutations related to inherited prion diseases in humans.

The mutants analysed in this work were the aspartate to asparagine exchange at position 178, shown to induce fatal familial insomnia (FFI), if the residue at position 129 is a methionine (*Figure 2.4.5.1*) (Gambetti et al., 1995), the phenalanine to serin exchange at position 198, shown to be responsible for the Gerstmann-Sträussler-Scheinker syndrome (*Figure 2.4.5.1*) (Unverzagt et al., 1997), and the glutamate to lysine exchange at amino acid 200, linked to the inherited form of Creutzfeldt-Jakob disease (*Figure 2.4.5.1*) (Spudich et al., 1995).

The preparation of these mutants is discussed in chapter 4, paragraph 4.2.2.

Spectra were measured at pH 4 as well as at pH 6 and 7.4 in the presence of up to ten molar equivalents of $CuCl_2$, according to the stoichiometry found for the wild-type protein (*Figure 2.4.5.2*).

PrP(23-231) D178N mutant (*Figure 2.4.5.1*). This mutant does not present any change in the EPR spectra with respect to the wild-type protein at pH 4.0 and 6.0. Complex **1** and complex **2** are formed at both pH values at approximately the same ratio as for the wild-type and the protein is shown to also bind up to ten molar equivalents $CuCl_2$ (*Figure 2.4.5.2*). Unfortunately, aggregation of the 0.1 mM protein at pH 7.4 in 10 mM MOPS/NaOH, which already occurs in the absence of copper(II),

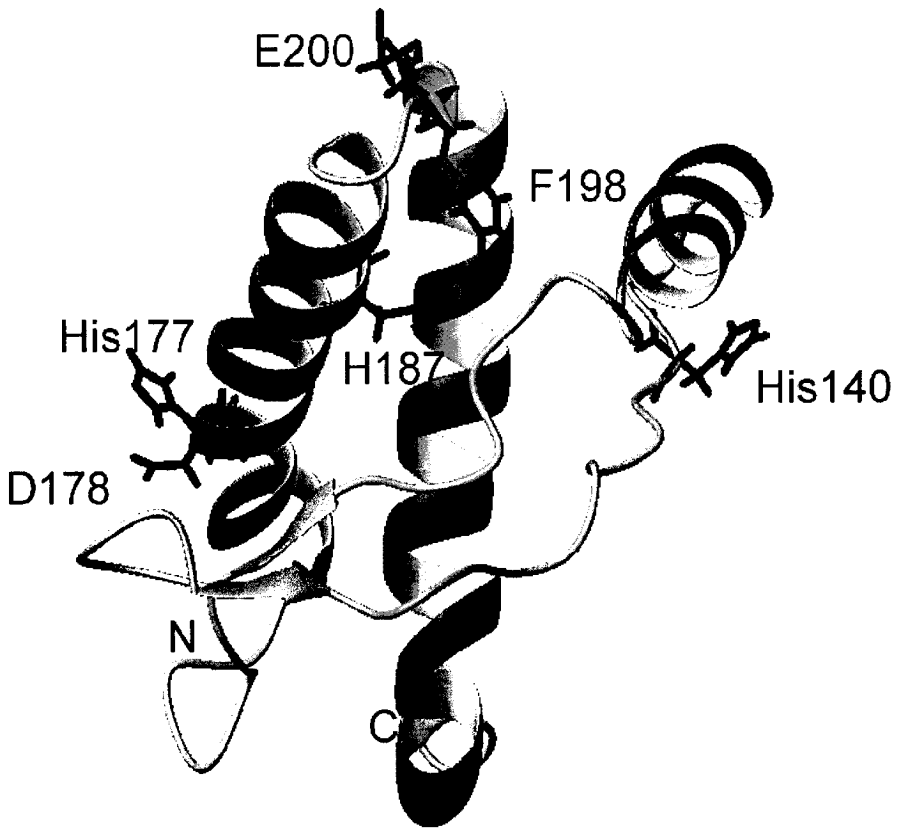

Figure 2.4.5.1. Location of residues D178, F198 and E200 relative to the three histidine residues in the three dimensional structure of PrP(121-231)

impeded EPR measurements on this mutant at pH 7.4.

PrP(23-231) F198S mutant (*Figure 2.4.5.1*). At pH 4.0 and 6.0 the spectra of this mutant strongly differ from those of the wild-type (*Figure 2.4.5.2*). At pH 4.0 complex **2** is clearly missing and the formate-copper(II) signal from the buffer still appears upon addition of four molar equivalents $CuCl_2$. Besides the buffer signal, a second component is present at this pH; interestingly, subtraction of the spectrum of the buffer-copper(II) complex from the spectrum of PrP(23-231) F198S results in a rest-spectrum which does not correspond to the spectrum typical for complex **1**. Also comparison with the corresponding subtraction-spectrum of the wild-type minus complex **2** at pH 4, which results in the spectrum of complex **1**, allows to conclude that the complex found for this mutant at pH 4.0 is different from complex **1**. The parameters of the two complexes are reported in *Table 2.4.5.1.*

Complex	$g_\perp$	$g_\parallel$	$A_\perp$ [MHz]	$A_\parallel$ [MHz]
1	2.068	2.332	12	452
F198S	2.058	2.32	35	480

Table 2.4.5.1. Comparison of the EPR parameters of complex 1 of wild type PrP(23-231) with the parameters found for the complex formed at pH 4.0 by the F198S mutant of PrP(23-231).

At pH 6.0 a complex different from that formed at pH 4.0 is found. The EPR parameters for this complex are:$g_\perp$ = 2.061, $g_\parallel$ = 2.295, $A_\perp$ = 10 MHz, $A_\parallel$ = 510 MHz. Clearly this complex is also not complex **2** as well.

In contrast to the case found for previous pH values, the spectrum of the F198S mutant at pH 7.4 is similar to that of the wild-type protein, although complex **3** does no longer dominate the spectrum; the second component of the spectrum possibly does not correspond to the complex **2** found for the wild-type.

Previous studies showed that the replacement F198S strongly destabilises the prion protein. The hydrophobic phenylalanine residue buried in the hydrophobic core of the protein in the region between helix 2 and 3 is replaced by the non-hydrophobic residue serin, and possibly induces local structural rearrangements within the protein

scaffold (Riek et al., 1996; Liemann and Glockshuber, 1999); perhaps these rearrangements within the C-terminal domain dislocate the copper(II) ligands within the region where complex **1** and complex **2** are located, therefore preventing complex formation, and maybe establishing novel copper(II) sites. The environment of complex **3** obviously remains unaffected from the mutation since complex **3** is observed at pH 7.4 as in the wild-type protein. In addition, if histidine 187 is responsible for copper(II) binding leading to the features of complex **2**, the fact that this residue comes in near vicinity to the site of mutation at position 198 in the three dimensional fold could also explain the absence of complex **2** due to an altered environment of His187.

PrP(23-231) E200K mutant (*Figure 2.4.5.1*). While the spectra at pH 4.0 and 6.0 of the E200K mutant of PrP(23-231) are almost identical to the spectra of the wild-type protein, the spectrum at pH 7.4 shows that complex **3** is clearly missing (*Figure 2.4.5.2*). The only complex formed by this mutant at pH 7.4 is found to have the same EPR parameters as the ones of complex **2**, allowing to argue that the histidine involved in complex **2** is not located in a site of the protein affected by this point mutation. On the contrary, since this is the only mutant in which lack of complex **3** is observed, complex **3** is likely to be close to residue 200 in the three dimensional fold of the C-terminal domain.

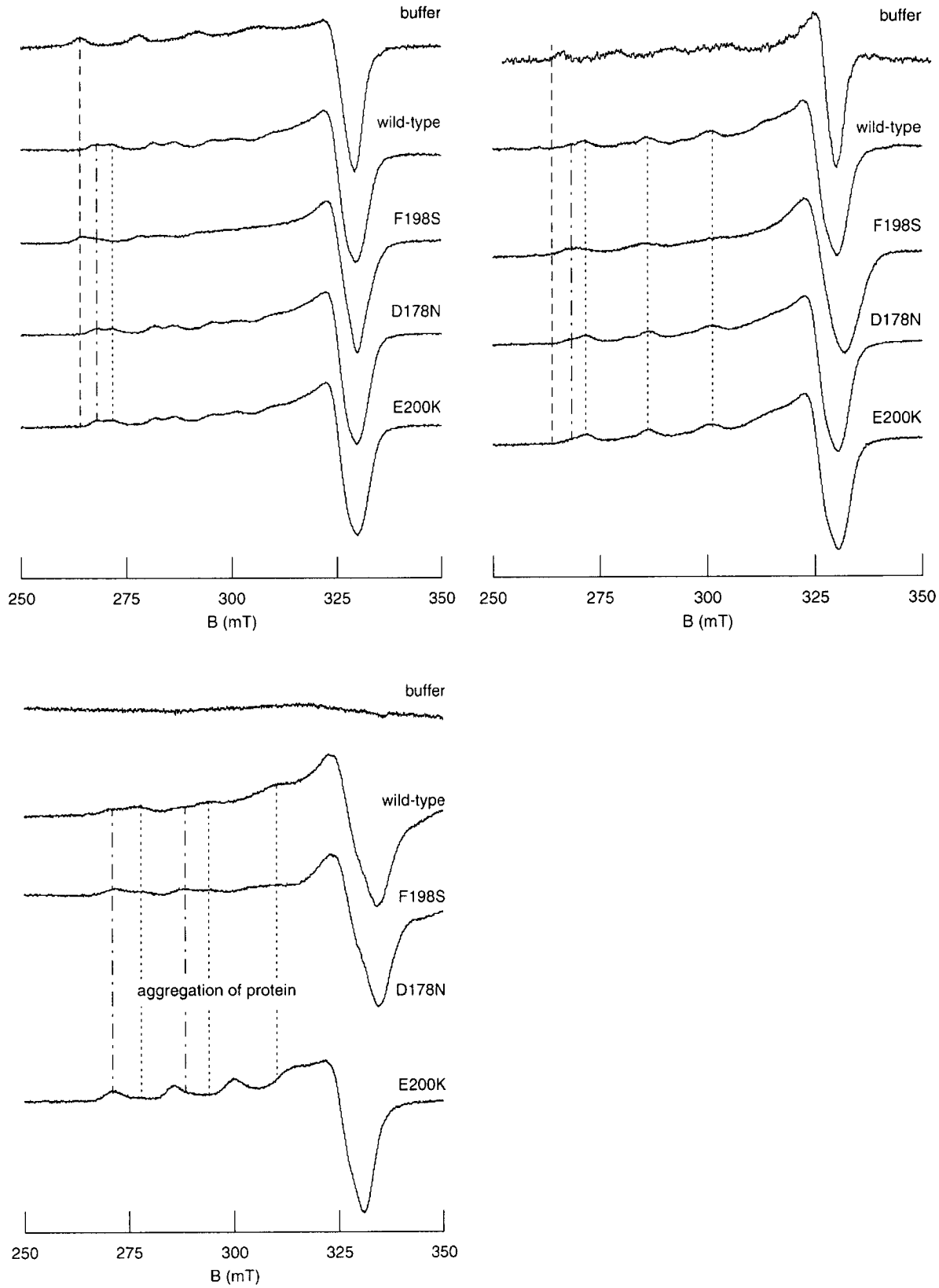

Figure 2.4.5.2. EPR spectra of PrP(23-231) and mutants at pH 4.0 (top left), pH 6.0 (top right) and pH 7.4 (bottom left) in the presence of 10 molar equivalents of Cu^{2+}.

In summary, the EPR measurements on the three point mutations D178N, F198S and E200K of PrP(23-231) allow one to speculate that His177 is not the one involved in copper(II) binding within complex **2**, because the loss of the negative charge of asparagine 178 would be expected to destabilise the coordination sphere of the metal ion in a way that complex **2** could no longer be formed. Instead, complex **2** is visible in the spectra of this mutant as well at pH 4.0 as at pH 6.0.

The clear loss of the signal attributable to the histidine-bound copper(II) of complex **2** in the F198S mutant is instead a good hint to argue that histidine 187 could be involved in complex **2**, since because of the vicinity of His187 and residue 198 and lack of complex **2** in the F198S mutant. The F198S mutant is known to be highly destabilised with respect to the wild-type, and perhaps a slightly changed conformation of this protein could also indirectly affect copper(II) binding. All experimental data however cannot entirely exclude that the solvent exposed histidine 140 is the copper(II) binding residue in complex **2**.

3. INFLUENCE OF COPPER(II) BINDING TO PrP ON THE DIGESTION PATTERN WITH SEVERAL PROTEASES

3.1. Copper(II) binding and structure of PrP(23-231)

The finding that fragments of the prion protein corresponding to the N-terminal region of PrP(23-231) are able to bind copper(II) ions via the histidine residues contained in each of the fourfold repeated octapeptide (PHGGGWGQ) prompted some investigators to suggest that binding of copper(II) could introduce a tertiary structure within the N-terminal region 23-120 of PrP (Viles et al., 1999; Stockel et al., 1998; Wong et al., 2000d; Wong et al., 2000a), which, as found by NMR spectroscopy, is otherwise highly flexible and disordered in all presently known PrP structures (Riek et al., 1997; Donne et al., 1997; Lopez Garcia et al., 2000; Zahn et al., 2000).

A further hint on the ability of the N-terminal region 90-120 to form a defined structure comes from the fact that the normal cellular isoform of PrP, PrP^{C}, is completely digested by proteinase K, while the scrapie isoform PrP^{Sc} is partially resistant to digestion and its N-terminal degradation stops approximately after residue 90 (Safar et al., 1990; Weissmann et al., 1996). Proteinase K resistance of this fragment is an indication that the region between residue 90 and residue 120 is no longer unstructured in PrP^{Sc}, in contrast to the NMR results on the PrP^{C} structure. Thus, copper(II) binding to the N-terminal PrP^{C} segment may principally induce tertiary structure in segment 90-120 and thereby this region facilitate the conformational transition to the infectious isoform PrP^{Sc}.

To further investigate this idea, we performed partial proteolysis of the recombinant mouse PrP(23-231) with five different proteases in the absence and presence of excess copper(II) ions at pH 7.4 and 37°C. If binding of copper(II) induces structure formation of the disordered N-terminal region, one would expect changes in the pattern of digestion upon binding, since the proteolysis should become more difficult due to lower accessibility for the proteases to the digestion sites. The proteolytic fragments were separated by SDS-PAGE and stained with Coomassie blue; resulting fragments were

further analysed by Edman sequencing after blotting onto PVDF membranes to identify the proteolytic fragments.

We found that upon limited digestion with the proteases proteinase K, subtilisin, thermolysin, trypsin and chymotrypsin in the presence of 1 up to 10 molar equivalents copper, the proteolytic patterns did not differ from those in absence of the metal ion. Both in the presence and absence of copper(II) a rapid and complete degradation of the flexible N-terminal region was observed for all proteases, followed by a slower attack of the folded C-terminal domain.

We also performed as control digestion of the fragment PrP(121-231) corresponding to the C-terminal, structured domain of murine PrP in absence and presence of copper(II) and found that the slower digestion in the presence of Cu^{2+} is attributable to the progressive aggregation of the protein induced by the copper(II) ions.

3.2. Limited proteolysis of PrP(23-231) and PrP(121-231) in absence and presence of Cu^{2+}

In order to analyse the dependence of the digestion pattern of PrP(23-231) and PrP(121-231) on the presence of Cu^{2+}, samples of 20 μM protein in 10 mM MOPS/NaOH, pH 7.4, 150 mM NaCl were digested for 1 hour at 37°C by one of the proteases: proteinase K (48.5 nM), subtilisin (0.4 μM), thermolysin (33.3 μM), trypsin (3.7 μM) and chymotrypsin (1.25 μM and 46.3 nM). 0, 1, 5 or 10 molar equivalents of $CuCl_2$ were added to the samples before addition of the proteases. The digestion was stopped by addition of 1 mM phenylmethylsulphonylfluoride (PMSF) or 1 mM EDTA in the case of thermolysin. The samples were then boiled in reducing SDS-PAGE sample buffer (62.5 mM Tris/HCl, 10% glycerol, 2% SDS, 0.005% bromphenol blue, 5 mM 2-mercaptoethanol) at 96°C for 5 minutes and the reaction products were separated by SDS-PAGE in 15% polyacrylamide gels. Protein bands were either stained with Coomassie blue or electrotransferred from the gel to a PVDF membrane and stained with Amido Black for Edmand sequencing.

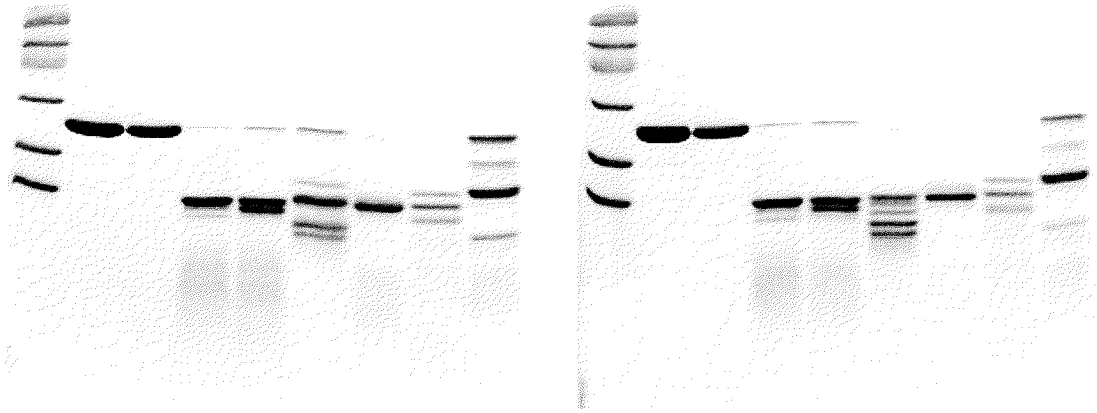

Figure 3.2.1. Coomassie blue stained gels of 20 μM PrP(23-231) digested at pH 7.4 and 37°C with several proteases in the absence (left) and presence (right) of 5 molar equivalents $CuCl_2$. The same digestion pattern is observed for 10 molar equivalents of $CuCl_2$. Lane 1: molecular mass standards; lane 2: PrP(23-231) in water, reference; lane 3: PrP(23-231) incubated without proteases; digested samples: lane 4 proteinase K, lane 5 subtilisin, lane 6 thermolysin, lane 7 trypsin, lane 8 and 9 chymotrypsin by two different concentrations (see text for details).

The results of the digestion studies on PrP(23-231) in the presence and absence of Cu^{2+} after Coomassie blue staining of the proteolytic fragments separated on 15% polyacrylamide gels are shown in *Figure 3.2.1.* A summary of the results from Edman

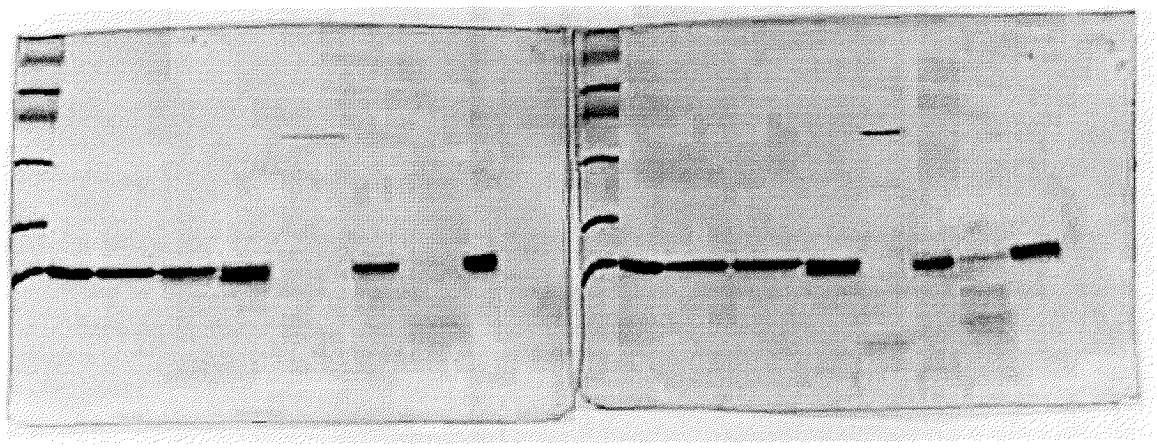

Figure 3.2.2. Coomassie blue stained gels of 20 μM PrP(121-231) digested at pH 7.4 and 37°C with several proteases in the absence (left) and presence (right) of 5 molar equivalents $CuCl_2$. Lane 1: molecular mass standards; lane 2: PrP(121-231) in water, reference; lane 3: PrP(121-231) incubated without proteases; digested samples: lane 4 proteinase K, lane 5 subtilisin, lane 6 thermolysin, lane 7 trypsin, lane 8 and 9 chymotrypsin by two different concentrations (see text for details).

sequencing of the digested bands is reported in *Table 3.2.1.*

Up to ten molar equivalents of $CuCl_2$ were added because the full-length PrP was shown in our EPR measurements to bind up to ten copper(II) ions per protein molecule (see chapter 2). As also shown in *Figure 3.2.1* for the limited digestion of mPrP(23-231) in the absence and presence of five molar equivalents Cu^{2+}, no difference can be seen in the pattern of digestion by all of the proteases. The same lack of proteolytic resistance is observed in the presence of 10 molar equivalents of Cu^{2+}. The digestion by thermolysin even appears faster by addition of 5 to 10 molar equivalents Cu^{2+}, but a time-dependent analysis of the proteolysis showed that exactly the same fragments are generated both in the absence and presence of $CuCl_2$. The somewhat faster digestion by thermolysin in the presence of 10 molar equivalents of $CuCl_2$ proved to be reproducible but was not analysed further. In contrast, digestion by all other proteases occurred at the same speed independent of the presence and concentration of $CuCl_2$.

Thereafter we analysed the digestion of the isolated C-terminal domain PrP(121-231) under exactly the same conditions. 0, 1 or 5 molar equivalents $CuCl_2$ were added before digestion at pH 7.4 and 37°C (*Figure 3.2.2*). PrP(121-231) was found to slowly aggregate upon addition of $CuCl_2$: as the aggregation of PrP(121-231) restricted the digestion to the fraction of the protein which remained in solution, the digestion of the copper(II)-containing samples appeared to proceed slower than that of the copper(II)-free samples. This difference is however only an artifact caused by decreased concentration of soluble substrate protein so that a direct comparison of the digestion patterns of PrP(121-231) and the full-length protein was not possible.

Protease Used	**Cleavage sites (in both presence and absence of copper(II))**
Proteinase K	•A116-A117 as well as G119-A120 and A118-G119 •cleavage between W and G within the octapeptide repeat region •other fragments correspond to peptides starting at the N-terminus
Subtilisin	•A116-A117 and some A118-G119 •M134-S135

	•cleavage between W and G within the octapeptide repeat region •other fragments correspond to peptides starting at the N-terminus
Thermolysin	•N108-L109 •W99-N100 •H111-V112 •other fragments correspond to peptides starting at the N-terminus
Trypsin	•K110-H111 •cleavage between W and G within the octapeptide repeat region
Chymotrypsin (1.25 μM)	•W99-N100 •L109-K110 •M134-S135
Chymotrypsin (46.3 nm)	•W99-N100 •L109-K110 •M134-S135 •other fragments correspond to peptides starting at the N-terminus

Table 3.2.1. Cleavage sites of the proteases indicated by the degradation of PrP(23-231) in presence or absence of $CuCl_2$.

In conclusion, our analysis by limited proteolysis could not confirm the hypothesis that addition of Cu^{2+} to the full-length PrP(23-231) induce structure into its N-terminal, disordered region at least under our conditions. The digestion pattern of PrP(23-231) upon copper(II) binding does indeed not show substantial changes compared to the case of proteolysis of the apoprotein. The observed rapid and complete digestion of the N-terminal domain, followed by slower proteolysis of the C-terminal domain, is observed independent of the presence or absence of bound copper(II). The digestion results with many cleavage sites in segment 108-118 also fully confirm the domain border of the folded C-terminal domain PrP(121-231), which initially had been identified by periplasmic degradation in *E.coli* of secreted PrP constructs (Hornemann and Glockshuber, 1996). On the other side, the lability of the bond between methionine 134 and serine 135 in the C-terminal domain is explained by its location in the three dimensional structure in a solvent accessible loop which renders it easily accessible for digestion (*Figure 3.2.3*).

We conclude that the octapeptide repeats containing region of PrP(23-231) does not adopt a defined tertiary structure after copper(II) binding.

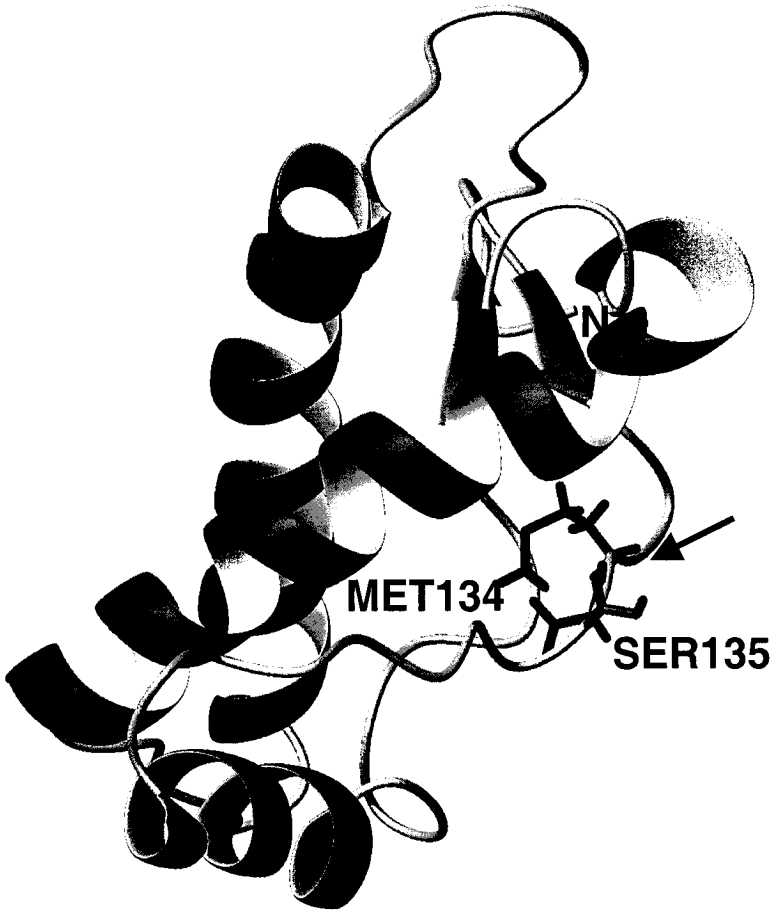

Figure 3.2.3. Location of the Met134-Ser135 cleavage site in the three dimensional structure of mPrP(121-231).

4. THERMODYNAMIC STABILITY OF PrP(23-231) AND PrP(121-231) WILD-TYPE AND MUTANTS

4.1. Concentration and Ionic strength dependence of folding intermediate formation of PrP(23-231) and PrP(121-231) at acidic pH

The finding that the folding of mPrP(121-231) (Hornemann and Glockshuber, 1998) as well as human PrP(90-231) (Swietnicki et al., 1997) at acidic pH occurs as a three-state transition with formation of a stable folding intermediate rich in β-sheet secondary structure raised the question about the conditions of formation of this intermediate in view to identify a possible relation between the generation of this folding intermediate and the conformational modification supposed to be at the origin of PrP^{Sc} formation.

We observed that the formation of the urea-induced folding intermediate was strongly dependent on the protein concentration and ionic strength of the medium. In this section we analysed the influence of these parameters on the folding of both recombinant mouse PrP(23-231) and PrP(121-231). The CD spectra of both proteins show two minima at 208 and 222 nm, as expected for proteins rich in α-helical structures, so equilibrium unfolding was monitored by measuring the ellipticity at 222 nm as a function of denaturant (urea) concentration at pH 4.0 in 50 mM formic acid/NaOH buffer at 22°C.

First, we found that formation of the intermediate at a ionic strength of 165 mM was only observed at higher protein concentrations: consequently, this intermediate state is oligomeric, possibly a dimer. Exactly the same concentration dependency was found for mPrP(23-231) and mPrP(121-231). In 50 mM formic acid/NaOH buffer at pH 4.0 and in the presence of 135 mM sodium chloride, the intermediate becomes evident only at protein concentrations ≥15 μM. The intermediate is no longer evident at a protein concentration lower than 10 μM. At a concentration of 28 μM, PrP(121-231) and the full-length protein show a very pronounced three-state transition with a strong population of the intermediate at about 3.5 M urea (*Figure 4.1.1* and *Figure 4.1.2*). Interestingly, the fact that the intermediate formation for both C-terminal domain and full-length protein

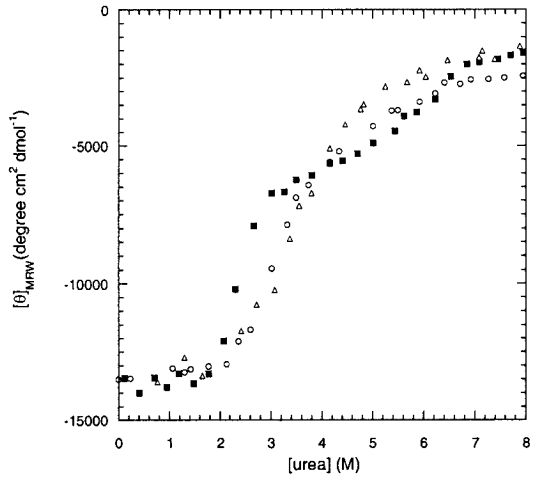

Figure 4.1.1. Unfolding transitions of mPrP(121-231) at 22°C and a ionic strength of 165 mM in the presence of urea at pH 4.0. Protein concentrations: (Δ) 8.8 μM; (O) 15 μM; (■) 28.5 μM.

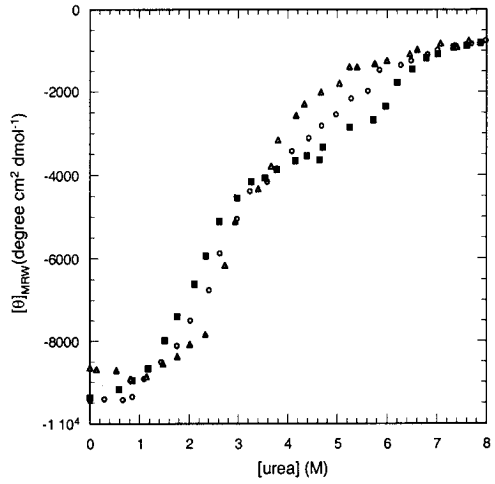

Figure 4.1.2. Unfolding transitions of mPrP(23-231) at 22°C and a ionic strength of 165 mM in the presence of urea at pH 4.0. Protein concentrations: (Δ) 8.8 μM; (O) 15 μM; (■) 28.5 μM.

shows the same concentration dependence indicates that the ability of PrP to form this intermediate is an intrinsic property of the C-terminal domain PrP(121-231) and is not hindered or facilitated by the N-terminal segment 23-120. The intermediate is maximally

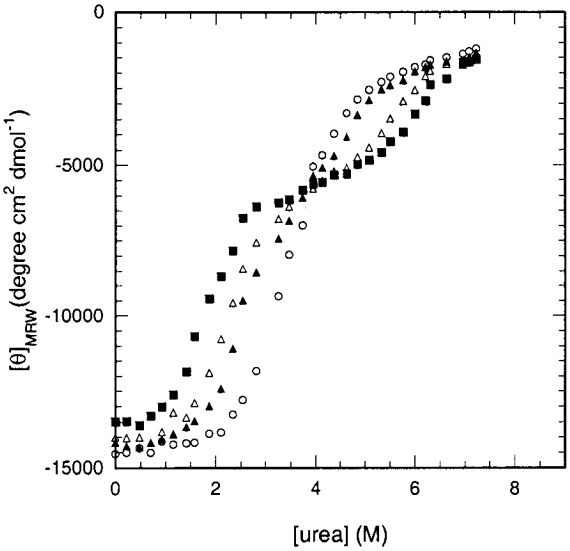

Figure 4.1.3. Dependence of urea-induced unfolding transitions of mPrP(121-231) at 22°C and pH 4.0 on ionic strength. Sodium chloride concentration: no NaCl (O); 50 mM NaCl (▲); 100 mM NaCl (Δ); 150 mM NaCl (■).

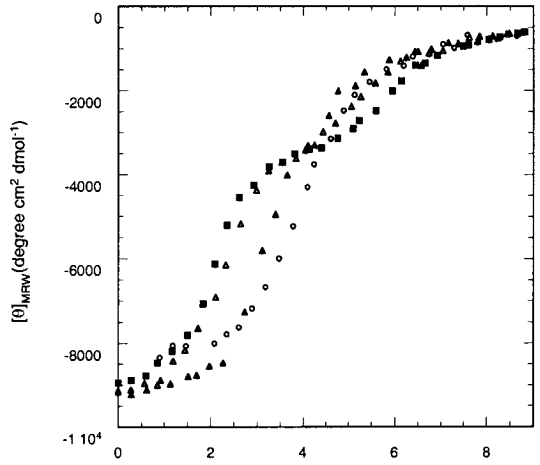

Figure 4.1.4. Dependence of urea-induced unfolding transitions of mPrP(23-231) at 22°C and pH 4.0 on ionic strength. Sodium chloride concentrations: no NaCl (O); 50 mM NaCl (▲); 100 mM NaCl (Δ); 150 mM NaCl (■).

populated at a urea concentration of 3.5 M and, as already found for PrP(121-231), also for PrP(23-231) the modified shape of the CD spectra with a minimum ellipticity at about 215 nm indicates that the conformation of this intermediate is rich in β-sheet elements at the expense of the α-helical domains, as found for the scrapie isoform PrP^{Sc}.

The observation that probably the formation of this folding transition state implicates dimerisation or oligomerisation of the protein is a good argument supporting the nucleation-polymerisation model in which a monomeric scrapie form PrP^{Sc} is supposed to be in fast equilibrium with the non-infectious isoform PrP^{C}; PrP^{Sc} is then itself in slow equilibrium with small PrP^{Sc} oligomers that act as nuclei and then grow irreversibly to larger aggregates (see paragraph 1.8 and Jarrett and Lansbury, 1993). Indeed our finding that dimerisation or oligomerisation of the protein under acidic conditions would induce the formation of an oligomeric PrP^{Sc}-like unfolding intermediate supports the idea that a pre-existing dimer or oligomer could trigger aggregation of the protein in scrapie amyloids.

Furthermore, the formation of the intermediate was shown to be dependent on the ionic strength of the medium. Sodium chloride concentrations of 0 to 150 mM were used in a 50 mM formic acid/NaOH buffer at pH 4.0 at a protein concentration of 28 μM where the intermediate is well populated at a ionic strength of 165 mM (*Figure 4.1.3* and *Figure 4.1.4*). Also in this case, the salt dependence was found to be practically the same for mPrP(121-231) and mPrP(23-231); the intermediate was observed only if at least 50 mM sodium chloride is added to the solution. In the presence of 100 or 150 mM sodium chloride urea-induced unfolding clearly progresses through the intermediate. Thus, ionic strengths in the physiological range are optimum for intermediate formation, indicating that the intermediate may indeed be relevant as a low-molecular weight precursor of prions.

In summary the folding of the prion protein at acidic pH, independent on the presence or absence of the unstructured, N-terminal region, progresses through the formation of a β-sheet rich intermediate state *in vitro* only if the protein concentration is higher than 15 μM at ionic strengths above 80 mM.

As previously found for mPrP(121-231) (Hornemann and Glockshuber, 1998) and human PrP(90-231) (Swietnicki et al., 1997), full-length mPrP(23-231) shows pH dependence for the formation of the intermediate. Only at pH values lower than 5 a three state model of folding can be observed. The apparent pK of this transition is 4.8 (*Figure 4.1.5*). Similarly, the apparent pK_a of the transition from the native to the

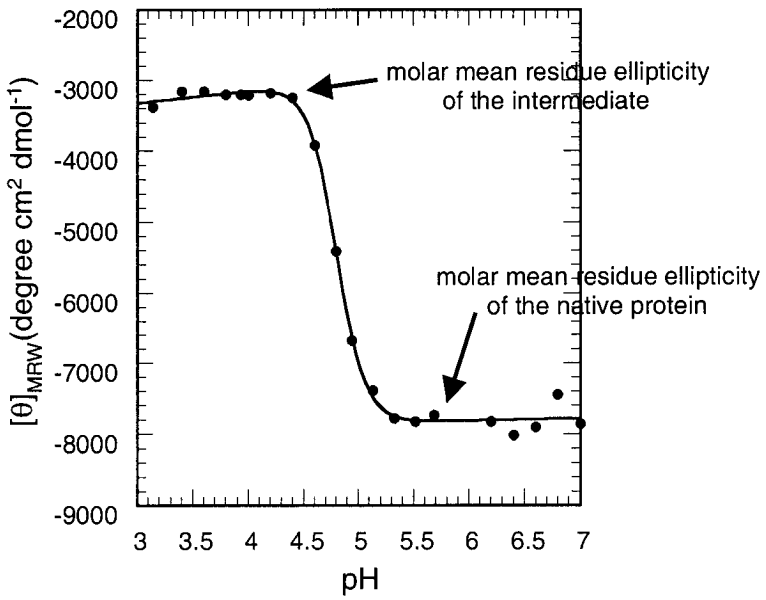

Figure 4.1.5. Acid-induced unfolding of 28 μM PrP(23-231), in the presence of 3.5 M urea and 135 mM NaCl, monitored by the mean residue ellipticity at 222 nm.

intermediate state was found to be 4.5 for PrP(121-231) (Hornemann and Glockshuber, 1998), and a value of 5.1 was reported for human PrP(90-231) (Swietnicki et al., 1997). Again those data indicate that the unstructured, N-terminal part of PrP(23-231) plays a minor role in the conformational change leading to the formation of this intermediate.

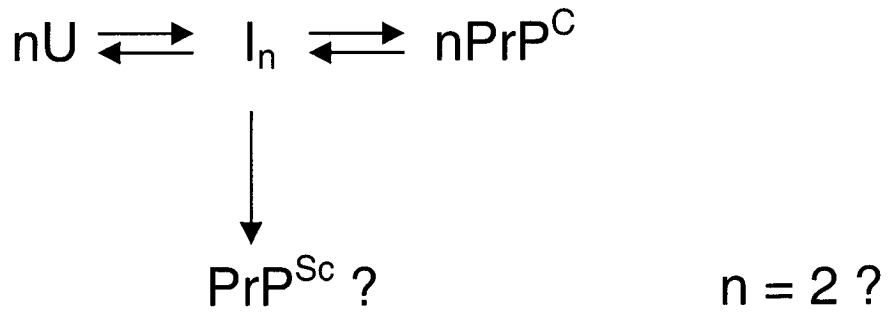

Figure 4.1.6. Proposed model for PrP^{Sc} formation at acidic pH from an oligomeric folding intermediate of PrP. U: unfolded PrP; I_n: oligomeric folding intermediate composed of n subunits. As proposed in the text, the intermediate could be a dimer of PrP, so n may be 2.

Since *in vivo* studies on scrapie-infected cells show that PrP^{Sc} formation and aggregation may take place during the endocytic pathway, in a milieu where the pH ranges values of 4.0 to 6.0, the observed propensity of the protein to form this scrapie-like intermediate state *in vitro* only under strictly defined conditions in this same pH range, relates our *in vitro* model to the physiological situation (*Figure 4.1.6*). We can demonstrate that a suitable cellular environment, perhaps in concert with other factors such as the hypothesised protein X (see paragraph 1.5, Telling et al., 1994 and Telling et al., 1995) is able to support an efficient conversion of PrP^{C} to an oligomeric, scrapie-like isomer, which can possibly act as a precursor of PrP^{Sc} formation, nucleating further protein aggregation and disease manifestation as postulated by the protein-only hypothesis (Prusiner, 1982). On the other hand, it has to be pointed out that the intermediate found under our *in vitro* conditions is soluble and therefore very likely do not directly represent PrP^{Sc} itself. However, further aggregation of the intermediate may have been prevented by the realtively high urea concentrations required to populate the intermediate.

4.2 Thermodynamic stability of PrP(23-231) and PrP(121-231) wild-type and mutants related to inherited human TSEs

4.2.1 Inherited point mutations related to TSEs

Several point mutations in the human prion protein are known to be able to promote disease spontaneously. These inherited forms of prion diseases are rare causes of TSEs in humans and used as strong arguments in favour of the protein-only hypothesis. Three different phenotypes of inherited human TSEs are known, the inherited Creutzfeldt-Jakob disease (CJD), the Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI). Besides the longer known 12 variants in human PrP inducing prion diseases, three new point mutations related to inherited Creutzfeldt-Jakob disease were found very recently: E196K, V203I, E211Q (Peoc'h et al., 2000). The point mutations and corresponding phenotypes of disease are listed in *Table 4.2.1*.

All but the three point mutations P102L, P105L and A117V are located in the folded, C-terminal domain of the protein. The remaining three point mutations in the unstructured region are located within the fragment 90-120, which is supposed to be structured in PrP^{Sc}, because the digestion by proteinase K of extracts from diseased brains ends up in a protein fragment PrP(90-231), which is resistant to further digestion (Safar et al., 1990; Weissmann et al., 1996).

CJD	**GSS**	**FFI**
D178N (V129)	P102L	D178N (M129)
T183A	P105L	
E196K	A117V	
E200K	V180I	
V203I	F198S	
R208H	Q217R	
V210I		
E211Q		

Table 4.2.1. Point mutations in human PrP related to inherited prion diseases in human.

The role played by the point mutations in facilitating the process of disease formation, i.e. the conformational change leading from PrP^{C} to PrP^{Sc}, is not clear. Destabilisation of the three dimensional fold of PrP^{C} for the mutant proteins was hypothesised as cause of the increased propensity of the mutants to convert to the infectious isoform PrP^{Sc} (Prusiner, 1997; Cohen et al., 1994; Huang et al., 1994). Studies of the thermodynamic stability of the corresponding variants of murine PrP(121-231) at pH 7.0 (Liemann and Glockshuber, 1999), and of some variants of human PrP(90-231) at pH 3.6-7.2 (Swietnicki et al., 1998) excluded this possibility as general explanation for spontaneous prion formation in inherited TSEs as they showed that not all of the mutants were apparently destabilised by the point mutation. So,

thermodynamic destabilization of the variant proteins responsible for the hereditary forms of prion disease may be a mechanism for those mutations that indeed proved to be destabilizing, but other mechanisms obviously apply in the case of non-destabilizing mutations.

Since the process of PrP^{Sc} formation likely takes place during the endocytic pathway, the question about the thermodynamic stability of the proteins at acidic pH is fundamental; furthermore, since the formation of an intermediate state of folding is observed in the pH range 4.0-6.0 for wild-type PrP(23-231) (Hornemann and Glockshuber, 1998; paragraph 4.2), it had to be tested whether the prion protein variants related to inherited TSEs are more prone to the formation of this intermediate state. In this section we try to approach these questions by measuring equilibrium folding transitions by urea denaturation of both PrP(121-231) and PrP(23-231) wild-type and variants under conditions of acidic and neutral pH, in absence and presence of sodium chloride.

4.2.2 Modified purification protocols

Purification of the variant PrP(121-231) E200K was performed from periplasmic extracts as previously described for the wild-type and soluble variants of PrP(121-231) (Liemann and Glockshuber, 1999).

The insoluble variants of PrP(121-231) D178N, F198S and T183A were purified from periplasmic inclusion bodies as in (Liemann and Glockshuber, 1999) but cell growth was allowed at 26°C instead of 33°C (12 hours) to avoid major degradation of the protein; in addition, after applying the protein to the DE52/CM52 tandem column, no gradient was applied, but the flow-through was directly concentrated for further purification by gel filtration as previously described (Liemann and Glockshuber, 1999). These modifications allowed to improve the yield by a factor of 10 (from about 0.5 mg/L cell culture to more than 5 mg/L cell culture).

The PrP(23-231) variants E200K, D178N and F198S where purified as described for the wild-type full-length protein (Liemann and Glockshuber, 1999), but in the case of the D178N and F198S variants a protein loss of about 95% occured at the last purification

step when the oxidized and refolded protein was applied to a CM52 cation exchange column. This problem was circumvented by applying the oxidized protein again onto a gel filtration column under denaturing conditions, removing this way covalent aggregates due to unspecific intermolecular disulfide bridge formation. The eluted fractions of pure protein were then collected and refolding was achieved by dialysis against 10 mM Mes/NaOH buffer (pH 6.0), 1 mM EDTA at 4°C. Precipitate was removed by centrifugation (20.000 rpm, 30 min, 4°C) and the supernatant was dialysed against distilled water before freezing in liquid nitrogen for storage at –20°C. HPLC analysis confirmed that the protein was uniformly oxidized. This protocol improved the purification yield of the F198S mutant from 0.5 mg/L bacterial culture to 29 mg/L and of the D178N variant from 1 mg/L to 10 mg/L.

4.2.3. Equilibrium folding transition of PrP(121-231) and PrP(23-231) wild-type and mutants

The thermodynamic stability of PrP wild-type and variants was analysed by measuring under denaturing conditions the ellipticity at 222 nm, where a minimum of absorption is detected in the PrP^C circular dichroism spectra. Urea at concentrations of 0 to 9 M was used as denaturant. The buffer used at pH 7.0 was 45 mM sodium phosphate/NaOH, whereas at pH 4.0 50 mM formic acid/NaOH was used. In addition to the samples in the absence of salt, measurements were also performed on samples, where to increase the ionic strength shown to be essential for the formation of the intermediate of folding at acidic pH (see Hornemann and Glockshuber, 1998 and paragraph 4.2) 135 mM NaCl was added to the PrP(23-231) solutions and 50 mM NaCl to the PrP(121-231) solutions. The protein concentration of both wild-type and mutants was about 30 μM for PrP(23-231) and about 20 μM for PrP(121-231). All of the transitions were measured at 22°C.

For recombinant mouse PrP(121-231) wild-type and mutants D178N, T183A, F198S, E200K measurements at pH 4.0 in presence and absence of sodium chloride were done and compared to the previous results found for the same proteins at pH 7 in the absence of salt (Liemann and Glockshuber, 1999). The thermodynamic parameters are reported in *Table 4.2.2.*

pH 4.0 **I = 30 mM**	**ΔG°** **(kJ/mol)**	**ΔΔG° (kJ/mol)**	**m (kJ/mol M)**	**$[urea]_{1/2}$ (M)**
WT	-16.9 ± 1.4	-	5.0 ± 0.4	3.4
E200K	-17.6 ± 1.0	0.7±2.4	5.5 ± 0.3	3.2
F198S	-12.2 ± 1.1	4.7±2.5	4.3 ± 0.3	2.8
D178N	-12.0 ± 0.9	4.9±2.3	4.5 ± 0.3	2.7
pH 7.0 **I = 80 mM***				
WT	-29.7 ± 1.0	-	4.8 ± 0.2	6.2
E200K	-29.1 ± 1.5	0.6±2.4	4.7 ± 0.1	5.9
F198S	-19.4 ± 0.8	10.3±1.7	4.9 ± 0.3	4.5
D178N	-22.5 ± 0.8	7.2±1.7	4.7 ± 0.1	4.8

Table 4,2,2. Thermodynamic stability of mPrP(121-231) wild-type and variants at pH 7.0 and 4.0 without NaCl. I: Ionic strength. * from (Liemann and Glockshuber, 1999).

The thermodynamic stability of PrP(23-231) wild-type and mutants D178N, F198S and E200K was analysed at pH 7.0 and 4.0 in the presence and absence of 135 mM NaCl. The corresponding thermodynamic parameters are summarized in *Table 4.2.3.*

The position of the point mutations analysed is reported in *Figure 4.2.1.*

The folding was found to be a reversible process for all the mutants also at pH 4.0 for both PrP(121-231) and PrP(23-231).

pH 4.0 **I = 30 mM**	**ΔG° (kJ/mol)**	**ΔΔG° (kJ/mol)**	**m (kJ/mol M)**	**$[urea]_{1/2}$ (M)**
WT	-17.3 ± 0.6	-	4.9 ± 0.2	3.53
E200K	-16.4 ± 0.6	0.9±1.2	5.2 ± 0.2	3.15
F198S	-9.5 ± 0.2	7.8±0.8	3.8 ± 0.1	2.50
D178N	-9.4 ± 0.2	7.9±0.8	4.0 ± 0.1	2.35
pH 7.0 **I = 80 mM**				
WT	-25.6 ± 1.6	-	4.2 ± 0.3	6.09
E200K	-25.3 ± 1.7	0.3±3.3	4.4 ± 0.3	5.75

F198S	aggr.	-	-	-
D178N	aggr.	-	-	-
pH 7.0 **I = 215 mM**				
WT	-26.4 ± 1.7	-	4.4 ± 0.3	6.00
E200K	-27.4 ± 1.5	-1±3.2	4.9 ± 0.3	5.59
F198S	aggr.	-	-	-
D178N	aggr.	-	-	-

Table 4.2.3. Thermodynamic stability of mPrP(23-231) wild-type and variants at pH 7.0 and 4.0 (22°C) at different ionic strengths. Aggr.: could not be evaluated quantitatively due to partial aggregation during the transition. I: Ionic strength

The replacement T183A of PrP(121-231) destabilises the native protein that much at pH 4.0, that a strong aggregation prevents measurement of the transition at a sufficiently high concentration to get an acceptable accuracy of the data. This same problem was still observed at pH 7.0 (Liemann and Glockshuber, 1999), but at acidic pH the aggregation is even stronger, so that no measurement could be performed.

In general, the mutant E200K is found to be almost as stable as the wild-type protein under all conditions tested; on the contrary, the mutants D178N and F198S, which were found to be unstabilising at pH 7.0, are also less stable than the wild-type at pH 4.0. The replacement F198S which proved to be destabilizing at pH 7.0 for the domain PrP(121-231) (Liemann and Glockshuber, 1999), was also destabilizing for the full-length protein under all conditions investigated.

Furthermore, supporting the finding by NMR, that the N-terminal region spanning residues 23-124 is unstructured, the thermodynamic stability of both the C-terminal domain alone, PrP(121-231), and the full-length protein, PrP(23-231), for the wild-type as well as for the mutants, is approximately the same under all the conditions used, indicating that the N-terminal tail is not implicated in the folding process.

A major change in folding and stability of all the proteins investigated is observed by increasing the ionic strength at acidic pH, and the formation of the folding intermediate described in the previous paragraph is observed also for the PrP variants. As explained

in paragraph 4.2, no determination of $\Delta G°$ can be obtained, because of the concentration dependence of the formation of the PrP^{Sc} like folding intermediate, and the unknown oligomeric state of the intermediate.

Albeit no general destabilisation of the mutants with respect to the wild-type can be

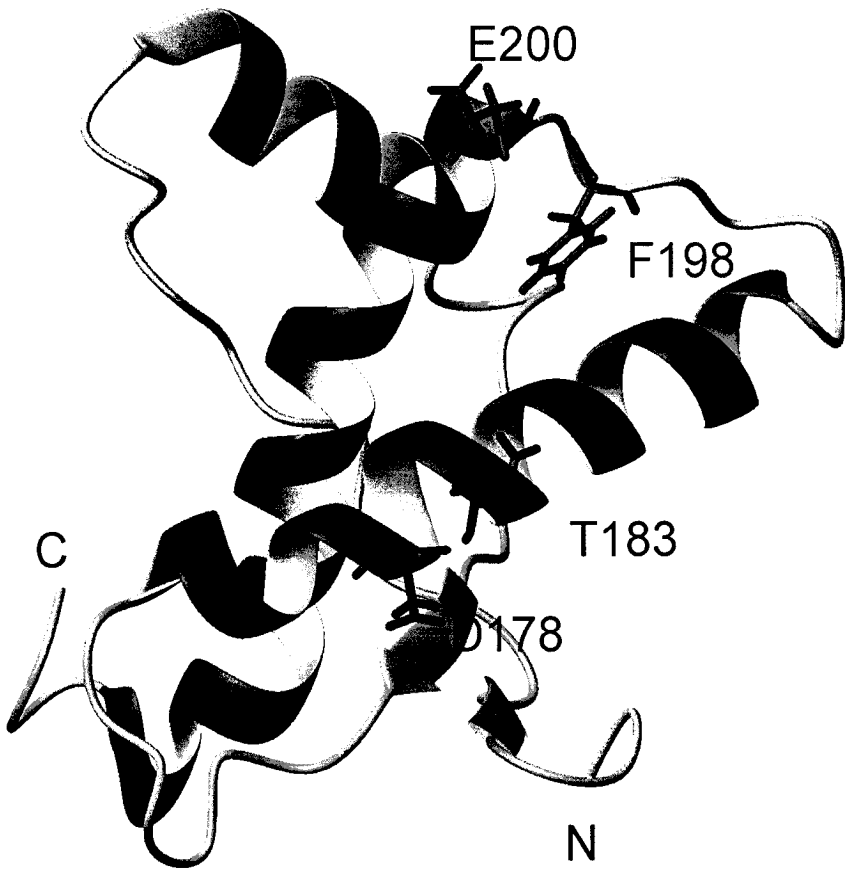

Figure 4.2.1. NMR solved structure of PrP(121-231) with amino acids D178, T183, F198 and E200 indicated. This amino acid are mutated in some prion disease related variants, D178N, T183A, F198S and E200K.

the cause of the increased facility of these ones to convert to the scrapie isoform PrP^{Sc}, all the proteins are destabilised at acidic pH, indicating that the conversion process may indeed occur and be facilitated in the endocytic compartments, where the pH is in the range 4.0-6.0 (Lee et al., 1996). Anyway, also at acidic pH, the thermodynamic stability data indicate that the mutants are not uniformly destabilised with respect to the wild-type, as for example the E200K variant is found to be as stable as the wild-type.

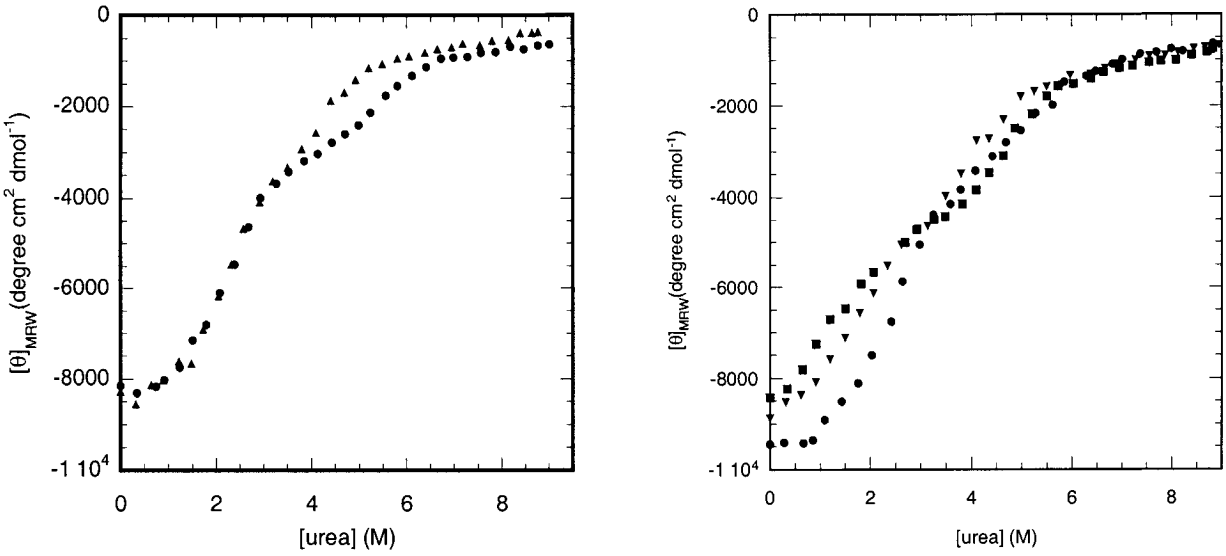

Figure 4.2.2. Unfolding transitions of mPrP(23-231) at pH 4.0 and at a ionic strength of 165 mM (22°C). On the left: 28 μM mPrP(23-231) wt (●) and 28 μM E200K (▲). On the rigth: 14.5 μM mPrP(23-231) wt (●), 20 μM mPrP(23-231) F198S (▼) and 20 μM mPrP(23-231) D178N (■).

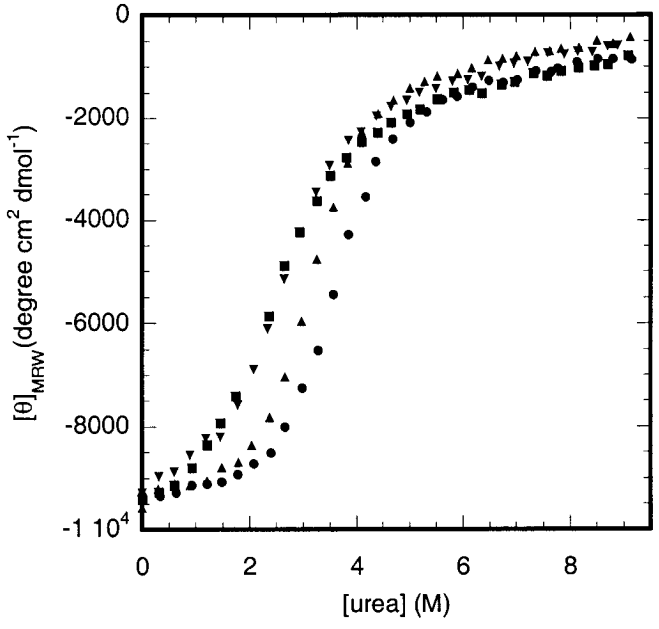

Figure 4.2.3. Unfolding transitions of mPrP(23-231) at pH 4.0 and at a ionic strength of 30 mM (22°C). The protein concentration was 28 μM. mPrP(23-231) wt (●), variant E200K (▲), variant F198S (▼) and variant D178N (■).

Interestingly, the PrP(23-231) variants F198S and D178N are found to aggregate at

pH 7.0, where no unfolding intermediate is found for the wild-type protein: this aggregation is most likely unspecific and is attributable to the decreased stability of the variants with respect to the wild-type at neutral pH. On the other side, no aggregation of these two mutant proteins is observed at pH 4 and at a ionic strength above 100 mM, i.e under the conditions where the unfolding intermediate is formed in wild-type PrP(23-231). The soluble unfolding intermediate found may be therefore a precursor of PrP^{Sc} analogous to the one formed during unfolding of wild-type PrP(23-231).

The intermediate formation occurs at pH 4.0 and in the presence of 165 mM ionic strength at the same urea concentration for all of the mutants as it was found for the wild-type: indeed, at 3.5 M urea an intermediate state with CD spectra identical to that of the wild-type and reminescent of PrP^{Sc} is found. It seems therefore that the oligomeric unfolding intermediate is also populated in the case of the variants related to prion diseases (*Figure 4.2.2*). No intermediate formation is found at ionic strength below 50 mM, as shown in *Figure 4.2.3*. Interestingly, the dependence of intermediate formation on the protein concentration appears to vary for the different protein variants used. As depicted in *Figure 4.2.2*, the D178N variant of PrP(23-231) presents a strongly populated intermediate still at 20 μM protein concentration, whereas the E200K variant shows minimal intermediate population at 3.5 molar urea concentration even at a concentration of about 30 μM. This observation prompt us to the assumption that the point mutation may facilitate or hinder oligomerization of the protein on the way to PrP^{Sc} formation. This may perhaps influence the interaction with other factors involved in normal PrP metabolism, if eventually temporary oligomerization of PrP plays a functional role in the cell.

From the data reported in this work, we conclude that the thermodynamic stability of the prion protein variants do not unambiguously explain their propensity to form the infectious isoform PrP^{Sc} spontaneously, since no general destabilisation is observed at both neutral and acidic pH. A different oligomerization pattern may be instead responsible for the increased propensity of the variants to spontaneously convert to the scrapie isoform PrP^{Sc}. In addition, as other factors are supposed to be determinant in the formation of PrP^{Sc}, the mutant proteins may differ in their interaction with other

cellular components in such a way, that the formation of the infectious isoform could be facilitated. Alternatively the mutations could also affect the kinetics of PrP^{Sc} formation or the stability of PrP^{Sc}. In any case, it has to be pointed out that since the PrP variants correlate with different forms of prion diseases as mentioned above, it is likely that in each case a different pathway of disease induction is followed, ending up in the specific pathologic phenotype.

5. CONCLUSIONS

In the research field on transmissible spongiform encephalopathies, the prion-transmitted infectious diseases, the open questions are certainly much more numerous than the solved problems. Starting from the question whether prion diseases are really transmitted only by a cellular host protein in its changed conformation, without involvment of nucleic acids, as is postulated in the protein-only hypothesis (Prusiner, 1982), to the uncertainty about the mechanism of conversion leading from the normal prion protein form, PrP^{C}, to its infectious isoform PrP^{Sc} (Jarrett and Landsbury, 1993; Prusiner 1991; Caughey et al., 1997), to the exact mechanism of propagation of the infectious prions to the central nervous system (Aguzzi, 1998; Montrasio et al., 2000; Mabbott et al., 2000), up to the unknown biological function of this protein, the prion appear to remain after more than a century of research an obscure and fascinating area in the context of infectious diseases.

In this work, we have tryied to approach some of the crucial questions on the biophysics of the prion protein.

First, detailed investigation by electron paramagnetic resonance (EPR) spectroscopy of the copper(II) binding behaviour of recombinant murine full-length PrP, mPrP(23-231), and its C-terminal, structured domain, mPrP(121-231), allowed to determine that not only the N-terminal region of full-length PrP, shown by NMR to be unstructured, is able to coordinate copper(II) by the histidine residues contained in its fourfold octapeptide repeat region (Brown et al., 1997a; Stockel et al., 1998; Viles et al., 1999; Whittal et al., 2000; Aronoff-Spencer et al., 2000), but also the C-terminal domain itself is able to specifically bind up to four Cu(II) ions. This 4 copper(II) ions, added to the 6 copper(II) ions coordinated by the N-terminal region, increase the stoichiometry of binding to 10 molar equivalents of copper(II) per prion protein molecule. Up to now, the proposed physiological role of copper(II) bound to PrP was not properly reliable with the infectivity of this protein, because on the one side, the N-terminal region was found to be non-essential for the propagation of infectivity (Shmerling et al., 1992; Flechsig et al., 2000), on the other side, oxidative damages related to a wrong copper(II) metabolism were assumed to be at the origin of the pathological phenotype of disease (Wong et al., 2000c). The discovery that PrP(121-231) is also able to bind copper(II) would

simultaneously explain why the same pathological degeneration is observed both in absence and presence of the N-terminal, octapeptide containing region, and why copper(II)-related oxidative damages are maybe the cause of cell death in prion diseases. As found for some variants of the enzyme superoxide dismutase (SOD), an enzyme shown to bear a copper(II) ion in its acitve site, the pathological phenotype has to be attributed to oxidative damages induced by copper(II) coordinated in an active site which is rendered more flexible by the mutation. Perhaps, the conformational change at the basis of the PrP^{C} to PrP^{Sc} conversion could modify the copper(II) binding sites in a similar way, so that the bound copper(II) ions may no more only play their physiologically relevant role, but also induce the unspecific oxidative damages found after disease onset. Furthermore, copper(II) binding may facilitate the interaction of the prion protein with other cellular components essential for its biological function, and modified copper(II) binding sites as perhaps in PrP^{Sc} or a precursor thereof may hinder the function of PrP by avoiding these interactions or by favouring the docking of factors responsible for the PrP^{C} to PrP^{Sc} conversion as the proposed "protein X". In view to find out which one may be the most likely picture *in vivo* it should be determined, whether the infectious PrP^{Sc} is able to bind copper(II) as well: surprisingly, this has not yet been determined.

Second, on the way to determine a possible structural relevance of copper(II) binding to the N-terminal region of PrP(23-231) (Viles et al., 1999; Stockel et al., 1998; Wong et al., 2000d; Wong et al., 2000a), we performed limited proteolysis of recombinant murine PrP(23-231) with several proteases in the absence and presence of up to ten molar equivalents copper(II) ions: our analysis consented us to exclude that the N-terminal, disordered domain becomes structured upon Cu(II) binding and moreover we could further confirm the domain border of the folded C-terminal domain also after saturation with Cu(II). This result consolidates the above assumption that only copper(II) binding to the structured C-terminal domain may be recruited in the conversion process from the normal PrP^{C} to its infectious PrP^{Sc} isoform, since the N-terminal region does not show any defined three dimensional structure even after copper(II) binding.

Third, we investigated the thermodynamic stability of recombinant mouse PrP(23-231) and PrP(121-231). Our unfolding equilibrium transition measurements under different protein concentration and ionic strength conditions allowed to determine that the formation of the unfolding intermediate with PrP^{Sc}-like tertiary structure characteristica found at acidic pH (Hornemann and Glockshuber, 1998; Swietnicki et al., 1997) is only observed at protein concentrations above 15 μM and at ionic strengths above 80 mM, which correspond to physiological ionic strengths; these results allow to assume that our scrapie-like intermediate may represent a precursor of PrP^{Sc}. In addition, the protein concentration dependence observed prompt us to argue that the intermediate state is an oligomeric form of PrP, most likely a dimer, giving a good argument in favour of the nucleation-polymerization model proposed for PrP^{Sc} formation and propagation (Jarrett and Lansbury, 1993). After that, our analysis of the thermodynamic stability of three PrP variants related to inherited TSEs in humans under the concentrations and ionic strength conditions found to favour the formation of the intermediate at acidic pH consented us to confirm that also for the PrP(23-231) variants, no general pattern of destabilization may be at the origin of the facilitated formation of the scrapie isoform in these inherited forms of TSEs. The interesting finding that the population of the unfolding intermediate is variable depending on the variant investigated recruits the possibility that perhaps a modified behavior at the level of the oligomerization in the variants with respect to the wild-type may favour the spontaneous disease appearence in the individui bearing the mutation.

In summary, our work allows to give some more insight into the specificity and stoichiometry of copper(II) binding to the prion protein, provides arguments in favour of a physiological role of copper(II) binding to the prion protein and of a relevance of binding in the context of prion infectivity, albeit copper(II) is shown not to induce structure into the N-terminal region of PrP^{C} as was previously postulated. Our finding that oligomerization of PrP under acidic conditions and at physiological ionic strengths may be essential in the formation of PrP^{Sc} or a precursor thereof further support the nucleation-polymerization model of prion formation and propagation during the endocytic pathway. Furthermore, the finding that the formation of the oligomeric unfolding intermediate in variants of PrP related to inherited TSEs in humans helps elucidating the

altered tendency of these variants to produce the infectious isoform PrP^{Sc} and thus induce disease spontaneously.

Concluding, this work should be a small contribute on the way towards an understanding of the function and malfunction of prions, in the hope that one day the human efforts and intelligence may defeat this... protein.

References

Aguzzi, A. 1998. Grafting mouse brains: from neurocarcinogenesis to neurodegeneration. *Embo J.* 17:6107-14.

Alper, T., W. A. Cramp, D. A. Haig, and M. C. Clarke. 1967. Does the agent of scrapie replicate without nucleic acid? *Nature.* 214:764-6.

Aronoff-Spencer, E., C. S. Burns, N. I. Avdievich, G. J. Gerfen, J. Peisach, W. E. Antholine, H. L. Ball, F. E. Cohen, S. B. Prusiner, and G. L. Millhauser. 2000. Identification of the Cu(2+) binding sites in the N-terminal domain of the prion protein by EPR and CD spectroscopy [In Process Citation]. *Biochemistry.* 39:13760-71.

Balter, M. 2000. Spongiform disease. Experts downplay new vCJD fears [news]. *Science.* 289:1663-6.

Basler, K., B. Oesch, M. Scott, D. Westaway, M. Walchli, D. F. Groth, M. P. McKinley, S. B. Prusiner, and C. Weissmann. 1986. Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell.* 46:417-28.

Bell, J. E., and J. W. Ironside. 1993. Neuropathology of spongiform encephalopathies in humans. *Br Med Bull.* 49:738-77.

Bertrand, J., H. Carre, and F. Lucam. 1937. La 'tremblante' du mouton (Recherche histopathologique). *Ann. Anat. Path.* 14:565-586.

Borchelt, D. R., M. Scott, A. Taraboulos, N. Stahl, and S. B. Prusiner. 1990. Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. *J Cell Biol.* 110:743-52.

Brown, D. R., and A. Besinger. 1998. Prion protein expression and superoxide dismutase activity. *Biochem J.* 334:423-9.

Brown, D. R., F. Hafiz, L. L. Glasssmith, B. S. Wong, I. M. Jones, C. Clive, and S. J. Haswell. 2000. Consequences of manganese replacement of copper for prion protein function and proteinase resistance. *Embo J.* 19:1180-6.

Brown, D. R., K. Qin, J. W. Herms, A. Madlung, J. Manson, R. Strome, P. E. Fraser, T. Kruck, A. von Bohlen, W. Schulz-Schaeffer, A. Giese, D. Westaway, and H. Kretzschmar. 1997a. The cellular prion protein binds copper in vivo. *Nature.* 390:684-7.

Brown, D. R., B. Schmidt, and H. A. Kretzschmar. 1997b. Effects of oxidative stress on prion protein expression in PC12 cells. *Int J Dev Neurosci.* 15:961-72.

Brown, D. R., B. Schmidt, and H. A. Kretzschmar. 1998. Effects of copper on survival of prion protein knockout neurons and glia. *J Neurochem.* 70:1686-93.

Brown, D. R., B. S. Wong, F. Hafiz, C. Clive, S. J. Haswell, and I. M. Jones. 1999a. Normal prion protein has an activity like that of superoxide dismutase [published erratum appears in Biochem J 2000 Feb 1;345 Pt 3:767]. *Biochem J.* 344 Pt 1:1-5.

Brown, K. L., K. Stewart, D. L. Ritchie, N. A. Mabbott, A. Williams, H. Fraser, W. I. Morrison, and M. E. Bruce. 1999b. Scrapie replication in lymphoid tissues depends on prion protein- expressing follicular dendritic cells. *Nat Med.* 5:1308-12.

Brownlee, A. 1940. Histopathological studies of scrapie, an obscure disease of sheep. *Vet. J.* 96:254-264.

Bruce, M. E., R. G. Will, J. W. Ironside, I. McConnell, D. Drummond, A. Suttie, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Fraser, and C. J. Bostock. 1997. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent [see comments]. *Nature.* 389:498-501.

Bueler, H., A. Aguzzi, A. Sailer, R. A. Greiner, P. Autenried, M. Aguet, and C. Weissmann. 1993. Mice devoid of PrP are resistant to scrapie. *Cell.* 73:1339-47.

Bueler, H., M. Fischer, Y. Lang, H. Bluethmann, H. P. Lipp, S. J. DeArmond, S. B. Prusiner, M. Aguet, and C. Weissmann. 1992. Normal development and behaviour of mice lacking the neuronal cell- surface PrP protein [see comments]. *Nature.* 356:577-82.

Calzolai, L., D. A. Lysek, P. Guntert, C. von Schroetter, R. Riek, R. Zahn, and K. Wuthrich. 2000. NMR structures of three single-residue variants of the human prion protein. *Proc Natl Acad Sci U S A.* 97:8340-5.

Cashman, N. R., R. Loertscher, J. Nalbantoglu, I. Shaw, R. J. Kascsak, D. C. Bolton, and P. E. Bendheim. 1990. Cellular isoform of the scrapie agent protein participates in lymphocyte activation. *Cell.* 61:185-92.

Caughey, B., D. A. Kocisko, G. J. Raymond, and P. T. Lansbury, Jr. 1995. Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease- resistant state. *Chem Biol.* 2:807-17.

Caughey, B. W., A. Dong, K. S. Bhat, D. Ernst, S. F. Hayes, and W. S. Caughey. 1991. Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy [published erratum appears in Biochemistry 1991 Oct 29;30(43):10600]. *Biochemistry.* 30:7672-80.

Chesebro, B. 1998. BSE and prions: uncertainties about the agent. *Science.* 279:42-3.

Chesebro, B., K. Wehrly, B. Caughey, J. Nishio, D. Ernst, and R. Race. 1993. Foreign PrP expression and scrapie infection in tissue culture cell lines. *Dev Biol Stand.* 80:131-40.

Cohen, F. E., K. M. Pan, Z. Huang, M. Baldwin, R. J. Fletterick, and S. B. Prusiner. 1994. Structural clues to prion replication [see comments]. *Science.* 264:530-1.

Collinge, J., K. C. Sidle, J. Meads, J. Ironside, and A. F. Hill. 1996. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD [see comments]. *Nature.* 383:685-90.

Cuille, J., and P. L. Chelle. 1936. Pathologie animale. La maladie dite tremblant du mouton est-elle inocuable? *Compt. Rend. Acad. Sci. (Paris).* 203:1552-1554.

DebBurman, S. K., G. J. Raymond, B. Caughey, and S. Lindquist. 1997. Chaperone-supervised conversion of prion protein to its protease- resistant form. *Proc Natl Acad Sci U S A.* 94:13938-43.

den Blaauwen, T., and T. G. Canter. 1993. Creation of type-1 and type-2 copper sites by addition of exogenous ligands to the *Pseudomonas aeruginosa* azurin His117Gly mutant. *J. Am. Chem. Soc.* 115:1121-1129.

Donne, D. G., J. H. Viles, D. Groth, I. Mehlhorn, T. L. James, F. E. Cohen, S. B. Prusiner, P. E. Wright, and H. J. Dyson. 1997. Structure of the recombinant full-length hamster prion protein PrP(29- 231): the N terminus is highly flexible [see comments]. *Proc Natl Acad Sci U S A.* 94:13452-7.

Dormont, D. 1999. Bovine Spongiform Encephalopathy and the new Variant of Creutzfeldt-Jakob disease. *In* Prions Molecular and Cellular Biology. h. s. press, editor.

Endo, T., D. Groth, S. B. Prusiner, and A. Kobata. 1989. Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. *Biochemistry.* 28:8380-8.

Flechsig, E., D. Shmerling, I. Hegyi, A. J. Raeber, M. Fischer, A. Cozzio, C. von Mering, A. Aguzzi, and C. Weissmann. 2000. Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. *Neuron.* 27:399-408.

Gabriel, J. M., B. Oesch, H. Kretzschmar, M. Scott, and S. B. Prusiner. 1992. Molecular cloning of a candidate chicken prion protein. *Proc Natl Acad Sci U S A.* 89:9097-101.

Gambetti, P., P. Parchi, R. B. Petersen, S. G. Chen, and E. Lugaresi. 1995. Fatal familial insomnia and familial Creutzfeldt-Jakob disease: clinical, pathological and molecular features. *Brain Pathol.* 5:43-51.

Ghetti, B., S. R. Dlouhy, G. Giaccone, O. Bugiani, B. Frangione, M. R. Farlow, and F. Tagliavini. 1995. Gerstmann-Straussler-Scheinker disease and the Indiana kindred. *Brain Pathol.* 5:61-75.

Gibbs, C. J., Jr., and C. L. Bolis. 1997. Normal isoform of amyloid protein (PrP) in brains of spawning salmon. *Mol Psychiatry.* 2:146-7.

Goldfarb, L. G., R. B. Petersen, M. Tabaton, P. Brown, A. C. LeBlanc, P. Montagna, P. Cortelli, J. Julien, C. Vital, W. W. Pendelbury, and et al. 1992. Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. *Science.* 258:806-8.

Gordon, W. S. 1946. *Vet. Res.* 58:516-520.

Griffith, J. S. 1967. Self-replication and scrapie. *Nature.* 215:1043-4.

Harris, D. A., A. Gorodinsky, S. Lehmann, K. Moulder, and S. L. Shyng. 1996. Cell biology of the prion protein. *Curr Top Microbiol Immunol.* 207:77-93.

Herms, J., T. Tings, S. Gall, A. Madlung, A. Giese, H. Siebert, P. Schurmann, O. Windl, N. Brose, and H. Kretzschmar. 1999. Evidence of presynaptic location and function of the prion protein. *J Neurosci.* 19:8866-75.

Hill, A. F., M. Antoniou, and J. Collinge. 1999. Protease-resistant prion protein produced in vitro lacks detectable infectivity. *J Gen Virol.* 80:11-4.

Hill, A. F., S. Joiner, J. Linehan, M. Desbruslais, P. L. Lantos, and J. Collinge. 2000. Species-barrier-independent prion replication in apparently resistant species [In Process Citation]. *Proc Natl Acad Sci U S A.* 97:10248-53.

Holman, H. H., and I. H. Pattison. 1943. Further evidence on the significance of vacuolated nerve cells in the medulla oblongata of sheep affected with scrapie. *J. Comp. Path.* 53:231-236.

Hornemann, S., and R. Glockshuber. 1996. Autonomous and reversible folding of a soluble amino-terminally truncated segment of the mouse prion protein. *J Mol Biol.* 261:614-9.

Hornemann, S., and R. Glockshuber. 1998. A scrapie-like unfolding intermediate of the prion protein domain PrP(121-231) induced by acidic pH. *Proc Natl Acad Sci U S A.* 95:6010-4.

Hornshaw, M. P., J. R. McDermott, and J. M. Candy. 1995a. Copper binding to the N-terminal tandem repeat regions of mammalian and avian prion protein. *Biochem Biophys Res Commun.* 207:621-9.

Hornshaw, M. P., J. R. McDermott, J. M. Candy, and J. H. Lakey. 1995b. Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides. *Biochem Biophys Res Commun.* 214:993-9.

Hsiao, K., H. F. Baker, T. J. Crow, M. Poulter, F. Owen, J. D. Terwilliger, D. Westaway, J. Ott, and S. B. Prusiner. 1989. Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. *Nature.* 338:342-5.

Huang, Z., J. M. Gabriel, M. A. Baldwin, R. J. Fletterick, S. B. Prusiner, and F. E. Cohen. 1994. Proposed three-dimensional structure for the cellular prion protein. *Proc Natl Acad Sci U S A.* 91:7139-43.

Jarrett, J. T., and P. T. Lansbury, Jr. 1993. Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell.* 73:1055-8.

Keshet, G. I., H. Ovadia, A. Taraboulos, and R. Gabizon. 1999. Scrapie-infected mice and PrP knockout mice share abnormal localization and activity of neuronal nitric oxide synthase. *J Neurochem.* 72:1224-31.

Kimberlin, R. H., and C. A. Walker. 1979. Pathogenesis of scrapie: agent multiplication in brain at the first and second passage of hamster scrapie in mice. *J Gen Virol.* 42:107-17.

Kitamoto, T., T. Muramoto, S. Mohri, K. Doh-Ura, and J. Tateishi. 1991. Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. *J Virol.* 65:6292-5.

Klein, M. A., R. Frigg, E. Flechsig, A. J. Raeber, U. Kalinke, H. Bluethmann, F. Bootz, M. Suter, R. M. Zinkernagel, and A. Aguzzi. 1997. A crucial role for B cells in neuroinvasive scrapie [see comments]. *Nature.* 390:687-90.

Klein, M. A., R. Frigg, A. J. Raeber, E. Flechsig, I. Hegyi, R. M. Zinkernagel, C. Weissmann, and A. Aguzzi. 1998. PrP expression in B lymphocytes is not required for prion neuroinvasion [see comments]. *Nat Med.* 4:1429-33.

Kocisko, D. A., J. H. Come, S. A. Priola, B. Chesebro, G. J. Raymond, P. T. Lansbury, and B. Caughey. 1994. Cell-free formation of protease-resistant prion protein [see comments]. *Nature.* 370:471-4.

Kocisko, D. A., S. A. Priola, G. J. Raymond, B. Chesebro, P. T. Lansbury, Jr., and B. Caughey. 1995. Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. *Proc Natl Acad Sci U S A.* 92:3923-7.

Kretzschmar, H. A., S. B. Prusiner, L. E. Stowring, and S. J. DeArmond. 1986. Scrapie prion proteins are synthesized in neurons. *Am J Pathol.* 122:1-5.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc Natl Acad Sci U S A.* 82:488-92.

Kunkel, T. A., K. Bebenek, and J. McClary. 1991. Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol.* 204:125-39.

Lasmezas, C. I., J. P. Deslys, O. Robain, A. Jaegly, V. Beringue, J. M. Peyrin, J. G. Fournier, J. J. Hauw, J. Rossier, and D. Dormont. 1997. Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science.* 275:402-5.

Lee, R. J., S. Wang, and P. S. Low. 1996. Measurement of endosome pH following folate receptor-mediated endocytosis. *Biochim Biophys Acta.* 1312:237-42.

Lehmann, S., and D. A. Harris. 1995. A mutant prion protein displays an aberrant membrane association when expressed in cultured cells. *J Biol Chem.* 270:24589-97.

Liemann, S., and R. Glockshuber. 1999. Influence of amino acid substitutions related to inherited human prion diseases on the thermodynamic stability of the cellular prion protein. *Biochemistry.* 38:3258-67.

Lopez Garcia, F., R. Zahn, R. Riek, and K. Wuthrich. 2000. NMR structure of the bovine prion protein. *Proc Natl Acad Sci U S A.* 97:8334-9.

Mabbott, N. A., F. Mackay, F. Minns, and M. E. Bruce. 2000. Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie [letter]. *Nat Med.* 6:719-20.

Manuelidis, L., and W. Fritch. 1996. Infectivity and host responses in Creutzfeldt-Jakob disease. *Virology.* 216:46-59.

Masters, C. L., and E. P. Richardson, Jr. 1978. Subacute spongiform encephalopathy (Creutzfeldt-Jakob disease). The nature and progression of spongiform change. *Brain.* 101:333-44.

McKenzie, D., J. Bartz, J. Mirwald, D. Olander, R. Marsh, and J. Aiken. 1998. Reversibility of scrapie inactivation is enhanced by copper. *J Biol Chem.* 273:25545-7.

McKinley, M. P., R. K. Meyer, L. Kenaga, F. Rahbar, R. Cotter, A. Serban, and S. B. Prusiner. 1991. Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis. *J Virol.* 65:1340-51.

Miura, T., A. Hori-i, H. Mototani, and H. Takeuchi. 1999. Raman spectroscopic study on the copper(II) binding mode of prion octapeptide and its pH dependence. *Biochemistry.* 38:11560-9.

Montrasio, F., R. Frigg, M. Glatzel, M. A. Klein, F. Mackay, A. Aguzzi, and C. Weissmann. 2000. Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. *Science.* 288:1257-9.

Moore, R. C., I. Y. Lee, G. L. Silverman, P. M. Harrison, R. Strome, C. Heinrich, A. Karunaratne, S. H. Pasternak, M. A. Chishti, Y. Liang, P. Mastrangelo, K. Wang, A. F. Smit, S. Katamine, G. A. Carlson, F. E. Cohen, S. B. Prusiner, D. W. Melton, P. Tremblay, L. E. Hood, and D. Westaway. 1999. Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. *J Mol Biol.* 292:797-817.

Morillas, M., W. Swietnicki, P. Gambetti, and W. K. Surewicz. 1999. Membrane environment alters the conformational structure of the recombinant human prion protein. *J Biol Chem.* 274:36859-65.

Moser, M., R. J. Colello, U. Pott, and B. Oesch. 1995. Developmental expression of the prion protein gene in glial cells. *Neuron.* 14:509-17.

Oesch, B., D. Westaway, M. Walchli, M. P. McKinley, S. B. Kent, R. Aebersold, R. A. Barry, P. Tempst, D. B. Teplow, L. E. Hood, and et al. 1985. A cellular gene encodes scrapie PrP 27-30 protein. *Cell.* 40:735-46.

Pan, K. M., M. Baldwin, J. Nguyen, M. Gasset, A. Serban, D. Groth, I. Mehlhorn, Z. Huang, R. J. Fletterick, F. E. Cohen, and et al. 1993. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A.* 90:10962-6.

Parry, H. B. 1983. Scrapie disease in sheep- historical, clinical, epidemiological, pathological and practical aspects of the natural disease. Academic Press, London.

Pattison, I. H. 1965. Resistance of the scrapie agent to formalin. *J. Comp. Pathol.* 75:159-164.

Pattison, I. H., and J. N. Jebbett. 1973a. Clinical and histological recovery from the scrapie-like spongiform encephalopathy produced in mice by feeding them with cuprizone. *J Pathol.* 109:245-50.

Pattison, I. H., and J. N. Jebbett. 1973b. Unsuccessful attempts to produce disease with tissues from mice fed on a diet containing cuprizone. *Res Vet Sci.* 14:128-30.

Pauly, P. C., and D. A. Harris. 1998. Copper stimulates endocytosis of the prion protein. *J Biol Chem.* 273:33107-10.

Peoc'h, K., P. Manivet, P. Beaudry, F. Attane, G. Besson, D. Hannequin, N. Delasnerie-Laupretre, and J. L. Laplanche. 2000. Identification of three novel mutations (E196K, V203I, E211Q) in the prion protein gene (PRNP) in inherited prion diseases with Creutzfeldt- Jakob disease phenotype. *Hum Mutat.* 15:482.

Prusiner, S. B. 1982. Novel proteinaceous infectious particles cause scrapie. *Science.* 216:136-44.

Prusiner, S. B. 1991. Molecular biology of prion diseases. *Science.* 252:1515-22.

Prusiner, S. B. 1995. The prion diseases. *Sci Am.* 272:48-51, 54-7.

Prusiner, S. B. 1997. Prion diseases and the BSE crisis. *Science.* 278:245-51.

Prusiner, S. B., M. Scott, D. Foster, K. M. Pan, D. Groth, C. Mirenda, M. Torchia, S. L. Yang, D. Serban, G. A. Carlson, and et al. 1990. Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell.* 63:673-86.

Raymond, G. J., J. Hope, D. A. Kocisko, S. A. Priola, L. D. Raymond, A. Bossers, J. Ironside, R. G. Will, S. G. Chen, R. B. Petersen, P. Gambetti, R. Rubenstein, M. A. Smits, P. T. Lansbury, Jr., and B. Caughey. 1997. Molecular assessment of the potential transmissibilities of BSE and scrapie to humans [see comments]. *Nature.* 388:285-8.

Riek, R., S. Hornemann, G. Wider, M. Billeter, R. Glockshuber, and K. Wuthrich. 1996. NMR structure of the mouse prion protein domain PrP(121-321). *Nature.* 382:180-2.

Riek, R., S. Hornemann, G. Wider, R. Glockshuber, and K. Wuthrich. 1997. NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231). *FEBS Lett.* 413:282-8.

Saborio, G. P., C. Soto, R. J. Kascsak, E. Levy, R. Kascsak, D. A. Harris, and B. Frangione. 1999. Cell-lysate conversion of prion protein into its protease-resistant isoform suggests the participation of a cellular chaperone. *Biochem Biophys Res Commun.* 258:470-5.

Safar, J., P. P. Roller, D. C. Gajdusek, and C. J. Gibbs, Jr. 1993. Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein. *J Biol Chem.* 268:20276-84.

Safar, J., W. Wang, M. P. Padgett, M. Ceroni, P. Piccardo, D. Zopf, D. C. Gajdusek, and C. J. Gibbs, Jr. 1990. Molecular mass, biochemical composition, and physicochemical behavior of the infectious form of the scrapie precursor protein monomer. *Proc Natl Acad Sci U S A.* 87:6373-7.

Sakaguchi, S., S. Katamine, N. Nishida, R. Moriuchi, K. Shigematsu, T. Sugimoto, A. Nakatani, Y. Kataoka, T. Houtani, S. Shirabe, H. Okada, S. Hasegawa, T. Miyamoto, and T. Noda. 1996. Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. *Nature.* 380:528-31.

Scott, M., D. Foster, C. Mirenda, D. Serban, F. Coufal, M. Walchli, M. Torchia, D. Groth, G. Carlson, S. J. DeArmond, and et al. 1989. Transgenic mice expressing hamster prion protein produce species- specific scrapie infectivity and amyloid plaques. *Cell.* 59:847-57.

Scott, M., D. Groth, D. Foster, M. Torchia, S. L. Yang, S. J. DeArmond, and S. B. Prusiner. 1993. Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. *Cell.* 73:979-88.

Scott, M. R., R. Will, J. Ironside, H. O. Nguyen, P. Tremblay, S. J. DeArmond, and S. B. Prusiner. 1999. Compelling transgenetic evidence for transmission of

bovine spongiform encephalopathy prions to humans. *Proc Natl Acad Sci U S A.* 96:15137-42.

Shmerling, D., I. Hegyi, M. Fischer, T. Blattler, S. Brandner, J. Gotz, T. Rulicke, E. Flechsig, A. Cozzio, C. von Mering, C. Hangartner, A. Aguzzi, and C. Weissmann. 1998. Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. *Cell.* 93:203-14.

Shyng, S. L., M. T. Huber, and D. A. Harris. 1993. A prion protein cycles between the cell surface and an endocytic compartment in cultured neuroblastoma cells. *J Biol Chem.* 268:15922-8.

Simonic, T., S. Duga, B. Strumbo, R. Asselta, F. Ceciliani, and S. Ronchi. 2000. cDNA cloning of turtle prion protein. *FEBS Lett.* 469:33-8.

Spudich, S., J. A. Mastrianni, M. Wrensch, R. Gabizon, Z. Meiner, I. Kahana, H. Rosenmann, E. Kahana, and S. B. Prusiner. 1995. Complete penetrance of Creutzfeldt-Jakob disease in Libyan Jews carrying the E200K mutation in the prion protein gene. *Mol Med.* 1:607-13.

Stahl, N., M. A. Baldwin, A. L. Burlingame, and S. B. Prusiner. 1990. Identification of glycoinositol phospholipid linked and truncated forms of the scrapie prion protein. *Biochemistry.* 29:8879-84.

Stahl, N., D. R. Borchelt, K. Hsiao, and S. B. Prusiner. 1987. Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell.* 51:229-40.

Stockel, J., J. Safar, A. C. Wallace, F. E. Cohen, and S. B. Prusiner. 1998. Prion protein selectively binds copper(II) ions. *Biochemistry.* 37:7185-93.

Swietnicki, W., R. Petersen, P. Gambetti, and W. K. Surewicz. 1997. pH-dependent stability and conformation of the recombinant human prion protein PrP(90-231). *J Biol Chem.* 272:27517-20.

Swietnicki, W., R. B. Petersen, P. Gambetti, and W. K. Surewicz. 1998. Familial mutations and the thermodynamic stability of the recombinant human prion protein. *J Biol Chem.* 273:31048-52.

Taraboulos, A., M. Scott, A. Semenov, D. Avrahami, L. Laszlo, S. B. Prusiner, and D. Avraham. 1995. Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie

isoform [published erratum appears in J Cell Biol 1995 Jul;130(2):501]. *J Cell Biol.* 129:121-32.

Telling, G. C., M. Scott, K. K. Hsiao, D. Foster, S. L. Yang, M. Torchia, K. C. Sidle, J. Collinge, S. J. DeArmond, and S. B. Prusiner. 1994. Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proc Natl Acad Sci U S A.* 91:9936-40.

Telling, G. C., M. Scott, J. Mastrianni, R. Gabizon, M. Torchia, F. E. Cohen, S. J. DeArmond, and S. B. Prusiner. 1995. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell.* 83:79-90.

Turk, E., D. B. Teplow, L. E. Hood, and S. B. Prusiner. 1988. Purification and properties of the cellular and scrapie hamster prion proteins. *Eur J Biochem.* 176:21-30.

Unverzagt, F. W., M. R. Farlow, J. Norton, S. R. Dlouhy, K. Young, and B. Ghetti. 1997. Neuropsychological function in patients with Gerstmann-Straussler-Scheinker disease from the Indiana kindred (F198S). *J Int Neuropsychol Soc.* 3:169-78.

Viles, J. H., F. E. Cohen, S. B. Prusiner, D. B. Goodin, P. E. Wright, and H. J. Dyson. 1999. Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc Natl Acad Sci U S A.* 96:2042-7.

Waggoner, D. J., B. Drisaldi, T. B. Bartnikas, R. L. Casareno, J. R. Prohaska, J. D. Gitlin, and D. A. Harris. 2000. Brain copper content and cuproenzyme activity do not vary with prion protein expression level. *J Biol Chem.* 275:7455-8.

Weissmann, C., and A. Aguzzi. 1997. Bovine spongiform encephalopathy and early onset variant Creutzfeldt- Jakob disease. *Curr Opin Neurobiol.* 7:695-700.

Weissmann, C., M. Fischer, A. Raeber, H. Bueler, A. Sailer, D. Shmerling, T. Rulicke, S. Brandner, and A. Aguzzi. 1996. The role of PrP in pathogenesis of experimental scrapie. *Cold Spring Harb Symp Quant Biol.* 61:511-22.

Wells, G. A., A. C. Scott, C. T. Johnson, R. F. Gunning, R. D. Hancock, M. Jeffrey, M. Dawson, and R. Bradley. 1987. A novel progressive spongiform encephalopathy in cattle. *Vet Rec.* 121:419-20.

White, A. R., S. J. Collins, F. Maher, M. F. Jobling, L. R. Stewart, J. M. Thyer, K. Beyreuther, C. L. Masters, and R. Cappai. 1999. Prion protein-deficient neurons reveal lower glutathione reductase activity and increased susceptibility to hydrogen peroxide toxicity. *Am J Pathol.* 155:1723-30.

Whittal, R. M., H. L. Ball, F. E. Cohen, A. L. Burlingame, S. B. Prusiner, and M. A. Baldwin. 2000. Copper binding to octarepeat peptides of the prion protein monitored by mass spectrometry. *Protein Sci.* 9:332-43.

Wong, B. S., C. Clive, S. J. Haswell, R. A. Williamson, D. R. Burton, P. Gambetti, M. S. Sy, I. M. Jones, and D. R. Brown. 2000a. Copper has differential effect on prion protein with polymorphism of position 129 [published erratum appears in Biochem Biophys Res Commun 2000 May 19;271(3):842]. *Biochem Biophys Res Commun.* 269:726-31.

Wong, B. S., T. Pan, T. Liu, R. Li, P. Gambetti, and M. S. Sy. 2000b. Differential contribution of superoxide dismutase activity by prion protein in vivo. *Biochem Biophys Res Commun.* 273:136-9.

Wong, B. S., T. Pan, T. Liu, R. Li, R. B. Petersen, I. M. Jones, P. Gambetti, D. R. Brown, and M. S. Sy. 2000c. Prion disease: A loss of antioxidant function? *Biochem Biophys Res Commun.* 275:249-52.

Wong, B. S., C. Venien-Bryan, R. A. Williamson, D. R. Burton, P. Gambetti, M. S. Sy, D. R. Brown, and I. M. Jones. 2000d. Copper refolding of prion protein [In Process Citation]. *Biochem Biophys Res Commun.* 276:1217-24.

Young, K., P. Piccardo, S. Dlouhy, O. Bugiani, F. Tagliavini, and B. Ghetti. 1999. The Human Genetic Prion Diseases. *In* Prions Molecular and Cellular Biology. h. s. press, editor.

Zahn, R., A. Liu, T. Luhrs, R. Riek, C. von Schroetter, F. Lopez Garcia, M. Billeter, L. Calzolai, G. Wider, and K. Wuthrich. 2000. NMR solution structure of the human prion protein. *Proc Natl Acad Sci U S A.* 97:145-50.

Zlotnik, I., and J. L. Stamp. 1961. *World Neurology.* 2:895-907.

Acknowledgements

First, I would like to thank Prof. Rudi Glockshuber for the possibility to work in his lab and for the competent support.

I also heartily thank Prof. Arthur Schweiger from the physical chemistry laboratory for the fruitful collaboration and for accepting to be co-referent of this work.

Many thanks to Dr. Sabine Van Doorslaer from the A. Schweiger's lab for the co-work in the investigation on copper(II) binding by EPR.

Dr. Simone Hornemann, Dr. Rüdiger Jankowski and Dr. Gudrun Wildegger, former members of the Prof. Glockshuber's lab, are acknowledged for co-work in the analysis of concentration and ionic strength dependence of unfolding intermediate formation described in this work.

Heike Eberl, Dr. Eva Zobeley, Dr. Estelle LeClerc-L'Hostis, Dr. Susanne Liemann and all other present and former members of the Prof. Glockshuber's lab are also acknowledged for the many useful discussions. I wish also to thank René Brunisholz for Edman sequencing of the PrP proteolysis samples.

A special thank to Luigi Calzolai, Roberto Fattorusso, Marco Pieri, Angelo Vargas, Maurizio Pellecchia, Francesca Viti, Lorenzo Tarli and Salvatore Demartis for the nice atmosphere at and outside the ETHZ.

My parents, my sister Paola, her husband Ivan and my nephew Marco, my brothers Tommaso and Damiano as well as my relatives and friends are acknowledged for the support and for advice.

I'm especially grateful to Prof. Franco Zambelloni for providing a quiet island of literature and music in the stormy sea of science.

Curriculum vitae

Name:	Grazia Maria Cereghetti
Date of Birth:	17.6.1974
Place of Birth:	Mendrisio, Kanton Tessin
Nationality:	Swiss

Education

1980-1985:	Primary school Arzo (TI)
1985-1989:	Secondary school Mendrisio (TI)
1989-1993:	Liceo Mendrisio (TI); final examination: Matura with old Greek and Latin
1993-1997:	Student of Biology at the Swiss Federal Institute of Technology in Zurich (ETHZ), Switzerland Diploma thesis: "Molecular dynamics simulations of the LH2 antenna complex from *Rhodopseudomona acidophila* strain 10050" under the supervision of PD Dr. Ernesto E. Di Iorio
1998-2001:	Graduate student at the Institute of Molecular Biology and Biophysics, ETHZ Ph.D. thesis under the supervision of Prof. Dr. R. Glockshuber

Appendix: papers

Grazia M. Cereghetti, Arthur Schweiger, Rudi Glockshuber and Sabine Van Doorslaer, EPR evidence for binding of Cu2+ to the C-terminal domain of the murine prion protein. *Bioph. J.* **81** (2001): 516-525

Sabine Van Doorslaer, Grazia M. Cereghetti, Rudi Glockshuber and Arthur Schweiger, Unraveling the Cu2+ binding sites in the C-terminal domain of the murine prion protein: A pulse EPR and ENDOR study. *J. Phys. Chem. B* **105** (2001): 1631-39