Analysis of the Molecular Basis of the Conversion and Aggregation of Prion Proteins induced by Oxidative Stress

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To My Family

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List of Abbreviations

AD	Alzheimer's disease
APS	Ammonium peroxydisulfate
Asn	Asparagine
Asp	Aspartic
Αβ	Amyloid-β peptide
bp	Base pair
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathies
CD	Circular dichroism
cDNA	complementary DNA
СНО	Carbohydrate
CIAP	Culf intestinal alkaline phosphatase
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
0	
Cu^0	Copper metal
Cu ^o Cu-Zn SOD	Copper metal Copper-zinc SOD
Cu ^o Cu-Zn SOD Cys	Copper metal Copper-zinc SOD Cysteine
Cu ^o Cu-Zn SOD Cys ddH ₂ O	Copper metal Copper-zinc SOD Cysteine Double distilled water
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO DNA	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide Deoxy ribonucleic acid
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO DNA dNTPs	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide Deoxy ribonucleic acid 2'-deoxynucleoside-5'-triphosphate
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO DNA dNTPs D-PEN	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide Deoxy ribonucleic acid 2'-deoxynucleoside-5'-triphosphate D-(-)-penicillamine
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO DNA dNTPs D-PEN DpI	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide Deoxy ribonucleic acid 2´-deoxynucleoside-5´-triphosphate D-(-)-penicillamine Doppel protein
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO DNA dNTPs D-PEN DpI DTT	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide Deoxy ribonucleic acid 2'-deoxynucleoside-5'-triphosphate D-(-)-penicillamine Doppel protein Dithiothreitol
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO DNA dNTPs D-PEN DpI DTT <i>E. coli</i>	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide Deoxy ribonucleic acid 2´-deoxynucleoside-5´-triphosphate D-(-)-penicillamine Doppel protein Dithiothreitol <i>Escherechia coli</i>
Cu ^o Cu-Zn SOD Cys ddH ₂ O DLS DMSO DNA dNTPs D-PEN DpI DTT <i>E. coli</i> EDTA	Copper metal Copper-zinc SOD Cysteine Double distilled water Dynamic light scattering Dimethyl sulfoxide Deoxy ribonucleic acid 2´-deoxynucleoside-5´-triphosphate D-(-)-penicillamine Doppel protein Dithiothreitol <i>Escherechia coli</i> Ethylene diamine tetraacetic acid

EtBr	Ethidium bromide
fCJD	familial CJD
FFI	Fatal familial insomnia
GSS	Gerstmann-Sträussler-Scheinker syndrome
Gu-HCl	Guanidine hydrochloride
HD	Huntington's disease
His	Histidine
hPrP/Ct	C-terminal domain of human PrP
iCJD	iatrogenic CJD
IPTG	Isopropyl-β-D-thiogalactopyranoside
L	Litre
LB	Luria Broth
М	Molar
MCO	Metal catalyzed oxidation
MeSO	Methionine sulfoxide
Met/M	Methionine
mM	Millimolar
Mox	Methoxinine
mPrP/Ct	C-terminal domain of mouse PrP
N2a	Neuroblastoma cell
N-CAM	Neural cell adhesion molecule
NDP	Nucleation-dependent polymerization
Ni-NTA	Nickel nitrilotriacetic acid
Nle	Norleucine
NMR	Nuclear magnetic resonance
nvCJD	new variant CJD
0^{-2}	Superoxide radical
°C	Degree centigrade
OD ₆₀₀	Optical density at 600 nm

11 J 01 0/1 J 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
Hydroperoxyl radical
Open reading frame
Polyacrylamide gel electrophoresis
Polymerase chain reaction
Parkinson's disease
Phenylalanine
Proteinase K
Protein misfolding cyclic amplification
Gene expressing doppel protein
Human prion protein gene
Prion protein gene in mice
Prion protein knockout mice
Proline
Prion protein
Normal cellular prion protein
Scrapie prion protein
Rough endoplasmic reticulum
Radius of gyration
Hydrodynamic radius
Ribonucleic acid
Alkoxyl radical
Peroxyl radical
Reactive oxygen species
Revolution per minute
Recombinant prion protein
Response unit
Small angle X-ray scattering
sporadic CJD

ScN2a	Scrapie infected neuroblastoma cell
SDS	Sodium dodecyl sulfate
Ser/S	Serine
SOD	Superoxide dismutase
SPR	Surface plasmon resonance
SRP	Signal recognition particle
ssDNA	single strand DNA
T _{1/2}	Half life time
ТА	Template assisted
TEMED	N,N,N´,N´- Tetramethylethylenediamine
Thr/T	Threonine
Trp/W	Tryptophan
TSEs	Transmissible spongiform encephalopathies
Tyr/Y	Tyrosine
UV	Ultra violet
V	Volt
v/v	Volume/volume
Val	Valine
vCJD	variant CJD
w/v	Weight/volume
w/w	Weight/weight
α-CD	α-cyclodextrin
β-CD	β-cyclodextrin

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ABSTRACT

Prion diseases are a group of fatal neurodegenerative disorders, characterized by the autocatalytic conversion of the normal cellular prion protein PrP^C into the infectious PrP^{Sc} isoform. Since the mechanism of $PrP^{C} \rightarrow PrP^{Sc}$ conversion still remains unknown, growing evidence suggests a central role of oxidative stress in the pathology of prion diseases. The site-specific oxidative modification of the surface exposed Met residues in the globular C-terminal domain of PrP is suggested to represent the initial event for the lethal $PrP^{C} \rightarrow PrP^{Sc}$ structural conversion. Therefore, the effect of the surface exposed Met residues on the oxidative-induced aggregation of PrP by MCO and UVB radiation was investigated in terms of the thesis presented. As revealed by circular dichroism and dynamic light scattering measurements, the observed oxidative induced PrP aggregation follows two independent pathways: (i) complete unfolding of the protein structure associated with precipitation or (ii) specific structural conversion into distinct soluble β -oligomers. It has been revealed that the entire replacement of the surface exposed Met-residues (M129, M134, M154, M166, and M213) in the folded C-terminal domain of human PrP (residues 121-231) by Ser residues resulted in: (i) enhancement of PrP stability towards the oxidativeinduced aggregation by MCO (ii) inhibition of $\alpha \rightarrow \beta$ transition, but formation of soluble α -oligometric intermediates. Moreover, the site specific substitution of Met 129 polymorphism by Thr showed significant decrease of the oxidative aggregation rate of PrP induced by MCO and inhibition of $\alpha \rightarrow \beta$ transition, suggesting that Met 129 represents one of the most important amino acids that share a significant contribution to the cellular $PrP^{C} \rightarrow PrP^{Sc}$ conversion. Moreover, the effect of β -CD on the *in vitro* oxidative aggregation of mouse and human PrP induced by MCO was investigated. β-CD gained attention in the field of anti-prion compounds due to its ability to clear PrP^{Sc} from infected cell cultures. Here it was shown that the delaying effect of β -CD on the structural conversion of human PrP is rather due to the caging of copper ions generated by MCO than to a direct interaction with PrP. Moreover, the observed pathway switch in the presence of β -CD from unspecific denaturation to specific oligomerization strongly supports the theory that aggregation pathways are determined by the population of specific intermediate states. The results obtained in this study provide new insights to understand the mechanism of prion conversion and the onset of associated neurodegenerative disorders, particularly of the sporadic form of CJD.

Zusammenfassung

Prion-Krankheiten eine umfassen Gruppe von schwerwiegenden neurodegenerativen Erkrankungen, welche auch als übertragbare spongiforme Enzephalopathien (TSEs) bezeichnet werden. Sie zeichnen sich durch eine autokatalytische Umwandlung des normalen zellulären Prion-Proteins (PrP^C) in eine falsch gefaltete infektiöse Isoform (PrPSc) aus. Der Mechanismus der Konformationsänderung des Prion-Proteins ist bisher weitestgehend unbekannt. Es wird aber vermutet, dass zellulärer oxidativer Stress eine entscheidende Rolle in der Pathologie von Prion-Erkrankungen spielt. Insbesondere die spezifische Oxidation von zugänglichen Methionin-Resten in der gefalteten C-terminalen Domäne der Prion-Struktur kann vermutlich signifikant zu der tödlichen $PrP^{C} \rightarrow PrP^{Sc}$ Umfaltung beitragen. Deshalb sollte in dieser Arbeit der spezifische Einfluss von Methionin-Resten auf die oxidativ-induzierten Aggregation von Prion-Proteinen mittels Metall-katalysierter Oxidation und UVB-Strahlung systematisch untersucht werden.

Durch Kombination der Analyse der Sekundärstruktur mittels CD-Spektroskopie und dynamischer Laserlichtstreuung wurde nachgewiesen, dass die oxidativ-induzierte Aggregation von Prion- Proteinen zwei unabhängigen Mechanismen folgt. Dabei handelt es sich zum einen um eine vollständige Strukturentfaltung mit nachfolgendem Ausfall des denaturierten Proteins sowie zum anderen um eine Prion-spezifische Konformationsänderung, die mit der Bildung löslicher Oligomere verbunden ist, welche durch einen hohen β-Faltblattanteil charakterisiert sind. Ein direkter Zusammenhang der Aggregationstendenz mit der Anzahl der vorhandenen Methionin-Reste in der Prion-Struktur konnte durch Untersuchung eines mutierten humanen Prion-Proteins hergestellt werden, bei dem fünf oberflächlich lokalisierte Met-Reste der C-terminalen Domäne gegen Serin ausgetauscht wurden. Nach Expression und Reinigung zeigte das vPrP 121-231 eine signifikant erhöhte Stabilität gegenüber oxidativ induzierter Umfaltung mittels Metall-katalysierter Oxidation

(MCO). Zur Bestimmung der lokalen Beiträge der einzelnen Met-Reste muss eine systematische Mutation erfolgen. Im Rahmen dieser Arbeit wurde in einem ersten Schritt gezeigt, dass das Methionin an Position 129 einen signifikanten Einfluss auf die Stabilität des Pion-Proteins gegenüber der oxidativ-induzierten Aggregation aufweist. Nach entsprechendem Austausch gegen Threonin war die Halbwertszeit des mutierten PrP im MCO-Test deutlich erhöht. Anstatt einer spezifischen $\alpha \rightarrow \beta$ Konformationsänderung wurde die vollständige Denaturierung der PrP-Moleküle nachgewiesen.

Zur Bestätigung der direkten Korrelation der Art des oxidativ-induzierten Umfaltungsmechanismus mit dem Ausmaß der Oxidation der Proteinstruktur des Prion-Proteins wurde der Einfluss von β -Cyclodexdrin (β -CD) auf die oxidative *in vitro* Aggregation von humanem und murinem PrP untersucht. β -CD hat aufgrund seiner Fähigkeit, den PrP^{Sc}-Gehalt infizierter Zellkulturen zu reduzieren, für Aufmerksamkeit im Bereich der Wirkstoffentwicklung zur Behandlung von Prion-Erkrankungen gesorgt. In der Tat verringerte die Zugabe von β -CD das oxidative Potential im MCO-Test, so dass ein Wechsel des Aggregationsmechanismus von vollständiger Denaturierung zu spezifischer Umfaltung unter Ausbildung oligomerer Strukturen detektiert wurde. Dieser Effekt beruhte allerdings nicht auf einer in vorigen Arbeiten postulierten Interaktion von β -CD mit den PrP-Molekülen, sondern auf der Komplexierung von freien Cu(II)-Ionen, die während des MCO-Tests gebildet werden und zur Entstehung freier Radikale signifikant beitragen.

Die Ergebnisse dieser Arbeit bekräftigen die Hypothese, dass die unterschiedlichen Aggregationswege des Prion-Proteins von spezifischen intermediären Übergangszuständen gesteuert werden, deren Existenz von der Destabilisierung der Protein-Struktur durch Energiezuführung, in diesem Fall durch Oxidation, abhängt. Ein derartiger detaillierter Einblick in die Umfaltungsmechanismen ist zum Verständnis der assoziierten Prion-Krankeiten zwingend erforderlich.

1 Introduction

1.1 Protein misfolding and disease

Protein folding is the process by which a group of amino acids of a synthesized polypeptide chain folds into its unique three-dimensional structure (1, 2). The synthesized proteins can attain their native conformation as well as their functions by the help of different cellular proteins known as chaperones that are usually localized in the endoplasmic reticulum (ER). Correctly folded proteins are then transported to the Golgi apparatus and exported to the extracellular compartment. On the other hand, incorrectly folded polypeptides are detected by a quality control mechanism that results in ubiquitination for proteasomal degradation in the cytoplasm (Fig. 1).



Fig. 1: The mechanism of protein folding. Synthesized nascent polypeptides interact via their N-terminal signal peptides with signal recognition particles (SRPs). The SRP drives the whole complex (ribosome, RNA, and polypeptide) to the ER membrane. The folding of proteins occurs by the action of molecular chaperones and enzymes that are involved in the regulation of this process. Incorrectly folded proteins are recognized by a quality control system, tagged by multiple ubiquitin molecules, and finally targeted for degradation by cytosolic proteasomes (1).

According to the energy landscape theory proteins have to pass different unfolded states that can be represented by folding funnels to reach their native conformation (Fig. 2). On the highest energy level, proteins do not comprise ordered structures. However, proteins find their energy minimum, as they gain their completely native conformation characterized by a unique set of secondary structure motifs (3, 4).



Fig. 2: Energy states of protein folding. Folding funnel illustrates many different folding pathways that can be used by the unfolded protein to reach the energy minimum (native state) that is located at the bottom of the funnel. Unfolded proteins possess a high energy level and occupy the top of the funnel (2).

During the folding process a failure can occur, resulting in destabilization of the peptide and the inability to adopt or retain its functional conformational state. This type of defects represents the basis of a variety of human diseases such as cystic fibrosis (5). Furthermore, in certain cases some partially unfolded protein intermediates can assemble into oligomeric complexes followed by formation of extremely stable and highly ordered fibrils called amyloid. These amyloids are considered to be pathogenic to the cell, which represents the molecular basis of a growing list of protein misfolding diseases, e.g. human neurodegenerative disorders and systemic amyloidosis (6-8). Amyloids are defined as extracellular depositions of protein fibrils with characteristic appearance in electron microscopic analysis, typical X-ray diffraction pattern, and affinity for Congo red dye with concomitant green birefringence (9). Biophysical studies on the

structure of amyloid fibrils have shown that amyloids do not have universal tertiary or quaternary structure, but their structure consist of parallel (10,11) or anti-parallel β -sheet conformation (12, 13).

Neurodegenerative disorders share common characteristic features concerning the mechanism of disease initiation and progression. Protein misfolding has been considered to be the central aspect, classifying these diseases as protein conformational disorders (14, 15). Examples are the Alzheimer's disease (AD), transmissible spongiform encephalopathies (TSEs), diabetes type 2, Huntington's disease (HD), and Parkinson's disease (PD) (Tab. 1). Protein conformational disorders display a high degree of similarity at the molecular level, although they have different clinical manifestations. The causative agent is well known to consist mainly of β -sheet structure, representing a misfolded isoform of the associated cellular protein.

Disease	Protein	Type of	Affected	Proposed function of
	associated	aggregates	organ	normal cellular protein
Alzheimer	Amyloid-β/	Amyloid	Brain	Neurite outgrowth, synaptic
	Tau	plaques/oligomers		vesicle transport
TSEs	Prion protein	Oligomers/amyloid	Brain	Signal transduction,
		plaques		antioxidant, copper binding
Huntington	Huntingtin	Not detected	Brain	Transcriptional regulation
Parkinson	α-Synuclein	Lewy bodies	Brain	Regulation of membrane
				stability or turnover

Table 1: Conformational disorders and their associated disease-causative proteins.

Within the pathogenesis of protein misfolding diseases, three steps are suggested in the structural conversion and aggregation of the associated proteins, (i) structural conversion (ii) a nucleus formation (iii) a fibril extension. Although the molecular mechanism of the conversion is still enigmatic, the native cellular structure of the disease-associated proteins changes into a β -sheet enriched structure via an energetically unfavourable transition (Fig. 3A). It has been reported that specific mutations within the disease-associated proteins as well as interactions with other biological molecules reduce the free energy barrier, facilitating the transition process (16-18). The misfolded proteins are finally assembled into amyloid fibrils, however during the conversion process unstable intermediates are supposed to be formed. When a nucleus of suitable size is formed (Fig. 3B), further addition of monomers to the nucleus becomes energetically favourable, followed by a rapid extension of the amyloid fibrils that obey a first order kinetic reaction (19, 20).



Fig. 3: Protein misfolding, aggregation, and amyloid fibril formation. (A) Transition of natively folded proteins into β -sheet enriched disease-associated proteins via an energetically unfavourable process. During the transition reaction unstable intermediates are supposed to be formed. Mutations or interactions of the proteins with other cellular components facilitate the transition process by reducing the free energy barrier. (B) When a nucleus of suitable size is formed, further incorporation of monomers into the nucleus becomes energetically favourable followed by an extension of the amyloid fibrils (21).

The aggregation state of the toxic protein isoform in neurodegenerative disorders is still in discussion. The presence of highly ordered amyloid fibrils in the brains of affected patients led to the postulation that the amyloid fibrils are the pathogenic agent. Moreover, the *in vitro* preparation of the fibrillar amyloid- β aggregates (A β) associated with AD was observed to be toxic to neuronal cell

cultures, resulting in initiation of membrane depolarization and alteration of the frequency of their action potentials (22, 23). Neuronal damage was also demonstrated by injection of $A\beta$ fibrils into the cerebral cortex of aged rhesus monkeys (24). However, recent studies suggested that soluble oligomeric intermediates formed on the pathway of amyloid synthesis are the pathogenic species that mediate cytotoxicity and cell damage. The severe memory loss in patients suffering from AD was found to be closely related to the presence of soluble oligomers and other low molecular weight species of A β (25, 26). Transgenic mice exhibited a marked defect in cognitive impairment, cell function, and neuronal plasticity before detection of sufficient quantities of amyloid fibrils of A β (27, 28). Similarly, it has been reported that mutants of α synuclein associated with the early-onset form of PD resulted in neuronal degeneration without accumulation of lewy bodies (29). Transgenic rats overexpressing a-synuclein showed neuronal loss without detection of intracellular deposits (30), and injection of nonfibrillar α -synuclein deposits in various brain regions exhibited substantial motor deficiencies and loss of dopamenergic neurons in transgenic mice (31). A mechanism by which the soluble oligomers induce cell damage was proposed. Disruption of the cell membrane via insertion of the oligomers into the lipid bilayer, alteration of the normal ion gradients following loss of the intrinsic biological function of the native protein, and blocking the proteasome components or association of chaperone to the misfolded protein are supposed (32, 33).

1.2 Prion diseases

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), comprise a group of fatal neurodegenerative disorders that affect both humans and animals. Human forms of prion disease include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and kuru. Prion diseases affecting animals include scrapie in

sheep and goat as well as bovine spongiform encephalopathies (BSE) in cattle (34-36). The histopathological features of TSEs such as spongiform degeneration of the brain, neuronal vacuolation, and astrocytic gliosis, represent the main consequences of the cerebral deposition of the misfolding isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) into amyloid plaques. Although both isoforms share the same amino acid sequence, their physical properties are completely different. PrP^{C} is a mainly α -helical-folded monomer that shows significant sensitivity towards Proteinase K (PK) digestion, whereas PrP^{Sc} resembles an assembled protein multimer characterized by an enhanced resistance toward PK-digestion and an increased amount of β -sheet conformation (37).

Prion diseases have been found to be infectious, mainly resulting from feeding animals with already scrapie-contaminated materials, familial due to mutations in the gene encoding the human prion protein PRNP, and sporadic, arising spontaneously without any apparent cause. The infectious origin has initially been elucidated for kuru (38), an endemic disease affecting the fore people of New Guinea that was found to be transmitted among women and children by ritual cannibalism. In iatrogenic CJD (iCJD), the infectious agent is transmitted by Dura mater grafts, one of the outermost three layer membranes covering the brain, administration of cadaveric growth hormone, and the use of scrapie contaminated equipments in neurosurgery (39). In 1996 the first case of new variant CJD (nvCJD) has been described, affecting young people in United Kingdom as a result of consuming beef or beef products contaminated with PrP^{Sc} (40). More than 80% of all CJD cases reported are sporadic (sCJD), also a few cases of GSS (41). Inherited or familial TSEs represent about 10% of all CJD cases. In contrast, GSS and FFI are entirely related to germline mutations in the *PRNP* gene located on chromosome 20 (42).

The nature of the infectious pathogen in prion diseases is still a matter of debate for approx. 20 years. The failure of inactivation of the scrapie agent by

UV irradiation together with the results of biophysical and biochemical studies have immediately neglected the idea of the coexistence of nucleic acids within the infectious agent (43). This leads to the theory that the pathogenic agent is a self-replicating protein that adopts an abnormal structure and has the ability to convert other proteins into the infectious isoform (44). Stanley Prusiner proposed this "protein only" hypothesis (45) after purification of the infectious agent from scrapie-infected hamster brains. Since the infectivity was significantly reduced by agents that denature proteins, he named the pathogenic agent "prion" (proteinaceous infectious particles) (46). Consequently, the agent responsible for the propagation of prion disease is the post-translationally misfolded isoform PrP^{Sc} of the cellular prion protein PrP^{C} . When PrP^{Sc} is formed and introduced to the host cell, it converts PrP^{C} molecules into the PrP^{Sc} isoform in an elusive autocatalytic process (47).

Several experiments strongly support the validity of Prusiner's prion hypothesis to date. PrP^{sc} has been co-purified with infectivity and the concentration of the protein correlated well with the infectivity titer. Furthermore, the highly purified PrP^{Sc} molecules free from any other detectable components have retained their activity (48). Büeler et al. (49) reported the importance of PrP-expression in the host cell for the propagation of the infectious agent and the development of disease. It has been shown that mice lacking the prion protein gene (Prnp^{0/0} mice) were resistant to prion infection. Strong evidence for the "protein only" hypothesis resulted from the observation that most of the inherited cases of TSE are directly related to mutations within the PRNP gene (35, 36). Overexpression of the PRNP gene carrying specific mutation related to GSS syndrome in mice resulted in the onset of a scrapie-like disease as well as some neurological signs like spongiform degeneration of the brain and astrocytic gliosis (50). The propagation of infectivity in neuroblastoma cells (ScN2a) infected with brain homogenate containing the infectious pathogen was also reported (51, 52).

The reconstitution of infectivity in vitro is another great evidence for the validity of Prusiner's "protein only" hypothesis. Regarding the generation of infectious PrP^{Sc}, two different strategies have been suggested: (i) conversion of PrP^C or recombinant prion protein (rPrP) into PrP^{Sc} in the absence of nascent PrP^{Sc}, and (ii) template-assisted amplification of PrP^{Sc}. The first strategy is only related to the sporadic form of prion diseases rather than to the acquired forms. Moreover, additional factors such as salts and chaotropic agents (53-55), pressure, (56), and heat (57) have been reported to induce transformation of PrP in different cell free conversion assays. The product formed in theses studies exhibited a high similarity to the native PrP^{Sc} molecule with respect to its physical properties (58, 59). Most of the researchers have detected an increase in PK resistance and β -sheet conformation. Since the PrP^{Sc} template is absent during the conversion process, the products possessed a large diversity of β sheet conformations, which acquired some, but not all the features of PrP^{Sc}. PrP adopted different conformations depending on the solvent conditions and the cofactors supplemented in the reactions (60) and no infectivity observed so far.

Considering the template-assisted amplification of PrP^{Sc}, several protocols have been developed. Recently, Saborio *et al.* (61) reported that PrP^{Sc} can be amplified similar to DNA when it is mixed with a large excess of PrP^C followed by successive cycles of amplification and sonication. This method is called "protein misfolding cyclic amplification" (PMCA). This approach indicated that PrP^{Sc} molecules generated *in vitro* were able to catalyze the formation of new PrP^{Sc} molecules, supporting their autocatalytic properties. However, the use of the whole brain homogenate in PMCA to generate infectivity raises the possibility of the participation of some unidentified cellular factors in the conversion process. The intracerebral inoculation of the synthetic amyloid fibrils from the recombinant mPrP89-230 in transgenic mice results in development of neurologic dysfunction (62). The brain extract of these mice exhibited PK resistance and transmitted the disease when inoculated into both *wild type* and transgenic mice, suggesting the prion infectivity. Despite the prion hypothesis elucidate the key role of PrP in TSEs, it has one particular weakness that has long been used as a strong argument against it. Prion infection occurs via different strains. These strains are characterized by different incubation times, clinical features, and pathological profiles in a single host, which are difficult to reconcile with the "protein only" hypothesis (35, 63). The presence of PrP^{Sc} in the host cell is usually correlated with infectivity (35, 48). However, it has been reported that infectivity can be propagated in mice injected with brain homogenate from BSE infected cattle where PrP^{Sc} is absent or barley detected in the serial passage (64). In contrast, no or little infectivity was detected in animals harbouring sufficient amount of PrPSc. This finding raises several questions about the nature of the neurotoxic molecule responsible for the widespread neuronal cell loss and spongiosis, which are the hallmarks of prion diseases. The conversion of PrP^C to PrP^{Sc} with involvement of nucleic acids or other cellular factors has only been accepted by some of the researchers. Narang (65) has isolated a viral single strand DNA (ssDNA) from scrapie-infected hamster brains. The DNA encodes a specific protein, which is supposed to play a role in the conversion of PrP^C to PrP^{Sc}. These results contradict the concept that prion is deprived of DNA (43). In addition, the interaction of RNA with PrP and its involvement in the conversion of PrP^{C} to PrP^{Sc} have been reported (66).

1.3 Structure and function of the cellular prion protein (PrP)

The cellular human PrP^{C} is encoded by *PRNP* gene on chromosome 20. *PRNP* consists of two exons, whereas *Prnp* gene in mice comprises three exons (67). The entire open reading frame (ORF) is encoded in one exon (exon 2 in human and exon 3 in mice). Human PrP^{C} is a highly conserved 253 amino acid sialoglycoprotein, mainly expressed in neurons and galial cells, but also expressed in a variety of non-neural tissues (68). The first 22 N-terminal amino acids encode a signal peptide that directs the translocation into the rough

endoplasmic reticulum (RER). PrP^C is anchored to the outer surface of the plasma membrane via a glycosyl phosphatidylinositol (GPI) moiety after cleavage of the C-terminal signal peptide. Nuclear magnetic resonance (NMR) and two X-ray crystallographic studies revealed that the N-terminal domain comprising the amino acids 23-120 is highly flexible and therefore considered to be unstructured. Five octapeptide repeats with the consensus sequence PHGGGWGQ, representing coordination sites of divalent metal ions are located between residues 50 and 90. The C-terminal domain (residues 121-230) forms a globular structure, containing three α -helices (H1, H2, and H3) formed by residues 144-154, 173-194, and 200-228, as well as two small antiparallel β sheets (S1 and S2) at residues 128-131 and 161-164 (Fig. 4A) (69, 70). Two Nglycosylated moieties are attached to the aspragine residues Asn 181 and 197 and one disulfide bond is formed between cysteine residues Cys 179 and 214 that links H2 and H3 (Fig. 4B). The mature full length human PrP(23-231) contains nine Met residues, which are either surface exposed to the solvent (Met 109, Met 112, Met 129, Met 134, Met 154, Met 166, and Met 213) or completely buried (Met 205 and Met 206) in the hydrophobic core of the PrP molecule (71).



Fig 4: (A) Three-dimensional structure of the globular C-terminal domain (residues121-231) of human PrP. Three α-helices and two antiparallel β-sheets are formed (PDB code. 1QM2, 50). (B) Scheme illustrating the primary structure of human PrP before and after maturation. The signal peptides at both termini are trimmed and the GPI anchor attaches the C-terminus to the surface of the plasma membrane. The mature PrP possesses two CHO moieties at residues Asn 181 and Asn 197 and a disulfide bond linking Cys 179 and Cys 214.

Despite a high number of investigations, the function of the *PRNP* gene within mammalian species is still a matter of controversy discussion. A study on *Prnp*^{0/0} mice did not reveal any neurological or behavioural changes. The mice were viable and developed normally (72). In contrast, Suehiro *et al.* (73) detected motor dysfunctions accompanied by extensive loss of Purkinje neurons in *Prnp*^{0/0} mice. A third study reported that PrP antagonize the neuorotoxic effect (loss of Purkinje cells and cerebellar ataxia) of doppel protein (DpI) in *Prnp*^{0/0} mice. DpI is a 179 amino acid protein encoded by the *Prnd*-gene that is located 16 kb downstream of the *Prnp*-gene. DpI exhibits a high degree of structural similarity to PrP. It mainly consists of α -helices, contains a disulfide bond, and two N-glycosylation sites, however it lacks the octapeptide repeat in the N-terminal domain (74, 75).

The tendency of PrP to bind copper ions via its N-terminal octapeptide repeats suggested an involvement of PrP in copper homeostasis. In this context, a decrease in the copper content as well as an increased susceptibility to oxidative stress was observed in $Prnp^{0/0}$ mice. The same research group reported that copper-bound PrP exhibits superoxide dismutase (SOD) enzyme activity and plays a role in the defence mechanism against cellular oxidative stress (76-78). In contrast, Waggoner et al. (79) stated that copper content of the brain and the enzymatic activity of Cu-Zn SOD is not affected in mice that either lack or overexpress PrP. Considering the localisation of PrP in the presynaptic membrane, a role of PrP in the regulation of the presynaptic copper concentration and of the synaptic transmission has been postulated (80). It was also demonstrated that PrP could participate in cell-cell adhesion and in the development of the central nervous system (CNS) by binding to the neuronal adhesion molecule N-CAM as a signalling receptor in neuroblastoma (N2a) cells (81). Furthermore, PrP was found to play a role in signal transduction in neuronal cell cultures by phosphorylation of the tyrosine kinase Fyn (82). Recently, it has been reported that PrP has a substantial contribution to the

suppression of apoptotic cell death (83-85). Neuronal cell culture deficient of PrP-expression (PrP^{-/-}) showed a high susceptibility to apoptosis, which was significantly decreased by the reintroduction of PrP into the cells (86).

1.4 Mechanism of prion replication

Based on the "protein only" hypothesis in which PrP^{Sc} serves as a template for the conversion of PrP^{C} into the infectious isoform, three models for the self replication mechanism have been proposed. Following the heterodimer or template assisted (TA) model (87, 88) the conversion of PrP^{C} into PrP^{Sc} is kinetically controlled. PrP^{Sc} occupies a higher energy minimum than PrP^{C} . Therefore, the formation of PrP^{Sc} is associated with the overcome of a high energy barrier, which is the rate limiting step. Once the PrP^{Sc} molecule is formed it binds to a PrP^{C} molecule to produce a heterodimer that is subsequently converted into a homodimer (Fig. 5). The homodimer undergoes dissociation into two PrP^{Sc} molecules to catalyse further conversion. According to this model highly ordered oligomers are formed in the course of aggregation followed by exponential propagation of the infectious pathogen. The assistance of chaperones or specific mutations within the prion protein gene sequence is proposed to facilitate the overcoming of the high activation energy barrier (75).



Fig. 5: Schematic diagram of the heterodimer model for prion replication (87). The process of conversion is kinetically controlled. The presence of a high activation energy barrier between both isoforms prevents the spontaneous conversion. Binding of PrP^{Sc} to the PrP^{C} molecule induced a conformational change that results in formation of PrP^{Sc} . Chaperones and specific mutations have been suggested to lower the energy barrier for the transition into the β -sheet conformation.

The nucleation-dependent polymerization model (NDP) by Lansbury (90) stated that PrP^{C} exists in a thermodynamic equilibrium with PrP^{Sc} . The formation of a PrP^{Sc} oligomer of suitable size is the rate limiting step. This oligomer serves as a nucleus that can promote the incorporation of further PrP^{Sc} molecules into the oligomer. Nucleation starts by shifting the equilibrium towards the formation of PrP^{Sc} , resulting in a rapid propagation by further addition of PrP^{Sc} into the growing nucleus (Fig. 6).



Fig. 6: Schematic diagram of the NDP model for prion replication (90). PrP^C and PrP^{Sc} exist in a thermodynamic equilibrium. PrP^{Sc} is stabilized when it forms an oligomeric stock (nucleus) like seed. Once the seed is formed further PrP^{Sc} molecules are incorporated into the oligomer, resulting in the formation of large aggregates.

The NDP model offers a simple explanation for the propagation of different scrapie strains due to the formation of infectious seed in a crystallization-like process. Therefore, it has been proposed that the various PrP^{Sc} strains presumably consist of PrP molecules packed together in a different orientation, arising from nuclei of different sizes (91).

The basic concept of a third model, known as the dimerization model (92) is that two PrP^{Sc} molecules form a dimer, stabilized by two intermolecular disulfide bridges. The recruitment of a PrP^{C} dimer that formed native intramolecular disulfide bridges is the rate limiting step of the PrP^{Sc} replication (Fig. 7). The binding of the PrP^{Sc} dimer partially unfolds the native structure of the PrP^{C} dimer and destabilizes its intramolecular disulfide bridges. As a result, a transient complex is formed followed by rearrangement of the disulfide bridges into intermolecular orientation. Finally, the transient complex undergoes rapid conformational changes into the β -sheet enriched scrapie isoform that either diffuses to catalyze another conversion reaction or remains in the complex to serve as a nucleus for association into amyloid structures. This model combines thermodynamic and kinetic parameters from the two models previously described. For instance the PrP^{Sc} dimer is supposed to exhibit a catalytic character as a monomer in the TA model (87, 88). On the other hand, the binding energy and the intermolecular disulfide bridges achieve a state of stabilization on the scrapie form, which is a characteristic feature of amyloid formation following the NDP model (90).



Fig. 7: Replication of PrP^{Sc} based on dimer formation and rearrangement of disulfide bonds (92). The lowered energy barrier resulting from the binding of the PrP^{Sc} dimer to a PrP^C dimer induces structural changes within the PrP^C dimer. Subsequently, the disulfide bonds are rearranged into an intermolecular form. The transient complex diffuses to catalyze further conversion reactions or remains to serve as a nucleus for the formation of amyloid aggregates.

1.5 Polymorphism of the PRNP gene

Familial forms of prion disease are associated with mutations in the *PRNP* gene on human chromosome 20. The human *PRNP* is characterized by the presence of two polymorphic alleles at codon 129 that encode either Met or Val (93-95). This polymorphism plays a central role in the determination of the genetic susceptibility of humans to prion diseases. Individuals affected by familial CJD (fCJD) possess Met homozygotes (Met/Met) at position 129, whereas hetero- and homozygote Val are rarely found (94, 96). Moreover, the polymorphic site in the *PRNP* gene is suggested to influence the

neuropathologic pattern and the mechanism of lesion formation in sporadic CJD (sCJD). In this context, the formation of amyloid plaques in sCJD patients is strongly associated with the presence of Val at position 129 (97, 98). Transgenic mice that express Met homozygotic human PrP are shown to be prone to develop variant CJD (vCJD). In contrast, mice expressing Val homozygotic human PrP are resistant towards this disease (99). Expression of PrP carrying the pathogenic mutation D178N and Met at position 129 results in FFI, whereas the same mutation with Val at the polymorphic site is strongly associated with fCJD (100). Val 129 homozygotes carrying the F198S mutation are considered to be the main cause of Indiana Kindred variant of GSS (101). In addition, Dermaut et al. (102) observed a significant correlation between the Val 129 homozygotes and the early onset of AD. The molecular analysis of some sCJD cases indicated a tight connection between Val homozygotes at codon 129 and the deposition of the monoglycosylated prion species (type I). Furthermore, the results also illustrated that codon 129 polymorphism and the physicochemical properties of protease resistant PrP are the major determinants of the clinical phenotypic variability in sCJD cases such as ataxia and myoclonus (103).

Despite strong efforts to understand the consequences of codon 129 polymorphism in terms of disease susceptibility and pathogenesis, the molecular mechanism by which these effects are mediated still remains unknown. The substitution of Met at position 129 against Val in the C-terminal domain of recombinant mPrP(121-231) did not affect its thermodynamic stability (104). An NMR study on PrP mutant revealed that changes in the hydrogen bond pattern at residues Tyr 128 and Asp 178 induced by the mutation D178N, which is linked to GSS, are influenced by the polymorphism at position 129 (105). Recently, the effect of polymorphism on the β -oligomer (β^{O}) formation and the type of interaction were investigated. The results revealed that the core region of PrP (residues 127-228) is involved in the formation of β^{O} . The ability to form stacks and the number of oligomers was reduced if Val is present at position 129 (106).

A molecular dynamics simulation on the effect of pH induced PrP conformation indicated a contribution of the Met 129 side chain in the formation of β -sheet structures at low pH. The interaction of Met 129 with Val 122 results in the contribution of N-terminal amino acids for the expansion of β -sheet structure (107). Moreover, Met at position 129 increases the tendency of PrP to form β sheet rich oligomers compared to Val, which directs PrP to α -helix rich monomers. The maturation of oligomer structures was found to be a timedependent process that proceeds with a higher rate if Met is present at position 129 (108). A mixture of oligomers containing both allelic forms significantly decreases the rate of amyloid formation compared to a homogenous oligomer preparation of each allele (109).

1.6 Oxidation of prion protein

It has been reported that elevated levels of reactive oxygen species (ROS) during oxidative stress, aging, and in certain pathological cases have a significant impact on the oxidative modification of proteins (110-112). In oxygenated biological systems the superoxide radical (O_2) is present in equilibrium with its protonated form, the hydroperoxyl radical (OH_2) . These radicals are produced by ionizing radiation and leakages from the electron transport chains of mitochondria, chloroplasts, and ER. O⁻² is relatively unreactive in comparison with many other radicals, but it has the ability to convert into more reactive species such as peroxyl (ROO), akoxyl (RO), and hydroxyl (OH⁻) radicals (112). Several studies (110-114) reported that OH⁻ radicals are the most reactive species responsible for the oxidative modification of proteins. The *in vivo* generation of OH⁻ radicals is considered to be associated with the decomposition of hydrogen peroxide (H_2O_2) by redox active metals such as iron (Fe) and copper (Cu), which is the basic concept of Fenton's reaction. The reaction is initiated by the reduction of the redox active metal by O_2^{-1} radical followed by the oxidation of its dismutation product H₂O₂ by the
reduced metal (115, 116). OH radicals can oxidize the side chains of certain amino acids and can oxidize the back bone of the polypeptide chain that leads to protein fragmentation (117). The oxidative damage of proteins is an irreversible process. Although oxidized proteins can be eliminated by the normal cellular proteolytic pathways, the heavily oxidized proteins become more resistant to proteolytic degradation. The protease resistant proteins undergo structural perturbation followed by aggregation and accumulation in the target organ (110, 118).

All amino acids in the peptide chain of proteins are vulnerable to oxidation, particularly sulphur-containing Cys and Met as well as aromatic amino acids [tryptophan (Trp), histidine (His), tyrosine (Tyr), phenylalanine (Phe), and proline (Pro)]. The sulphur-containing amino acids, particularly Met, are of significant importance due to their high sensitivity towards oxidative modification. Met residues are easily oxidized to the more hydrophilic Metsulfoxide (MeSO), which can be reversely reduced by the enzyme MeSO reductase. Along with Cys oxidation this is the only oxidative modification of proteins that can be repaired. Met residues at the surface of proteins are more susceptible to oxidation, resulting in a more hydrophilic protein. In contrast, partially or totally buried Met residues are less or not susceptible to oxidation (119, 120). Consequently, Met residues have been shown to act as endogenous antioxidants in the protein structure to protect other vital residues from oxidation (110, 120). It has been reported that oxidative stress is increasing with increasing age (116). Furthermore, the proteosomal function responsible for the degradation of oxidized proteins and the enzymatic activity of the cellular antioxidant system are decreased with age (121).

Considering the number of Met residues in the PrP molecule, it represents an excellent target for *in vitro* oxidation investigation by ROS, resulting in different structural conformations that depend on the applied system (116). Several *in vitro* oxidation assays (116, 122, 123) have been applied to understand the role

of Met residues in the PrP molecule and their effect on its structural conversion. The refolding of recombinant mouse and chicken PrP in the presence of Cu(II) induced a selective Met oxidation, resulting in a unique structural conformation compared to PrP refolded in the absence of Cu(II) (124). Oxidation of the surface exposed Met residues of recombinant Syrian hamster prion protein rSHaPrP(29-231) by H₂O₂ was investigated. Mass spectroscopy analysis showed a high susceptibility of Met 109 and Met 112 located in the non-structured Nterminal region towards oxidation. Additionally, the toxic fragment of PrP(106-126) and the polymorphic site Met 129 were also susceptible to oxidation followed by extensive aggregation and precipitation (122). Oxidation of His residues in the octarepeat region of rSHaPrP(29-231) to 2-oxohistidine by metal catalyzed oxidation (MCO) followed by extensive aggregation was also reported (123). Furthermore, it has been observed that the histidine enriched octarepeat region of rmPrP(58-91) has a protective role by decreasing Cu-catalyzed oxidation of the accessible residues in the C-terminal domain (125). Breydo et al. (126) stated that oxidation of Met residues by H_2O_2 in the central region of rSHaPrP(90-140) inhibit the formation of amyloid fibrils that adopt PrP^{Sc}-like conformation. The influence of MCO on the in vitro aggregation and the structural conversion of recombinant human prion protein rhPrP(90-231) has been analysed (116). The oxidation process was monitored by mass spectroscopy. The results showed a distinct increase in the molecular mass of the peptides due to the incorporation of oxygen into His and Met residues, resulting in the formation of 2-oxohistidine and MeSO. The oxidized PrP molecule is rapidly converted into a β -sheet enriched conformation. Moreover, two distinct oligomers consisting of 25 and 100 monomeric PrP molecules were detected, which are similar in size to those that have been reported to induce infectivity in brain tissues of hamsters (127). Oxidized Met residues have also been found at high levels in the senile plaques of AD patients. Raman spectral analysis in the same study indicated the binding of Zn(II) and Cu(II) to His residues in the senile plaques. This binding has been reversed by addition of the chelator ethylenediaminetetraacetate, resulting in the disappearance of the β sheet features of the senile plaques (128). Colombo et al. (71) reported applying molecular dynamic simulations that the replacement of Met 213 in helix-3 of hPrP(125-229) with MeSO (i) destabilizes the native state of the molecule, (ii) increases the flexibility in specific regions, and (iii) increases the probability to acquire alternative conformations, which is required for the pathogenic conversion. Recently, the impact of Met-oxidation on the conversion of PrP was investigated by replacing the nine Met residues of the full length rhuPrP (23-231) completely with two chemically stable non-oxidizable amino acid analogues norleucine (Nle) and methoxinine (Mox). The results revealed that PrP mutants containing the more hydrophobic Nle have an increased α -helix content and possess high resistance to sodium periodate induced oxidative aggregation and a structural transition into a β -sheet enriched isoform was inhibited. Conversely, PrP mutants containing the more hydrophilic Mox showed an increased β -sheet content and exhibited proaggregation features (129).

1.7 Therapeutic approaches against TSEs

At present, there is no effective therapy to treate prion diseases. However, several compounds have been tested for their anti-prion activity using scrapie infected neuroblastoma (ScN2a) cells as a model system (130). These compounds are distinguished and classified by their mechanism of action.

Some of these compounds prevent the propagation of PrP^{Sc} by binding PrP^{C} such as Congo red (131), quinacrine, chloropromazine (132), as well as several polyanionic glycans like dextran and pentosan sulfate. Pentosan sulfate also prolongs the incubation time in scrapie infected animal models (133). Furthermore, it reduces the amount of PrP^{C} on the surface of N2a cells by stimulating its endocytosis. This results in a redistribution of the protein from

the plasma membrane to the cell interior, thus preventing the formation of PrP^{Sc} (134). Suramin inhibits the formation of PrP^{Sc} in infected animal models by inducing a posttranslational misfolding of PrP^{C} and bypassing the route of the protein to the acidic compartment. Consequently, the protein does not reach the surface of the plasma membrane, where the conversion presumably occurs (135).

Another group of compounds are known as chemical chaperones, which have the tendency to stabilize the native conformation of PrP^{C} . Therefore, the incubation of ScN2a cells with protein stabilizing agents like dimethylsulfoxide (DMSO), glycerol, and trimethylamine n-oxide (TMAO) did not affect the amount of pre-existing PrP^{Sc} . However, these compounds inhibited the formation of PrP^{Sc} from freshly expressed PrP^{C} (136). In contrast, cationic lipopolyamines bind to PrP^{Sc} on the surface of ScN2a cells and stimulate its degradation via an unknown mechanism (137). Recently, β -cyclodextrin (β -CD) was categorized as an efficient anti-prion compound (138). It has the ability to clear the pathogenic isoform PrP^{Sc} to undetectable levels in ScN2a cell culture within two weeks of treatment. Additionally, β -CD has also been reported to inhibit the toxic effect of the amyloid- β peptide (A β 1-40) known to be associated with AD in cell cultures (139).

Another strategy of TSE treatment is the inhibition of PrP^{Sc} replication by synthetic peptides that are designed to bind and to stabilize PrP^{C} in cell free conversion systems (140-142). A novel therapeutic approach is the β -sheet breaker peptide, a conserved amino acid sequence of PrP that is involved in the formation of the abnormal isoform PrP^{Sc} . This peptide has been shown to reverse PrP^{Sc} formation into PrP^{C} -like molecules (143). Moreover, the treatments with monoclonal anti-PrP antibodies and recombinant Fab fragments have shown a marked effect in PrP^{Sc} replication in ScN2a cell cultures. Since PrP^{C} is an endogenous protein, there is no immune response against both PrP^{C}

and PrP^{Sc} isoforms, which represents the main impediment in the development of suitable vaccines against the infectious isoform (144, 145).

One of the major obstacles in the treatment of TSEs by anti-prion compounds is the low ability of these compounds to cross the blood brain barrier (BBB) that renders their accessibility to the CNS. In addition, most of these compounds did not exhibit any therapeutical effect when administered after the appearance of neurologic signs into animal model (125). Therefore, the development of optimized and effective therapies against TSEs is urgently needed.

1.8 Aim of this work

Up to now the molecular mechanism by which the infectious isoform of the prion protein PrP^{Sc} is formed and propagated still remains unknown. However, Oxidative stress has been reported to play a central role in the pathogenesis and transmission of prion diseases via oxidative modification of specific amino acid residues such as Met, His, Tyr and Trp (116, 122, 123, 129), particularly oxidation of the surface exposed Met residues in the PrP molecule by ROS is supposed to significantly contribute to this pathogenic process. The human prion protein contains nine Met residues, most of them are surface exposed and localized in the folded C-terminal domain (residues 121-230). Oxidative damage of PrP induces in vitro structural conversion, which is similar in physicochemical properties to the PrP^{Sc} isoform. Therefore, the aim of this study was to identify the key amino acids within the surface exposed Met residues that have a significant contribution to the oxidative conversion and aggregation of prion proteins. In the study the C-terminal domain of mouse and human prion proteins was analyzed, which share 90% of overall sequence identity and the same number of Met residues; however the localization of Met residues differs slightly, enabling the investigation of the impact of the local structure environment to the oxidative conversion. Following cloning and recombinant expression in E. coli, the oxidative induced aggregation of both proteins was

investigated by MCO and irradiation using UV light. The structural changes that occur in the course of aggregation were characterized by circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), and PK-resistance. To investigate the effect of Met residues on the oxidative conversion, the entire surface exposed Met residues (M129, M134, M154, M66, and M213) were mutated into Ser residues by site directed mutagenesis of the human PrP domain. After cloning, recombinant expression, and full characterization of the human PrP mutant, the oxidative aggregation behaviour was analyzed by MCO and compared to its wild type form. To assign the observed effect to the individual Met residues, stepwise substitution of all Met was finally required. In this study the systematic replacement starts with the mutation of Met 129 against Thr. Met at position 129 is the site of polymorphism in several species and therefore of specific interest. Following mutation, cloning, and recombinant expression of mouse and human PrP domains, the oxidative aggregation behaviour of the variant proteins was analyzed comparative towards the *wild* type form and the human PrP mutant lacking the entire surface exposed Met residues. The obtained results provide new insights to understand the mechanism of prion conversion induced by oxidative damage and therefore will reveal the impact of cellular oxidative stress to the pathological transition of PrP, particularly for the sporadic forms of prion diseases.

On searching for ideal candidates for the treatment of TSEs β -CD has been reported to remove the infectious isoform of prion protein (PrP^{Sc}) in scrapie infected neuroblastoma (ScN2a) cell cultures (138). Therefore, the effect of β -CD on the oxidative *in vitro* aggregation of the recombinant mouse and human prion protein domains induced by MCO was characterized and the binding affinity of β -CD was analyzed by small angle X-ray scattering (SAXS) and surface plasmon resonance (SPR) measurements. The results are supposed to support the development of new lead structures for TSE drugs.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Company	Chemicals
Applichem Darmstadt, Germany	DTT, EDTA, glycine, glycerol, glucose, guanidine hydrochloride, imidazole, nickel (II) sulphate hexahydrate, IPTG, TEMED, tryptone, yeast extract, 2x YT-medium
Fluka Taufkirchen, Germany	Agar, chloramphenicol, copper metal
Merck Darmstadt, Germany	Ammonium sulfate, calcium chloride dihydrate, disodium hydrogen phosphate, ethanol, hydrochloric acid, methanol, potassium dihydrogen phosphate, proteinase K, SDS, sodium acetate, sodium hydroxide
Qiagen Hilden, Germany	Factor Xa protease, Ni-NTA agarose affinity chromatography resin
Roth Karlsruhe, Germany	Acetic acid, agarose, ampicillin, dipotassium hydrogen phosphate, sodium citrate dihydrate, sodium chloride, Tris- hydrochloride

2.1.2 Enzymes and kits

Company	Enzymes and Kits
Fermentas St. Leon, Rot	BamHI, EcoRI, DpnI, CIA phosphatase, T4 ligase,
Germany	Taq polymerase
Macherey Nagel, Düren	NucleoSpin Extarct II kit
Germany	
PeqLab Erlangen,	PeqGold plasmid miniprep kit I, peqGold gel
Germany	extraction kit
Stratagene, Heidelberg	Pfu turbo® DNA polymerase, site directed
Germany	mutagenesis kit

2.1.3 Instruments

Instrument	Source	
Autoclave VX-120	Systec GmbH, Wettenberg, Germany	
Balance CP224S-OCE	AG Sartorius, Göttingen Germany	
Centrifuges:		
Sorvall RC-5B Plus	Kendro Laboratory Products,	
	Lagenselbold, Germany	
Bench Centrifuge 5801	Eppendorf, Hamburg, Germany	
Bench Centrifuge 5415 R	Eppendorf, Hamburg, Germany	
CD-Spectroscopy:	Jacob Commonly	
Jasco J-715 spectropolarimeter	Jasco, Germany	
Electrophoresis equipment:		
Agarose gels	PeqLab, Erlangen, Germany	
Polyacrylamide gels	Hoefer Inc, USA	
Electroporator E. coli pulser	Bio-Rad Munich, Germany	
ELISA-GENios plate reader	Tecan, Crailsheim, Germany	
1		
GE-FPLC	Pharmacia Biotec, Freiburg, Germany	
GE-FPLC Gel documentation system	Pharmacia Biotec, Freiburg, Germany Intas, Göttingen, Germany	
GE-FPLC Gel documentation system	Pharmacia Biotec, Freiburg, Germany Intas, Göttingen, Germany	
GE-FPLC Gel documentation system Incubators:	Pharmacia Biotec, Freiburg, Germany Intas, Göttingen, Germany Heraeus, Hanau, Germany	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Inpoya 4330	Pharmacia Biotec, Freiburg, Germany Intas, Göttingen, Germany Heraeus, Hanau, Germany NewBrunswick Scientific, Nürtingen,	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330	Pharmacia Biotec, Freiburg, Germany Intas, Göttingen, Germany Heraeus, Hanau, Germany NewBrunswick Scientific, Nürtingen, Germany	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen,GermanyEppendorf, Hamburg, Germany	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal NanoDrop ND-1000	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen, GermanyEppendorf, Hamburg, GermanyPeqLab, Erlangen, Germany	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal NanoDrop ND-1000	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen, GermanyEppendorf, Hamburg, GermanyPeqLab, Erlangen, GermanyMettler-Toledo, Schwarzenbach,	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal NanoDrop ND-1000 pH-meter	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen, GermanyEppendorf, Hamburg, GermanyPeqLab, Erlangen, GermanyMettler-Toledo, Schwarzenbach, Switzerland	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal NanoDrop ND-1000 pH-meter Power supplies:	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen, GermanyEppendorf, Hamburg, GermanyPeqLab, Erlangen, GermanyMettler-Toledo, Schwarzenbach, Switzerland	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal NanoDrop ND-1000 pH-meter Power supplies: Agarose gelelectrophoresis	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen, GermanyEppendorf, Hamburg, GermanyPeqLab, Erlangen, GermanyMettler-Toledo, Schwarzenbach, SwitzerlandBio-Rad, Munich, Germany	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal NanoDrop ND-1000 pH-meter Power supplies: Agarose gelelectrophoresis Polyacrylamide gelelectrophoresis	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen, GermanyEppendorf, Hamburg, GermanyPeqLab, Erlangen, GermanyMettler-Toledo, Schwarzenbach, SwitzerlandBio-Rad, Munich, GermanyPeqLab, Erlangen, Germany	
GE-FPLC Gel documentation system Incubators: Incubator Incubator shaker Innova 4330 Mastercycler personal NanoDrop ND-1000 pH-meter Power supplies: Agarose gelelectrophoresis Polyacrylamide gelelectrophoresis Sonifier 250	Pharmacia Biotec, Freiburg, GermanyIntas, Göttingen, GermanyHeraeus, Hanau, GermanyNewBrunswick Scientific, Nürtingen, GermanyEppendorf, Hamburg, GermanyPeqLab, Erlangen, GermanyMettler-Toledo, Schwarzenbach, SwitzerlandBio-Rad, Munich, GermanyPeqLab, Erlangen, GermanyBranson, Danbury, CT USA	

2.1.4 Oligonucleotides

All oligonucleotides were purchased from Metabion, Martinsried, Germany

Oligonucleotide	Sequence (5'-'3)
mPrP-BamHI-Xaf	AAGGATCCATCGAGGGAAGGGGTGTAGTGGGGGGCCTT
	GGT
mPrP-EcoRIr	AAGAATTCCTAGGATCTTCTCCCGTCGTAATAGG
hPrP- <i>Ram</i> HI-Xaf	AAGGATCCATCGAGGGAAGGGGTGTGGGGGGGGGCCT
	Т
hPrP-EcoRIr	AAGAATTCCTACGATCCTCTCTGGTAATAGGCC
mPrPM129Tf	GGGCCTTGGTGGCTACACGCTGGGGAG
mPrP M129Tr	CTCCCCAGCGTGTAGCCACCAAGGCCC
hPrP M129Tf	GGCCTTGGCGGCTACACGCTGGGAAG
hPrP M129Tr	CTTCCCAGCGTGTAGCCGCCAAGGCC
hPrP-BamHI start	AAGGATCCATCGAGGGAAGGGGTGTGGT
hPrP-EcoRI stop	AAGAATTCCTACGATCCTCTCTGGTAATAGGCCTGAGA
	TTC
hPrP-M134Sf	TCAAGCAGGCCCATCATACATTTCGGCAGTG
hPrP-M134Sr	Pho-GGCACTTCCCAGCATGTAGCCGC
hPrP-M154Sf	TCACACCGTTACCCCAACCAAGTGTACTACAG
hPrP-M154Sr	Pho-GTTTTCACGATAGTAACGGTCCTCATAGTC ACT
hPrP-M166Sf	TCAGATGAGTACAGCAACCAGAACAACTTTGTGCAC
hPrP-M166Sr	Pho-GGGCCTGTACACTTGGTTGGGGGTAAC
hPrP_M2138f	TCATGTATCACCCAGTACCAGTACGAGAGGGAATCTCA
III II -1 v 121355	G
hPrP-M213Sr	Pho-CTGCTCAACCACGCGCTCCATCATCTT
hPrP-M129Sf	GATCCATCGAGGGAAGGGGGTGTGGGGGGGGGCCTTGG
	CGGCTACTCACTGGGAAGTGCC
hPrP-M129Sr	Pho-GGCACTTCCCAGTGAGTAGCCGCCAAG
	GCCCCCACCACACCCCTTCCCTCGAT

2.1.5 Plasmid

pRSETA plasmid (Invitrogen) is a pUC-derived expression vector designed for high level protein expression and purification. Expression of the gene of interest is controlled by the strong phage T7 promoter and provides an ampicillin resistance gene. In addition, the DNA inserts are located downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG translation initiation codon, a 6-fold polyhistidine tag that functions as a metal binding domain in the translated protein, a transcript stabilizing sequence from gene 10 of phage T7, the Xpress TM epitope, and the enterokinase cleavage recognition sequence (Fig. 8).



Fig. 8: Vector map of pRSETA

2.1.6 Constructs

pRSETA-hPrP/Ct (121-231):

pRSETA-hPrP/Ct M129T:

Coding for the C-terminal domain of human prion protein (residues 121-231). Coding for the C-terminal domain of hPrP (121-231) in which methionine at position 129 mutated into threonine (M129T).

pRSETA-hPrP/Ct M129S, M134S,	
M154S, M166S, M213S:	Coding for the C-terminal domain of
	hPrP (121-231) in which methionine at
	position 129, 134, 154, 166, and 213
	was mutated into serine (S).
pRSETA-mPrP/Ct (120-230):	Coding for the C-terminal domain of
	mouse prion protein (120-230).
pRSETA-mPrP/Ct M129T:	Coding for the C-terminal domain of
	mPrP (120-230) in which methionine at
	position 129 was mutated into threonine
	(M129T).

2.1.7 Escherichia coli strains

All E. coli Strains were purchased from Invitrogen, Karlsruhe, Germany

Strain	Genotype
BL21 (DE3)	F^- ompT gal dcm lon hsdS _B ($r_B^- m_B^-$) λ (DE3 [lac]
	lacUV5-T7 gene 1 ind1 sam7 nin5])
DI 21 (DE2) pl vos	F^{-} ompT gal dcm lon hsdS _B (r_{B}^{-} m_{B}^{-}) λ (DE3)
BL21 (DE3) pLyss	$pLysS(cm^{R}).$
	F- mcrA Δ (mrr-hsdRMS-mcrBC) φ 801acZ Δ M15
TOP 10	Δ lacX74 nupG recA1 araD139 Δ (ara-leu)7697 galE15
	galK16 rpsL(Str ^R) endA1 λ^{-} .

2.1.8 DNA and Protein Markers

pUC mix marker,8	
PageRuler TM unstained protein ladder	Fermentas, St. Leon, Rot, Germany
Unstained protein Mw marker	
1 kb DNA ladder	Invitrogen Karlsruhe, Germany

2.1.9 Media

2x YT-medium (1L)	16 g trypton, 10 g yeast extract, 5 g NaCl
LB-Broth medium	10 g trypton, 5 g yeast, 10 g NaCl
Agar medium (1L)	10 g trypton, 5 g yeast extract, 10 g NaCl, 15 g agar
Expression medium (1L)	10 g trypton, 5 g yeast extract, 7.7 g K_2 HPO ₄ 2 g
	KH_2PO_4 , 2 g (NH ₄) ₂ SO ₄ , 1 g sodium citrate (pH 7.5)

2.1.10. Buffers and solutions

Purification of recombinant prion protein	
Binding buffer	10 mM Tris-HCl (pH 8.0), 100 mM Na ₂ HPO ₄ , 10
	mM reduced glutathione
	10 mM Tris-HCl (pH 8.0), 100 mM Na ₂ HPO ₄ ,
Buffer A	10 mM reduced glutathione, 4 M guanidine
	hydrochloride (Gu-HCl)
Buffer B	10 mM Tris-HCl (pH 8.0), 100 mM Na ₂ HPO ₄
Buffer C	10 mM Tris-HCl (pH 8.0), 100 mM Na ₂ HPO ₄ , 50
	mM imidazole
Flution buffer	10 mM Tris-HCl (pH 8.0), 100 mM Na ₂ HPO ₄ ,
	150 mM imidazole
Factor Xa cleavage buffer	20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM
	CaCl ₂
Protein preserving buffer	5 mM sodium acetate (pH 5.0)
Agarose gelelectrophoresis	
50x Tris-Acetic acid	2 M Tris-HCl (pH 8.0), 5.7% (v/v) acetic acid, 50
EDTA (TAE) buffer	mM EDTA

Polyacrylamide gel electrophoresis (PAGE)		
Sample buffer (2x)	0.5 M Tris-HCl (pH 6.8), 30% (w/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue	
10x Electrode buffer (1L)	30.3 g Tris-HCl, 144 g glycine, 10 g SDS	
Coomassie staining solution	450 ml methanol, 100 ml acetic acid,1 g coomassie brilliant blue	
Coomassie destaining solution (1L)	200 ml acetic acid	

For the SDS-PAGE analysis of the C-terminal domain of both recombinant mouse and human (rmPrP120-230 & rhPrP121-231) prion proteins 15% resolving gels and 4% stacking gels were prepared.

- Resolving gel 15% (10 ml):

5 ml 30% acryl-bisacrylamide solution
2.5 ml 1.5 M Tris-HCl pH 8.8
0.1 ml 10% SDS
5 µl TEMED
50 µl 10% APS
2.4 ml H ₂ O

- Stacking gel 4% (10 ml):

1.3 ml 30% acryl-bisacrylamide mixture
 2.5 ml 0.5 M Tris-HCl pH 6.8
 0.1 ml 10% SDS
 5 μl TEMED
 50 μl 10% APS
 6.1 ml H₂O

2.2 Methods

2.2.1 Generation of electrocompetent E. coli cells

E coli cells were grown overnight in 3 ml LB-medium at 37 °C and 220 rpm. 200 ml LB-medium were inoculated with 2 ml from the overnight culture and an appropriate amount of the required antibiotic. The bacterial culture was incubated at 37 °C and 220 rpm up to an optical density of 0.3-0.4 at 600 nm wavelength (OD_{600}). Following 10 min. incubation on ice, the culture was centrifuged for 15 min. at 4300 rpm and 4 °C. Afterwards the pellet was suspended in 200 ml ice-cold water (ddH₂O) and then centrifuged at 4300 rpm for 15 min. at 4 °C. The pellet was again suspended in 100 ml ddH₂O and the centrifugation step was repeated. The cells were washed two times with ice-cold 10% glycerol followed by centrifugation at the same conditions. Finally, the cells were suspended in 1 ml of 10% glycerol and 100 µl aliquots were frozen immediately in liquid nitrogen and stored at -80 °C.

2.2.2 Transformation of E. coli cells by electroporation

A 100 µl aliquot of electrocompetent *E. coli* was thawed on ice for 30 min. and then mixed with ~1-2 µg of plasmid DNA carrying the gene of interest in an electroporation cuvette. The cell suspension was transformed by electroshock using elctroporation device (Bio-Rad, Munich). The cell suspension shocked by 1800 V and then 900 ml of LB-medium were added immediately followed by incubation at 37 °C and 220 rpm for 1h. Finally, the cell suspension was plated on LB-agar plate containing the appropriate antibiotic and incubated overnight at 37 °C.

2.2.3 Cloning of His₆-tagged C-termini of both *wild type* mouse (mPrP120-230) and human (hPrP121-231).prion proteins

The open reading frame (ORF) of mPrP120-230 and hPrP121-231 PrP genes were amplified by polymerase chain reaction (PCR) from pRSETA vectors that

encode for the cDNAs of both mPrP89-230 and hPrP90-231 PrP genes, respectively. The primers mPrP-*Bam*HI-Xa*f*, mPrP-*Eco*RI*r*, hPrP-*Bam*HI-Xa*f*, and hPrP-*Eco*RI*r* carrying engineered restriction sites as well as a Factor Xa protease cleavage site as indicated were used to flank the appropriate PrP gene sequence. To perform PCR, a 50 μ l reaction mixture was set up in a 0.5 ml tube containing DNA template (20-50 ng), 1 μ l dNTPs mix (12.5 mM), 1 μ l forward and reverse primers (100 nM), 10x DNA polymerase buffer (10% of total volume), *T*aq DNA polymerase (5-10 units), and the required volume of ddH₂O. The PCR amplification was performed on a mastercycler personal (Eppendorf, Hamburg, Germany) using the following cycling conditions:



2.2.4 Agarose gelelectrophoresis

Agarose gel electrophoresis is a method used to separate DNA fragments according to their sizes. PCR products were separated on 2% agarose gels using gel chamber filled with 1x TAE buffer. To prepare the gel, the appropriate amount of agarose is suspended in 1x TAE buffer and heated in microwave oven until the agarose is completely dissolved. After the solution had cooled down to $\sim 40 \,^{\circ}$ C, 5 µl ethidium bromide (10 mg/ml) solution was added and the solution was transferred to a gel cast tray provided with a comb to create slots for loading DNA samples on the gel, and then the agarose is allowed to solidify. The gel was run under constant voltage (90 V) using a power supply in order to separate DNA fragments. After electrophoresis, the DNA was visualized by UV-light and analysed in a gel documentation system (Intas, Göttingen, Germany).

2.2.5 Purification of DNA from agarose gel

Bands were excised from the gel and purified using the peqGold gel extraction kit (PeqLab, Erlangen, Germany) according to the manufacture's procedures.

2.2.6 Restriction digestion of DNA fragments

The amplified DNA fragments as well as the pRSETA vector were digested using 2 units of both *Bam*HI and *Eco*RI restriction endonucleases per μ g DNA and the recommended buffer in final volume of 50 μ l. The reaction was performed at 37 °C for 3h.

2.2.7 Dephosphorylation of plasmid DNA

After digestion, the pRSETA vector was dephosphorylated by addition of 1 μ l calf intestinal alkaline phosphatase (CIAP) followed by incubation at 37 °C for 1h, while the digested DNA fragments were stored on ice. The digestion products were purified using the nucleospin extract II kit (Macherey-Nagel, Düren, Germany) following the PCR product purification protocol of the supplier.

2.2.8 Ligation

Plasmid vector and DNA fragments were ligated using a molecular ratio of 1:5 by T4 ligase (2 units) and 10x ligation buffer in a total volume of 40 μ l. the reaction mixture was incubated overnight at 18 °C. Afterwards the ligation mixtures were directly transformed into TOP 10 electrocompetent *E. coli* cells. The cells were plated out onto an agar plates containing 100 μ g/ml ampicillin. The plates were incubated overnight at 37 °C.

2.2.9 Isolation of plasmid DNA

Following an overnight incubation at 37 °C, 10 colonies (5 of each construct) were picked from the plates and 3ml overnight cultures were inoculated. For

isolation of plasmid DNA, peqGold plasmid miniprep kit (PeqLab, Erlangen) was used according to the manufacturer's instructions.

2.2.10 DNA sequencing

DNA sequencing was carried out by dideoxynucleotide chain termination method. 250-500 ng of plasmid DNA was mixed with 3 μ l of big dye reaction mixture, 5 μ l of big dye buffer, and 1 μ l (100 nM) of the sequencing primer to result in a final volume of 20 μ l. The sequencing reaction was performed in thermal cycler using the following cycling conditions:

Denaturation96 °C30 sec.Annealing
$$54 °C$$
15 sec.Elongation $60 °C$ 4 min.

Following amplification the sequencing mixture was adjusted to 100 μ l with ddH₂O. The DNA was precipitated by addition of 300 μ l ethanol (96%) and 10 μ l 3 M sodium acetate (pH 5.2) and then centrifuged at 13000 rpm for 30 min. at 4 °C. The supernatant was discarded, the DNA pellet washed with 500 μ l ice-cold 70% ethanol, and then centrifuged at 13000 rpm for 15 min. at 4 °C. The supernatant was again discarded and the DNA pellet was dried at 37 °C for 30 min.. Sequencing analysis have been performed by the Institute of Pathology, University Hospital Eppendorf (UKE), Hamburg, Germany.

2.2.11 Mutagenesis

2.2.11.1 Site directed mutagenesis

Site directed mutagenesis was carried out to replace the surface exposed methionine at position 129 in the C-terminal domain of mPrP120-230 and hPrP121-231 by threonine. Two primers were designed for each mutation to change the methionine coding base pairs into threonine coding base pairs. The total length of each primer is recommended to be around 30 base pairs, ending with a GC-rich sequence. The amplification reaction was performed using *pfu*

DNA polymerase to reduce the risk of undesired random mutations. The reaction mixture was setup as follows:

10x reaction buffer	5 µl
Plasmid DNA	5-50 ng
Primer (sense)	125 ng
Primer (anti-sense)	125 ng
dNTPs (12.5 mM)	1 µl
Adjusted to 49 μ l with dd H ₂ O	

pfu DNA polymerase 1 µl

The primers mPrP-M129T*f*, mPrP-M129T*r*, hPrP-M129T*f*, and hPrP-M129T*r* were used to induce point mutation as well as to amplify the whole plasmid including the mutated genes using the following cycling conditions:

Initial denaturation95 °C30 sec.Denaturation95 °C30 sec.Annealing55 °C1 min.Elongation68 °C2 min.

Afterwards the PCR product was digested with 1 μ l *Dpn*I restriction endonuclease for 1h at 37 °C. *Dpn*I only removes methylated parental DNA, while the newly PCR generated DNA comprising the required mutation remained. The mutated DNA was transformed into *E. coli* TOP 10 cells and incubated overnight at 37 °C. The resulting colonies that carry the mutated plasmid DNA were verified by DNA sequencing.

2.2.11.2 Site directed mutagenesis of non-overlap extension

Mutation of the entire surface exposed methionine residues (M129, M134, M154, M166, and M213) in the hPrP/Ct(121-231) into serine residues has been performed by the PCR-based method of non-overlap extension. The linearized plasmids pRSETA-hPrP121-231-*Bam*HI and pRSETA-hPrP121-231-*Eco*RI

served as a template to introduce site specific mutation by PCR using the following cycling conditions:



The corresponding internal mutagenic primers as well as the forward and reverse primers that have been used to perform such mutation are listed in section 2.1.4. To allow blunt end ligation of the PCR products, one of each internal primer was modified by phosphate. The respective ligated product was served as a template for the next mutation. First, M134 was mutated into serine, followed by M154, M166, and finally M213. The mutant M129S was introduced by ligation of a double stranded oligonucleotide carrying this mutation with the PCR fragment that contained the other four mutations M134S, M154S, M166S, and M213S. The final product was cloned via *Bam*HI and *Eco*RI into pRSETA vector. All mutations were confirmed by DNA sequencing.

2.2.12 Expression of the C-terminal domain of human and mouse PrP

The expression and purification of *wild type* and all variant of mPrP/Ct(120-230) and hPrP/Ct(121-231) followed the same protocol.

Plasmid DNA containing the C-terminal domain of human or mouse PrP was freshly transformed into *E. coli* BL21 (DE3) cells. The cells were grown on agar plate containing 100 μ g/ml ampicillin and 30 μ g/ml chloramphenicol overnight. A single colony was picked to inoculate 3 ml culture that was grown for 8hrs at 37 °C and then kept at 4 °C up to the next morning. 1 ml of the pre-culture was transferred into 200 ml 2x YT-medium and the cells were incubated at 37 °C for 4hrs. The 200 ml culture was used to inoculate 1.8L of the expression medium (final volume 2L) containing 100 μ g/ml ampicillin, 30 μ g/ml chloramphenicol, 2 mM MgSO₄, 0.2 mM CaCl₂, and 0.1% glucose. Protein expression was induced at $OD_{600} = 1.8$ by addition of Isopropyl β -D thiogalactoside (IPTG) to a final concentration of 0.4 mM. After induction the cells were incubated for 4h. At the induction time and 2h after induction the cells were supplied with 20 ml 50% glucose, 20 ml 10% yeast extract, and 20 ml 20% trypton. Finally, the cells were harvested by centrifugation at 6000 rpm for 15 min. and stored at -20 °C.

2.2.13 Purification of the C-terminal domain of human and mouse PrP.

The C-terminal domain of human and mouse PrP was recombinantly expressed as inclusion bodies containing an N-terminal six-fold histidine tag and an engineered factor Xa protease cleavage site. For purification of the recombinant proteins, the bacterial pellets were resuspended in 35 ml binding buffer and disrupted by sonification (12000 microns and 45% output) for 10 min.. The inclusion bodies as well as the cell debris were pelleted by centrifugation at 16000 rpm for 30 min. at 4 °C. The supernatant containing the soluble E. coli proteins was discarded. Afterwards the inclusion bodies were washed with 40 ml buffer A diluted with binding buffer to a final concentration of 1 M Gu-HCl, followed by homogenization in an ultrasonic bath for 10 min.. The inclusion bodies were pellet down by centrifugation at 16000 rpm for 15 min. at 4 °C and solubilised in ~30 ml buffer A followed by homogenization as previously mentioned. To get rid off cell debris, the protein solution was centrifuged at 16000 rpm for 15 min. at 4 °C. The supernatant was applied onto a Ni-NTA agarose resin pre-equilibrated with buffer A and the protein was bound to the resin by incubation at room temperature for 30 min.. The resin was washed with 5 volumes of buffer A to remove unbound proteins and nucleic acids. Oxidative refolding of the protein was achieved via application of linear 400 ml gradient decreasing the Gu-HCl concentration gradually from 4 M to 0 M by replacing buffer A against B using an automated FPLC-system (Biotech, Freiburg, Germany) with flow rate of 0.5 ml/min.. The resin was washed with 50 ml buffer C containing 50 mM imidazole to remove impurities and the soluble, readily folded prion protein was finally eluted with elution buffer containing 150 mM imidazole.

2.2.14 Cleavage of histidine tag sequence by factor Xa protease

The purified proteins were dialysed against factor Xa cleavage buffer and the protein concentration was adjusted to 0.25 mg/ml. The histidine tag sequence was removed by overnight incubation in the presence of 20 units of factor Xa (Qiagen, Hilden, Germany) per mg prion protein at room temperature. Factor Xa was removed according to the instruction manual of the supplier. To remove the cleaved histidine tag peptide the protein was dialysed against 10 mM Tris-Hcl pH 8.0 and then incubated with Ni-NTA agarose resin pre-equilibrated with the same dialysis buffer at room temperature for 30 min.. The pure protein was collected from the flow through, dialysed against 5 mM sodium acetate pH 5.0, and finally stored at 4 °C.

2.2.15 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the recombinant proteins was analysed by SDS-PAGE. Protein samples (10-20 μ l) were heated in the presence of 2x sample buffer at 95 °C for 5 min. and loaded on 15% polyacrylamide resolving gels. A constant voltage (120 V) was applied for separation of proteins according to their molecular masses. The proteins were stained by coomassie brilliant blue for 1h and subsequently visualized by appropriate incubation in destaining solution.

2.2.16 Determination of protein concentration

The concentration of proteins was determined by analyzing the specific absorption at a wavelength of 280 nm using NanoDrop ND-1000 spectrophotometer (PeqLab, Erlangen). The corresponding extinction coefficients have been calculated to be $\varepsilon_{280} = 16,515 \text{ M}^{-1} \text{ cm}^{-1}$ for hPrP121-231, hPrP121-231 M129T, and hPrP121-231 M129S, M134S, M154S, M166S,

M213S as well as $\varepsilon_{280} = 22,015 \text{ M}^{-1} \text{ cm}^{-1}$ for both mPrP120-230 and mPrP120-230 M129T.

2.2.17 Circular dichroism (CD) spectroscopy

Far-UV (190-260 nm) CD-spectra of the recombinant prion proteins were measured on a Jasco J-715 spectropolarimeter (Jasco, Germany) using a 1 mm path length quartz cell. Typically, a scanning rate of 100 nm/min. was applied. Spectra of samples containing 0.1 mg/ml prion protein in 5 mM sodium acetate buffer (pH 5.0) were recorded at 20 °C. Normally, 5 individual scans were averaged per spectrum and the corresponding background spectrum of the buffer was subtracted from the final spectra. The experimental results were expressed as molar ellipticity units (deg. cm² dmol⁻¹) by using the conversion formula:

 $[\psi]: \Theta/(100 \text{ C l})$

 Θ : ellipticity (mdeg)

C: molar concentration (mol/l)

1: path length of the cuvette

2.2.18 Dynamic light scattering (DLS)

Dynamic light scattering measurements were carried out using the spectroscatter 201 (Molecular Dimension, UK) containing a helium-neon laser that operates at a wavelength of $\lambda = 690$ nm and a laser power of 10-50 mW. All measurements have been performed at 20 °C and a scattering angle of 90° using 40 µl sample in a quartz cuvette. An accumulation of 20 measurements per sample was recorded by using the autopilot function. The theoretic hydrodynamic radii and the molecular mass of both monomeric and aggregated PrPs were calculated, as previously described (146, 147) using the approximation for spherical proteins:

$$R_H^{calc} = \sqrt[3]{\frac{3M(Vs+h)}{4\pi N_A}}.$$

A value of 0.73 cm³ g⁻¹ was used for the specific particle volume (V_s). To account for the hydrodynamic behaviour of globular proteins, a value of 0.35 g H₂O/(g protein)⁻¹ was applied for the hydration (h) of the prion proteins.

2.2.19 Conversion and aggregation of prion protein by metal catalyzed oxidation (MCO)

The aggregation process was initiated by aerobic incubation of 44 µM PrP in the presence of 16 mg solid copper pellets (Cu⁰) in 5 mM sodium acetate buffer (pH 5.0) in a total reaction volume of 60 µl for different time periods at 37 °C. After incubation the copper pellets were immediately removed and the aggregated prion proteins were pelleted by centrifugation at 13000 rpm for 45 min. at 4 °C. The remaining concentration of soluble proteins in the supernatants was determined by the Bradford assay. Therefore, 10 µl of a 1:1, 1:5, and 1:10 dilution of each were mixed with 250 µl Bradford reagent in 96-well plate. The developed blue colour was determined by measuring the absorption of the samples at 595 nm using an automated ELISA reader (Tecan, Crailsheim, Germany). Additionally, the concentration of proteins in the supernatants was determined by analysis of specific absorbance at 280 nm. Values were referred to control samples that did not contain copper pellets. To investigate the inhibitory effect of β -cyclodextrin (β -CD) on the MCO-induced conversion and aggregation of PrP, the described assay was performed in the presence of β -CD at 10-fold excess.

2.2.20 Proteinase K (PK) digestion

Proteinase K resistance was assayed by incubating sample aliquots of monomeric and aggregated Prion proteins in 20 mM Tris-HCl (pH 8.0) for different time periods (30 and 60 min.) at 37 °C in the presence of PK. The PrP-PK ratio was adjusted to be 3:1 (w/w). Digestion reaction was terminated by addition of 2x SDS sample buffer and subsequent incubation at 95 °C for 5 min..

2.2.21 Conversion and aggregation of prion proteins by ultra violet (UV) radiation

UV radiation at a wavelength of 302 nm was generated using an argon-ion laser (2085 "Beamlok"; spectra physics, Mountain View CA, USA) with a laser tube optimized for application in the UV range. The laser was adjusted to approx 60 mW output power that was attenuated to ~10 mW by a neutral density filter (optical density 0.3; Newport Crop, Mountain View, CA, USA) positioned behind the laser. As the diameter of the laser beam was 2 mm, the illuminated sample volume was 16 μ l (approx 3.2% of the total sample volume at the beginning of each measurement). During all measurements the direct beam intensity behind the cuvette was measured using a laser power analyzer system (Ultima LabMaster; Coherent, Santa Clara, CA, USA) with a high-sensitivity thermal sensor (LM-1; Coherent). Between the measurements the laser beam was redirected by a movable mirror at a position in front of the cuvette into another sensor (LM-2; Coherent). The values of both sensors were recorded by a computer in 1-s intervals. All measurements were carried out in a clean room with constant temperature (22.0 ±0.5 °C) and humidity (40 ±3%).

Before and after individual measurements, the beam power was rescaled without a cuvette, with any empty cuvette, and with a cuvette filled with water (each for 1 min.) to obtain reference values for subsequent standardization. All power measurements were normalized to the mean value of transmissions through a cuvette filled with H₂O before and after measurement. Samples (500 µl) of various mouse and human PrPs (44 µM) buffered in 20 mM sodium acetate (pH 5.0) and 20 mM sodium phosphate (pH 7.4), respectively, were UV irradiated for up to 4 h. Generally, the sample solutions were measured under aerobic conditions in a Suprasil cuvette (dimension $33.5 \times 7.5 \times 7.5 \text{ mm}^3$; Helma GmbH, Mülheim, Germany). Continuous stirring ensured the homogeneity of the solution and the maintenance of the dissolved oxygen concentration, which was afterward verified by the Winkler method (148). After the desired periods of time (5, 15, 30, 60, 90, 120, 180, and 240 min.), aliquots of 40 μ l were removed for detailed analysis. Because the sample volume accordingly decreased after each period, corrected incubation times were calculated for the reduced volumes, resulting in measurement periods of 5, 15.9, 33.7, 73.7, 117.3, 167.3, 282.7, and 419.1 min.. These values were applied throughout the entire study.

Moreover, the PrPs were irradiated in the presence of the free oxygen radical scavengers dimethyl sulfoxide DMSO (200 mM), sodium azide (200 mM), and ascorbic acid (20 mM), as well as in absence of oxygen. Anaerobic conditions were established by using degassed buffers in a sealed cuvette, which was extensively flushed with argon. Aliquots were removed under continuous argon flow. To compare power transmission values and biochemical analysis the mean power value of the last 15 s per period was plotted against the irradiation time. For the evaluation of the observed aggregate formation, irradiated samples were centrifuged at 20.800 g for 45 min. at 4 °C, and the remaining protein concentration in the supernatants was determined using a coomassie protein assay reagent (Pierce, Rockford, IL, USA) and by analysis of the specific absorbance at 280 nm. The obtained values were referred to control samples that had not been irradiated.

2.2.22 Small angle X-ray scattering (SAXS)

The synchrotron SAXS data were collected on the X33 camera of the EMBL at DESY (Hamburg, Germany). Data were recorded using a MAR345 image plate detector at a sample detector distance of 2.7 m and a wavelength of λ =0.15 nm, covering the range of momentum transfer (*s*) from 0.08 to 5 nm⁻¹. The data were reduced, processed and the overall parameters were computed following standard procedures using the program package PRIMUS (149). The molecular mass of the proteins were computed by scaling against the scattering of bovine serum albumin (BSA) as a reference solution. Protein samples were buffered in

10 mM Tris-HCl (pH 8.0). To investigate the impact of β -CD on the PrP structure, β -CD was added in a 10-fold excess to the protein samples as indicated. The following samples have been analysed at the depicted concentrations:

Protein	Concentration (mg/ml)
hPrP121-231	2.0 3.3 5.0
hPrP121-231 in the presence of β-CD	1.8 2.9 3.9
mPrP120-230	1.5 3.6 5.5
mPrP120-230 in the presence of β -CD	1.5 3.1 4.3

2.2.18 Surface Plasmon Resonance (SPR)

The SPR experiments were carried out using Biacore T100 device (Biacor, Germany) equipped with CM5 sensor chip. The measuring chip is coated with a layer of dextran matrix that used for immobilization of the target protein. For the measurements, the enclosed buffer HBS-N (20 mM HEPES, 150 mM NaCl, pH 7.4) was used according to the instructions of the supplier. In this experiment the binding affinity of β -CD to hPrP121-230 was determined. To investigate the binding specificity of β -CD to PrP, the binding affinity of other related sugars

such as α -cyclodextrin (α -CD) and cellobiose to hPrP121-231 was also determined. Before immobilization, the surface of the measuring chip was activated by EDC/NHS solution (ratio 1:1) with a flow rate of 10 µl/min. for 15 min.. For immobilization the prion proteins stock solution was diluted with sodium acetate buffer (pH 5.0) to a final concentration of 1 μ M. Afterwards 5 μ l of 1 µM proteins were passed over the activated dextran matrix with a flow rate of 5 µl/min.. To block the free binding sites of the activated dextran matrix, both the flow cell carrying the protein and the control channel were capped with 1 M ethanolamine for 15 min. using a flow rate of 10 µl/min.. The flow cells were washed with HBS-N buffer pH 7.4, until a stable baseline was obtained. Approximately 218 fmol [3810 RU (response units)] for hPrP121-231, which was covalently attached to the sensor chip surface. A serial concentration of the sugars was prepared using the commercial HBS-N buffer of Biacore (pH 7.4). For absorption, the dissolved sugars were applied on the measuring chip for 300 s with a flow rate of 10 μ l/min.. Dissociation of the ligands can occur up to 300 s after the end of the injection. Therefore, the regeneration of the measuring chip is not necessary.

3. Results

3.1 Oxidative induced conversion of the C-terminal domain of mouse and human prion proteins

The C-terminal domain represents the folded part of the prion protein that contains most of the surface exposed methionine (Met) residues. Moreover, the structural differences between the infectious isoform PrP^{Sc} and the normal cellular prion protein PrP^{C} are restricted to the C-terminal part, which adopts α -helical fold in PrP^{C} and displays a high content of β -sheet structures in PrP^{Sc} (71, 129).

3.1.1. Cloning and recombinant expression

To clone the C-terminal domain of both mouse and human PrP genes, the corresponding DNA sequences coding for mouse (mPrP120-230) and human (hPrP121-231) prion proteins were amplified by PCR from plasmids harbouring the respective cDNAs of mPrP89-230 and hPrP90-231 prion proteins. The primers mPrP-*Bam*HI-Xaf, mPrP-*Eco*RIr, hPrP-*Bam*HI-Xaf, and hPrP-*Eco*RIr have been used to amplify both fragments, introducing the required restriction sites and an engineered factor Xa protease cleavage site as indicated. The expected size of the amplified DNA fragments was verified by agarose gel electrophoresis (Fig. 9).



Fig. 9: Gel electrophoretic analysis of the PCR amplification of mouse and human PrP gene sequences coding for the C-terminal domain using 2% agarose gels. (1) DNA ladder. pUC mix. (2) mPrP120-230 DNA (333 bp). (3) hPrP121-231 DNA (330 bp). The amplified fragments were cloned in frame with an N-terminal six-fold His tag via *Bam*HI and *Eco*RI restriction sites into the prokaryotic expression vector pRSETA (Invitrogen). The resulting bacterial expression plasmids pRSETA-hPrP/Ct(121-231) and pRSETA-mPrP/Ct(120-230) contain the PrP genes under the control of the T7 promoter. Following the ATG start codon, the six-fold His tag and the factor Xa site are connected to the N-terminal part of the respective PrP sequence. After cleavage of the N-terminal His tag by factor Xa, one additional glycine remains attached to the proteins. The correct sequence of the cloned genes was confirmed by dideoxy-mediated chain termination sequencing method.

The cloned DNA-sequence coding for the C-terminal domain of both mouse and human prion proteins was expressed in the cytoplasm of E. coli BL21 (DE3) cells. To avoid toxicity due to promoter leakage, the corresponding expression vector was always freshly transformed into E.coli cells. Based on the existing expression protocol for other PrP constructs (116), the specific conditions including temperature, IPTG concentration, and the expression time were varied. An initial analysis of the expression efficiency showed that the C-terminal prion protein constructs completely formed insoluble inclusion bodies. Best results in terms of quantity have been obtained after expression induced by 0.4 mM IPTG for 4h at 37 °C (Fig. 10). After expression the cells were disrupted by sonication and the inclusion bodies were dissolved in 4 M guanidine hydrochloride solution. Matrix-assisted refolding using Ni-NTA resin finally resulted in soluble and pure proteins. The elution of the refolded prion proteins from Ni-NTA resin was optimised from the already existing purification protocol (116, 147) by using serial concentrations of imidazole (100-500 mM) in the elution buffer. Complete elution of both proteins was achieved with 150 mM imidazole. The N-terminal His-tag was removed by factor Xa to avoid interference of the additional residues with the oxidative induced conversion process. Usually an amount of approx. 20 mg protein was obtained from 2L of cell culture. The purity and the completeness of the proteolytic cleavage of both mouse and human proteins were analyzed by SDS-PAGE. Pure protein bands appeared at the expected molecular weight of approximately 17 kDa including the Nterminal His-tag (Fig. 11A) and 13 kDa, which represents the mature recombinant PrPs (Fig. 11B). Protein concentrations were determined by absorbance at 280 nm using the calculated molar extinction coefficient $\varepsilon_{280} =$ 22,015 M⁻¹ cm⁻¹ for mPrP120-230 and $\varepsilon_{280} =$ 16,515 M⁻¹ cm⁻¹ for hPrP121-231. The secondary structure of the recombinant proteins was assessed by far UV (190-260 nm) circular dichroism (CD) spectroscopy in 5 mM sodium acetate at pH 5.0 (Fig. 12). The results revealed that the structure of both proteins predominantly consist of α -helices characterized by two distinct negative minima at 208 nm and 222 nm. Therefore, the folding of the recombinant prion proteins corresponds to the reported PrP^C structure (150).



Fig. 10: Non-reducing SDS-PAGE analysis of the recombinant expression of the C-terminal domain of (A) hPrP121-231 and (B) mPrP120-231 PrP. Samples of the crude *E. coli* extract were separated using a 15% SDS gel. (M) Page ruler unstained protein marker. The time of expression is indicated above the gel.



Fig. 11: (A) Non-reducing SDS-PAGE (15%) analysis of the purification of the recombinant C-terminal domain of mouse and human PrP (A) including the N-terminal His tag (~17 kDa) and (B) after cleavage of the N-terminal His tag (~ 13 kDa). (M): Page ruler unstained protein marker. Lane 1: mPrP120-230. Lane 2: hPrP121-231. All proteins have been stained by coomassie dye.



Fig. 12: Far-UV CD spectra of the recombinant C-terminal domain of mPrP120-230 and hPrP121-231 PrP. CD-spectra were recorded on samples containing a protein concentration of 0.1 mg/ml in 5 mM sodium acetate buffer (pH 5.0). All measurements were carried out at 20 °C in a 0.1 mm path length cuvette using a scanning rate of 100 nm/min.

3.1.2 Structural conversion by metal catalyzed oxidation (MCO)

It is already known that oxidative modifications of specific residues are able to induce the structural conversion of PrP^{C} into an isoform that shares its physicochemical properties with PrP^{Sc} (151). Particularly MCO has been shown *in vitro* to result in β -sheet enriched and aggregated isoforms of recombinant PrP (123, 125). Therefore, a novel *in vitro* cell-free conversion assay based on MCO that has recently been established in our group (116) was applied to investigate the effects of the oxidative damage on the C-terminal domain of mouse and human PrP. The assay mimics the physiological conditions of cellular oxidative stress by aerobic incubation of the recombinant PrP in the presence of the redox active copper metal (Cu⁰) in slightly acidic buffer. This results in generation of hydrogen peroxide (H₂O₂) and the corresponding copper ions. A combined mechanism of oxidative protein damage is proposed including direct oxidation by H₂O₂ as well as secondary oxidation by ROS that are formed by Cu²⁺-catalyzed H₂O₂ disproportionation.

To investigate the effect of MCO on recombinant mPrP120-230 and hPrP121-231, both proteins (44 μ M) buffered in 5 mM sodium acetate (pH 5.0) were incubated at 37 °C in the presence of Cu⁰ for different time periods (1-180 min. for mPrP120-230 and 1-60 min. for hPrP121-231). Following separation of precipitation and high molecular weight aggregates by centrifugation, the remaining concentration of soluble PrP molecules was determined as a marker for the rate of conversion and aggregation (Fig. 13). The results revealed that hPrP120-23 aggregated at a higher rate than mPrP120.230. The corresponding half life of hPrP121-231 at the applied conditions was determined to be T_{1/2} = 3 min., while the half life of mPrP120-230 was significantly increased by approx. 5-fold to T_{1/2} = 15 min.. In the absence of Cu⁰, no aggregation has been observed. The monomeric PrP molecules remained stable and soluble up to 60 and 180 min. for hPrP121-231 and mPrP120-230, respectively. In the course of hPrP121-231 aggregation heavy precipitation was observed, whereas only light precipitation was formed during the aggregation of mPrP120-230.



Fig. 13: Time-resolved monitoring of the *in vitro* aggregation of the recombinant C-terminal domain of mPrP120-230 and hPrP121-231 PrP induced by MCO. Protein aliquots (44μM) buffered at pH 5.0 were incubated in the presence and in the absence (control) of Cu⁰ at 37 °C for the indicated time periods. After removal of copper pellets, the protein samples were centrifuged and the remaining concentration of the PrP in the supernatants was determined. Error bars represent the standard deviations.

The protein aggregates formed by MCO and isolated by centrifugation have been used for structural characterization after separation from the supernatants. Initial non-reducing SDS-PAGE analysis of the hPrP121-231 aggregates showed the persistence of a dominating monomeric PrP band after 60 min. of incubation (Fig. 14A). After a few minutes of MCO, additional bands appeared, characterized by molecular weights of approx. 12 kDa, 16 kDa, and 26 kDa. The latter was attributed to a dimeric form of PrP that showed stability against heat denaturation in the presence of SDS, while the 12 and 16 kDa bands are supposed to result from alternative conformations and/or denaturation states of the monomeric molecule. High molecular weight aggregates of approx 39 kDa and more than 116 kDa have been initially detected after 60 min. of incubation. The 39 kDa protein band was ascribed to a trimeric form of PrP. Under reducing conditions (Fig. 14B) the dimers as well as the high molecular weight aggregates dissociated into the monomeric form (13 kDa). Consequently, the stability of the formed aggregates increases with the incubation time, obviously due to the continual formation of intermolecular disulfide bonds.



Fig. 14: Non-reducing (A) and reducing (B) SDS-PAGE analysis (15%) of hPrP121-231 aggregates formed by MCO after incubation for indicated time periods. The aggregated proteins were pellet down by centrifugation and resuspended in 5 mM sodium acetate pH 5.0. An appropriate amount of the suspension was mixed with sample buffer and incubated at 95 °C for 10 min. (M): Page ruler unstained protein marker. (C): hPrP121-231 incubated for 60 min. in the absence of copper (control). (NR) non-reducing conditions and (R) reducing conditions. The incubation time of the MCO assay is indicated above the gel.

It has also been revealed that the aggregated infectious isoform of prion proteins (PrP^{Sc}) showed a partial resistance to proteinase K (PK) digestion due to the strong interactions within the β -sheet structure of the aggregates, compared to the normal cellular form PrP^{C} (45, 152). Therefore, proteinase K resistance was assayed by incubating sample aliquots of monomeric and MCO-aggregated forms of human PrP121-231 for different time periods at 37 °C in the presence of PK. The corresponding SDS-PAGE analysis (Fig. 15) revealed that the monomeric recombinant PrP is highly sensitive to PK, shown by complete digestion after 60 min. of PK incubation. In contrast, after 1 min. of MCO, a weak monomeric PrP band persisted after 30 and even 60 min. of PK treatment that significantly increased in intensity after 60 min. of MCO-induced aggregation, indicating the increase of PK resistant molecules.

Incubation time MCO



Fig. 15: Non-reducing SDS-PAGE analysis (15%) illustrating the PK-resistance of hPrP121-231 aggregates formed by MCO. Sample aliquots of monomeric and aggregated PrP isolated by centrifugation buffered in 20 mM Tris-HCl pH 8.0 were incubated with PK at 37 °C for the indicated time periods. The PrP-PK ratio was adjusted to be 3:1 (*w/w*). (M): Page ruler unstained protein marker. The PK digestion time is indicated below the gel. The incubation time of the MCO assay is indicated above the gel.

The change in the secondary structure of recombinant mPrP120-230 and hPrP121-231 during MCO was monitored by CD-spectroscopy. For the isolated high MW aggregates of the proteins, no CD spectra have been obtained since these aggregates formed insoluble precipitation. In contrast, the CD spectra of the soluble fractions of mPrP120-230 exhibited a typical α -helical dichroic signal characterized by two distinct negative minima at 208 nm and 222 nm up to 10 min. of MCO (Fig. 16A). The intensity of the CD signal significantly decreased during MCO. After 20 min. of incubation, almost no CD signal was detected due to the strongly reduced concentration of soluble PrP (Fig. 12). An increase of the β -sheet specific signal was not observed in the soluble fractions. In contrast, within the first minute of incubation, the CD spectra of hPrP121-231 significantly changed. The typical α -helical signal transformed into a flattened curve with a characteristic single minimum at 215 nm, typical for a β -sheet structure (Fig. 16B). After 5 min. of incubation, no CD signal was detected anymore, confirming the rapid progression of hPrP121-231 aggregation.

Dynamic light scattering (DLS) analysis revealed that all prion proteins were monodisperse in the reaction buffer at the beginning of the MCO reaction, predominantly consisting in monomeric forms as indicated by hydrodynamic radii of 1.98 ± 0.1 nm and 1.94 ± 0.09 nm for mPrP120-230 and hPrP121-231, respectively. These determined hydrodynamic radii corresponded to the theoretical values calculated from the molecular masses (1.80 nm for mPrP120-230 and 1.78 nm for hPrP121-231), assuming a roughly globular shape of the molecules. In the course of oxidative-induced aggregation, stable and homogeneous soluble oligomeric forms have not been detected by DLS. Only high molecular weight aggregates of more than 100 nm in radius appeared in solution, which showed a high degree of heterogeneity. These aggregates disappeared immediately after centrifugation of the corresponding solutions resulting in appearance of the monomeric signal for the soluble fraction of both proteins.


(B)



Fig. 16: Far-UV CD spectra monitoring the secondary structure change of (A) mPrP120-230 and (B) hPrP121-231 on the pathway of MCO. Aliquots (44 μ M) buffered at pH 5.0 were incubated with Cu⁰ at 37 °C for different time periods. After removal of the copper metal, the samples were centrifuged and the supernatants were analyzed by CD spectroscopy. All measurements were performed at 20 °C using 0.1 mm path length cuvette and 100 nm/min. scan speed.

3.1.3 Structural conversion by ultra violet (UV) radiation

The structural effects induced by UV radiation on the C-terminal domains of mouse and human prion proteins were additionally studied. UV radiation is

known to oxidize proteins directly or by generation of ROS in aerobic solution. Since both proteins share a high degree (90%) of overall sequence identity, the observed differences in the effects caused by UV radiation can be referred to a limited number of deviant amino acids. The measurements were performed at pH 5.0, which resembles the acidified conditions within the endocytotic vesicles and lysozymes, and the typical physiological pH of 7.4. The selected buffers were determined to be stable under UV irradiation. Although sodium acetate can react with free radicals this buffer was reported to be appropriate for radiation experiments at low pH values (153).

For all measurements, an incident photon energy of 4.11eV (302 nm) was selected, which is in the middle of the UVB spectrum close to the highest solar energy reaching the earth's surface (154). A power dependency study was performed to estimate an appropriate measurement time using hPrP90-231, which already exhibited structural sensibility to oxidation (116). Various values of incident power ranging from 1 to 61.6 mW were applied. Initially, a transparency of about 75 to 85% compared to the transmission through pure H₂O was observed for all protein-containing samples (Fig. 17A). An increase in the light transmission within the first 10 s can be explained by technical issues, e.g. warming of the power sensor. However, because all measurements were consistently affected, this effect can be neglected. During the course of irradiation, distinct amounts of protein precipitated, exhibiting an exponential correlation between the protein concentration remaining in solution and the applied laser power (Fig. 17B). A comparable relation was also detected between the incident UV intensity and $T_{1/2}$ of the transmission caused by the increasing turbidity of the solution (Fig. 17C). Because the exponential decrease in both the protein concentration and the transmission of the samples proceeded on an appropriate time scale, the UV radiation power was adjusted to provide 8.6 mW within the cuvette for all subsequent measurements.



Fig. 17: Dependence of UV radiation power, sample transmission, and PrP aggregation. Samples (250 μ l) of hPrP90-230 (44 μ M) buffered at pH 7.4 were exposed to UV radiation at room temperature for 15 min.. (A) The transmission through the samples was continuously recorded. (B) After irradiation for 5 or 15 min., aliquots of 40 μ l were removed and centrifuged and the remaining PrP concentrations in the supernatants were determined. The radiation intensity was normalized to sample volume, irradiation time, and power. Error bars represent standard deviations. (C) The calculated half lives of the transmission are strongly correlated to the incident UV light intensity, following an exponential decrease function.

As expected from the investigations mentioned above, the C-terminal domain of the mouse prion protein was strongly affected by UV radiation at 302 nm. Within the first 10 min., the transmission of UV radiation through the samples containing mPrP120-230 at pH 5.0 was reduced by approx 93% ($T_{1/2} = 3.6$ min.), obviously caused by immediate protein precipitation (Fig. 18A, inset). The discrete edges within the transmission curves at 5 and 16 min. are due to the interruption of data recording during the removal of aliquots. After 20 min. of irradiation, the UV light transmission was completely blocked, apart from some leaps within the curve progression caused by adhesion of the precipitate into the voluminous particles that only temporarily allow the transmission of UV radiation, indicating a highly sticky nature of the formed aggregates. Although the overall protein concentration in the samples also decreased in an exponential manner, the time course was significantly extended (Fig. 18B). The corresponding half life was determined to be 10 min. for mPrP120-230 at pH 5.0. Complete protein precipitation was observed after 180 min. of irradiation.

Samples containing mPrP120-230 at pH 7.4 are characterized by a decreased aggregation rate ($T_{1/2} = 45$ min.). Even after maximum irradiation a remaining UV transmission of 8% ($T_{1/2} = 7.8$ min.) (Fig. 18A) and a solute protein concentration of 15% were still detected (Fig. 18B).

The pathway of UV-light induced aggregation of hPrP121-231 at pH 5.0 is characterized by a similar aggregation rate ($T_{1/2} = 47$ min.), which is accompanied by immediate precipitation and complete blocking of UV transmission after 200 min. ($T_{1/2} = 3.2$ min.) (Fig. 19). Leaps in the curve progression are as well indicative of the sticky nature of the precipitating protein. In contrast, hPrP121-231 at pH 7.4 displayed the highest resistance against UV radiation. Its soluble protein concentration remained at 80% even after 420 min. of irradiation (Fig. 19B). Only a marginal precipitation was observed, which was reflected by a significantly increased half life of UV light transmission of 182 min. (Fig. 19A).



Fig. 18: Time-resolved monitoring of mPrP120-230 aggregation induced by UV radiation. Aliquots (44 μ M) of mPrP120-230 buffered at pH 5.0 (\Box) and pH 7.4 (\blacktriangle) were exposed to UV radiation of 302 nm wavelength at room temperature for the indicated periods. (A) The transmission of UV radiation was continuously recorded behind the cuvette. The transmission curves of the initial 35 min. of irradiation are shown in detail (inset). (B) After irradiation, the samples were centrifuged and the remaining amounts of PrP in the supernatants were determined to estimate the degree of aggregation in percentage. Error bars represent standard deviations.



Fig. 19: Time-resolved monitoring of hPrP121-231 prion protein aggregation induced by UV radiation. Aliquots (44 μ M) of hPrP121-231 buffered at pH 5.0 (**•**) and pH 7.4 (**•**) were exposed to UV radiation (302 nm) at room temperature for the indicated periods. (A) Again the transmission of UV radiation was continuously recorded behind the cuvette. The transmission curves for the first 35 min. of irradiation are shown in detail (inset). (B) After irradiation, the samples were centrifuged and the remaining soluble fractions of PrP in the supernatants were determined to estimate the degree of aggregation. Error bars represent standard deviations.

Monitoring the secondary structure content of the C-terminal domain of mouse prion protein by CD spectroscopy revealed that mPrP120-231 is characterized by a predominant α -helical fold at both pH values before irradiation (Fig. 20A), which represents the typical conformation of cellular prion proteins (35, 155). In the course of aggregation, the CD spectra of mPrP120-230 at pH 5.0 were difficult to obtain, because the fraction of soluble protein rapidly decreased during irradiation. The corresponding weak curves were dominated by a minimum at 200 nm that is characteristic for random coil structures. Contributions of β -sheet structures have not been observed (data not shown). In contrast, both the intensity and the shape of the α -helical curve (minima at 208 and 222 nm) of mPrP120-230 at pH 7.4 significantly changed during UV-light induced aggregation, resulting in flattened curves with a single minimum at approx. 214 nm, typical for β -sheet structures (Fig. 20A). Time-dependent monitoring of the ellipticity at a wavelength of 222 nm revealed that the conversion reaction of mPrP120-230 was almost completed after 30 min. (Fig. 20C).

At pH 5.0, the initial CD spectra of hPrP121-231 are characterized by distinct minima at wavelengths of 208 and 222 nm, assigned to the high percentage of α helices within the protein (Fig. 20B). During irradiation, the spectra significantly changed, resulting in flattened curves showing a pronounced minimum at approx. 200 nm, a characteristic of random coil structures. The β -sheet content remained comparatively low, whereas the percentage of α helices notably decreased. However, at pH 7.4 the typical α -helical spectra of the monomeric protein consistently converted into flattened curves dominated by a single minimum at around 215 nm, resembling spectra characteristic for a β -sheet-rich structure (Fig. 20B). Regarding the associated mouse PrP, the structural conversion of hPrP121-230 at pH 7.4 was slightly decelerated as revealed by time-dependent monitoring of the ellipticity at a wavelength of 222 nm (Fig. 20C). Almost complete refolding was observed after UV irradiation for approx. 120 min.



Fig. 20: CD spectroscopy monitoring changes in the secondary structure content of the C-terminal domain of (A) mPrP120-230 and (B) hPrP121-231 induced by UV irradiation. Protein aliquots (44 μ M) were exposed to UV radiation at room temperature. CD spectra of each sample were recorded immediately at the beginning (0 min.) as well as after 419 min. of irradiation. (C) The time course of the structural conversion was illustrated at a wavelength of 222 nm for mPrP120-230 and for hPrP121-231 at pH 7.4.

The appearance of soluble intermediates on the pathway of UV-induced aggregation was also investigated by DLS measurements. Before irradiation, all solutions exhibited monodisperse behaviour, containing the monomeric prion proteins in solution. The corresponding hydrodynamic radii (1.98±0.1 nm for mPrP120-230 and 1.94±0.09 nm for hPrP121-231) were in a good agreement with the theoretical values calculated from the molecular masses, assuming a roughly globular shape of the molecules. Within the first 30 min. of irradiation, the monomeric peaks consistently disappeared from all samples, because the concentration of PrP monomers fell below the detection limit of the DLS device. For mPrP120-230 and hPrP121-231 at pH 5.0, evidence of specific oligomer formation was not obtained. Instead, high molecular weight aggregates (> 150 kDa) were detected by SDS-PAGE analyses that were covalently cross-linked. However, an additional sharp peak appeared in the solutions of mPrP120-230 at pH 7.4, with a radius of 13.89 ± 0.19 nm (O^{120}_{A}), indicating stable and soluble oligomers. Within the first 15 min. of irradiation mPrP120-230 (pH 7.4) formed a second stable and soluble oligomer characterized by a doubled hydrodynamic radius of approx. 28 nm (O_{B}^{120}). Both O_{A}^{120} and O_{B}^{120} persisted in solution up to 420 min.. On the pathway of hPrP121-231 aggregation at pH 7.4, a single stable and soluble oligomer was also detected (O¹²¹_A) within the first 30 min. of irradiation. This oligomer persisted throughout the entire incubation period and is characterized by a hydrodynamic radius of 8.31±0.35 nm.

Additional irradiation experiments were performed to investigate and to separate different mechanisms of UV-induced structural damage. The contribution of protein oxidation by ROS was evaluated by irradiation of the C-terminal mouse prion protein domain under anaerobic conditions and in the presence of the oxygen free radical scavengers dimethyl sulfoxide (DMSO) and sodium azide, as well as ascorbic acid (156-158). The most pronounced effects were observed in the presence of ascorbic acid (Fig. 21), which almost completely prevented

the aggregation and precipitation of mPrP120-230, at least up to 420 min. of incubation (90.2 \pm 3.7%) in solution at pH 5.0. Monomeric molecules characterized by the typical α -helical PrP^C fold persisted in the samples. An enhanced stabilization of mPrP120-230 was observed at pH 5.0 when oxygen was absent (79.2 \pm 4.6% in solution). This effect was mainly attributed to an inhibition of the oxidation process mediated by reactive oxygen species, because the addition of the specific scavengers sodium azide and DMSO also resulted in a significantly increased stabilization of PrP. However, inhibition of the indirect oxidation did not affect the fundamental aggregation pathway. Protein denaturation without formation of soluble oligomers was detected in the absence of oxygen as well, even if the rate of precipitation was significantly reduced. The UV-induced aggregation of human prion proteins is followed the same fundamental mechanisms of oxidative protein damage that have already been described for the mouse PrPs.



Fig. 21: Influence of oxygen free radical scavengers and anaerobic conditions on the aggregation rate of mPrP120-230 at pH 5.0.

3.2 Oxidative induced conversion of hPrP121-231 (M129S, M134S, M154S, M166S, M213S)

To specifically investigate the effect of Met residues within the oxidative induced conversion process of the prion proteins, the surface exposed Met residues (M129, M134, M154, M166, and M213) in the folded C-terminal domain of human PrP were mutated into Ser residues. Subsequently, the MCO-induced conversion was investigated and compared to the *wild type* hPrP121-231 conversion.

3.2.1 Mutation, cloning, and recombinant expression

All mutations were stepwise introduced by the PCR method of non-overlap extension. Therefore, five successive site-directed mutagenesis steps were performed to mutate the selected Met residues into Ser residues. The flanking primers hPrP-BamHI-start and hPrP-EcoRI-stop were used in all reactions. In the first step the M134 codon was mutated from ATG to TCA, thus turning it into a Ser codon. The specific primers hPrP-M134Sf and hPrP-M134Sr were used to amplify two separated fragments of 42 and 288 bp, which have been visualized by agarose gel electrophoresis (Fig. 22A). Ligation of the purified PCR fragments resulted in one fragment that comprises the desired mutation. The successful insertion of a M134S mutation was confirmed by sequence analysis and the ligation product was used as a template to introduce further mutations. In the second step amplification with the primers hPrP-M154Sr and hPrP-M154Sf resulted in generation of two specific PCR fragments at the expected size of 99 and 231 bp, respectively, introducing a M154S mutation (Fig. 22B). Mutation M166S was introduced using the primers hPrP-M166Sr and hPrP-M166Sf, resulting in two specific fragments of 132 and 198 bp (Fig. 22C), while the fourth mutation M213S was performed using the primers hPrP-M213Sr and hPrP-M213Sf in the amplification reaction. Agarose gel electrophoresis confirmed the expected size of the amplified fragments of 54

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and 276 bp, which were purified and ligated (Fig. 22D). Finally, M129 was mutated into Ser. This mutation has been achieved by ligation of double stranded oligonucleotides (hPrP-M129S*f* and hPrP-M129S*r*) carrying the mutation M129S with a 288 bp PCR amplified fragment comprising the other four mutations M134S, M154S, M166S, and M213S. The primers hPrP-M134S*f* and hPrP-*Eco*RI were used to amplify the 288 bp fragment. The expected size of the fragment was confirmed by agarose gel electrophoresis (Fig. 22E). Following purification and ligation into the prokaryotic expression vector pRSETA via *Bam*HI and *Eco*RI restriction sites, the insertion of all mutations was verified by dideoxy sequencing, resulting in formation of the corresponding plasmid pRSETA *v*-hPrP121-231.



Fig. 22: Gel electrophoretic analysis of the PCR amplification of the hPrP121-231 PrP gene carrying the mutations M129S, M134S, M154S, M166S and M213S, using 2% agarose gels. (A) M134S, (B) M154S, (C) M166S, (D) M213S and (E) M129S.

The expression as well as the purification of the mutant hPrP121-231 (M129S, M134S, M154S, M166S, M213S), subsequently referred to as v-hPrP121-231, was performed according to the established protocol of the wild type hPrP121-231. Strong protein bands with an apparent molecular weight of approx. 17 kDa appeared after 2h of expression at 37 °C (Fig. 23A). However, the refolding process of v-hPrP121-231 failed using the optimized conditions of the wild type protein. Moreover, the application of other refolding protocols that depend either on stepwise removal of the denaturant by dialysis (159) or addition of a stabilizing agent, which prevents the aggregation of proteins during refolding such as arginine (160) and α -cyclodextrin (161) was also not successful. Investigating the secondary structure of the resulting protein by CD spectroscopy revealed that the PrP mutant still mainly consists of random coil structures characterized by a single minimum at around 200 nm. Finally, a slight change in the flow rate from 0.5 to 0.2 ml/min. of the linear gradient that remove guanidine hydrochloride following the *wild type* refolding protocol resulted in successful refolding of the mutant PrP. The purity and the complete proteolytic cleavage of the N-terminal His tag using factor Xa was confirmed by SDS-PAGE analysis (Fig. 23B), resulting in a pure protein characterized by a molecular weight of approx. 17 kDa, corresponding to the His-tagged protein, and 13 kDa, representing the mature recombinant variant PrP. The protein concentration was determined by absorbance at 280 nm using the calculated molar extinction coefficient $\varepsilon_{280} = 16,515 \text{ M}^{-1} \text{ cm}^{-1}$ for *v*-hPrP121-231.

Investigating the secondary structure of mature soluble *v*-hPrP121-231 by CD spectroscopy (Fig. 24) showed two distinct minima at a wavelength of 208 and 222 nm, characteristic for α -helical structures (130). Compared to the *wild type* protein, the CD spectrum of the variant human PrP structure exhibited a slight decrease in the total α -helical content, associated by an increase of random coil structure, which can be attributed to the substitution of all surface exposed Met residues by hydrophilic Ser residues.

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Fig. 23: (A) Non-reducing SDS-PAGE analysis of the recombinant expression of *v*-hPrP121-231. Samples of the crude *E. coli* extract were separated using a 15% SDS gel. The time of expression is indicated above the gel. (B) Non-reducing SDS-PAGE (15%) analysis of the purification of recombinant *v*-hPrP121-231. Lane 1: *v*-hPrP121-231 including the N-terminal His tag (~17 kDa). Lane 2: *v*-hPrP121-231 after cleavage of the N-terminal His tag (~13 kDa). (M): Page ruler unstained protein marker. All proteins were stained by coomassie dye.



Fig. 24: Far-UV CD spectra of recombinant *v*-hPrP121-231 and *wild type* hPrP121-231. CDspectra were recorded for samples containing a protein concentration of 0.1 mg/ml in 5 mM sodium acetate buffer (pH 5.0). All measurements were carried out at 20 °C in a 0.1 mm path length cuvette using a scanning rate of 100 nm/min.

3.2.2 Structural conversion by metal catalyzed oxidation (MCO)

The effect of the substitution of all surface exposed Met residues of the recombinant *v*-hPrP121-231 was investigated applying the oxidative-induced aggregation by MCO. Protein samples (44 μ M) buffered in 5 mM sodium acetate (pH 5.0) were incubated at 37 °C in the presence as well as in the absence of Cu⁰ for different time periods. After separation of precipitation and high molecular weight aggregates by centrifugation, the remaining concentration of soluble PrP molecules was determined to analyse the rate of conversion and aggregation (Fig. 25). As expected, a significant enhancement in the stability of *v*-hPrP121-231 towards oxidative-induced aggregation has been observed. The variant PrP aggregated at a lower rate compared to *wild type* hPrP121-231, resulting in approx. 8-fold increase of the half life of the variant PrP ($T_{1/2} = 24$ min.) compared to that of the *wild type* protein ($T_{1/2} = 3$ min.). In the absence of Cu⁰, no aggregation has been observed. The monomeric PrP molecules remained stable and soluble up to 60 and 90 min. for *wild type* and the variant PrP, respectively.



Fig. 25: Time-resolved monitoring of the *in vitro* aggregation of recombinant *v*-hPrP121-231 and *wild type* hPrP121-231 induced by MCO. Protein aliquots (44μM) buffered at pH 5.0 were incubated in the presence and in the absence (control) of Cu⁰ at 37 °C for the indicated time periods. After removal of the copper pellet, the protein samples were centrifuged and the remaining PrP concentrations in the supernatants were determined. Error bars represent the standard deviations.

The aggregates of the variant human PrP formed by MCO that have been isolated from the supernatant by centrifugation were used for structural characterization. Non-reducing SDS-PAGE analysis indicated the persistence of the monomeric PrP band even after 24h of incubation (Fig. 26A). Within the first 5 min. of MCO, an additional band appeared characterized by a molecular weight of approx. 26 kDa, corresponding to the dimeric form of the variant PrP molecule. High molecular weight aggregates including a 39 kDa fraction and molecules of more than 116 kDa have been initially detected after 6h and 16h of MCO, respectively. The 39 kDa band attributed to the trimeric form of the variant PrP. To investigate whether copper ions are involved in the dimerization of the variant PrP, the reaction mixture was treated with EDTA to a final concentration of 10 mM at the end of each incubation period. However, the dimer formation was not affected by the addition of EDTA (Fig. 26A). Applying reducing conditions (Fig. 26B) all high molecular weight aggregates dissociated into monomeric PrP molecules (13 kDa) except the dimer. The high stability against reducing heat denaturation indicates a dimer formation via covalent cross linking rather than via intermolecular disulfide bonds, as it is suggested for the high molecular weight aggregates.

Monitoring the MCO-induced secondary structure changes of *v*-hPrP121-231 and *wild type* hPrP121-231 on the pathway of aggregation applying CD spectroscopy revealed that all proteins exhibited a typical α -helical secondary structure characterized by two pronounced minima at 208 nm and 222 nm before aggregation. In the course of *v*-hPrP121-231 aggregation, the CD signal corresponding to α -helical fold did not change up to 30 min. of MCO, confirming the enhanced stability of the variant protein (Fig. 27). After 30 min. of MCO, no CD signal was detected. Also the increase of β -sheet specific signal was not observed. In contrast, within the first minute of MCO the CD spectra of the *wild type* hPrP121-231 significantly changed into a flattened curve characterized by single minimum at 215 nm, typical for a β -sheet structure.

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Fig. 26: Non reducing (A) and reducing (B) SDS-PAGE analysis (15%) of the *v*-hPrP121-231 aggregates formed by MCO. The aggregated proteins were pellet down by centrifugation and resuspended in 5 mM sodium acetate pH 5.0. An appropriate amount of the suspension was mixed with sample buffer and incubated at 95 °C for 10 min.. (M): Page ruler unstained protein marker. (C): *v*-hPrP121-231 incubated for the indicated time periods in the absence of copper (control). (E): EDTA.



Fig. 27: Far-UV CD spectra monitoring the secondary structure change of *v*-hPrP121-231 and wild type hPrP121-231 on the pathway of MCO. Aliquots (44 μM) buffered at pH 5.0 were incubated with Cu⁰ at 37 °C for different time periods. After removal of the copper metal, the samples were centrifuged and the supernatants were analyzed by CD spectroscopy. All measurements were performed at 20 °C using 0.1 mm path length cuvette and 100 nm/min. scan speed.

The DLS analysis of v-hPrP121-231 revealed that the variant PrP possessed reaction buffer before monodisperse behaviour in the aggregation, predominantly consisting of its monomeric form, as indicated by a hydrodynamic radius of 1.69±0.48 nm. The determined hydrodynamic radius was in a good agreement with the theoretical value calculated from the molecular mass, assuming a roughly globular shape of the molecule. During aggregation, particularly within the first minute of incubation, v-hPrP121-231 formed a soluble oligomer with a hydrodynamic radius of 17.3 ± 2.68 nm. This oligomer persisted in the reaction mixture up to 30 min. of incubation. The recorded CD spectra of the soluble oligomer indicated α -helical fold characterized by double distinct negative minima at 208 and 222 nm (Fig. 27). Conversely, the wild type hPrP121-231 aggregated without formation of soluble oligomeric intermediates. Instead, high molecular weight heterogenic aggregates of more than 100 nm in radius appeared in solution. These aggregates immediately disappeared by centrifugation, resulting in appearance of the corresponding monomeric peak.

3.3 Oxidative induced conversion of hPrP121-231 M129T and mPrP120-230 M129T

To assign the observed reduced MCO-induced aggregation tendency of *v*-hPrP121-231, carrying the mutations M129S, M134S, M154S, M166S, and M213S to specific Met residues, the stepwise substitution of the surface exposed Met residues is finally required. In this study the systematic replacement starts with the mutation of M129 against Thr in both human and mouse prion proteins, followed by an investigation of the oxidative-induced conversion of the M129T mutant. Met at position 129 is the site of polymorphism in several species and therefore of specific interest.

3.3.1 Mutation, cloning, and recombinant expression

Mutation of Met at position 129 into Thr was performed by site directed mutagenesis of the recombinant C-terminal domain of human and mouse PrP. The primers hPrP-M129T and mPrP-M129T were constructed to induce a specific point mutation in the PrP gene. The expected mutation was generated at an annealing temperature of 55 °C and 12 cycles using *pfu* DNA polymerase for high fidelity amplification. Sequencing the DNA from three selected colonies of each construct identified two plasmids.with the required mutation (pRSETA-hPrP121-231-M129T and pRSETA-mPrP120-230-M129T).

Expression and purification of hPrP121-231 M129T and mPrP120-230 M129T were performed according to the established protocol of the associated *wild type* prion proteins. Again only insoluble inclusion bodies were obtained. Strong protein bands with an apparent molecular weight of approx. 17 kDa appeared after 2h of expression at 37 °C (Fig. 28). The purity and the complete proteolytic cleavage of the N-terminal His tag using factor Xa was confirmed by SDS-PAGE analysis. Pure protein bands appeared at the expected molecular weights of approx. 17 kDa, representing the His tag proteins and 13 kDa, corresponding to the mature recombinant PrPs (Fig. 29). Protein concentrations were determined by absorbance at 280 nm using the calculated molar extinction coefficient $\varepsilon_{280} = 16,515 \text{ M}^{-1} \text{ cm}^{-1}$ for hPrP121-231 M129T and $\varepsilon_{280} = 22,015 \text{ M}^{-1} \text{ cm}^{-1}$ for mPrP120-230 M129T.



Fig. 28: Non-reducing SDS-PAGE analysis of the recombinant expression of (A) hPrP121-231 M129T and (B) mPrP120-230 M129T. Samples of the crude *E. coli* extract were separated using a 15% SDS gel. (M) Page ruler unstained protein marker. The time of expression is indicated above the gel. All proteins have been stained by coomassie dye.

(A)														(B)
kI	Da	Μ	1	2					kDa	Μ	1		2	
1	16	-							66	-				
4	45								45	-				
	35	3							35	-				
	25 -	-							25	_		1		
	18	•	- 1						18	-				
	14			-					14	-			4	

Fig. 29: Non-reducing SDS-PAGE (15%) analysis of the purification of (A) hPrP121-231 M129T and (B) mPrP120-230 M129T. Lane 1: recombinant His tag PrP (~17kDa). Lane 2: the recombinant mature PrP after cleavage of the N-terminal His tag (~ 13 kDa). (M): Page ruler unstained protein marker. All proteins have been stained by coomassie dye.

The secondary structure of the recombinant variant proteins was investigated by CD spectroscopy (Fig. 30). The results showed that both proteins predominantly consist of α -helices characterized by two negative minima at 208 nm and 222 nm, highly similar to the spectra of the associated *wild type* proteins.



Fig. 30: Far-UV CD spectra of recombinant hPrP121-231 M129T and mPrP120-230 M129T compared to the *wild type* proteins hPrP121-231 and mPrP120-230. CD-spectra were recorded on samples containing a protein concentration of 0.1 mg/ml in 5 mM sodium acetate buffer (pH 5.0). All measurements were carried out at 20 °C in a 0.1 mm path length cuvette using a scanning rate of 100 nm/min..

3.3.2 Structural conversion by metal catalyzed oxidation (MCO)

The effect of the mutated surface exposed Met residue at position 129 of hPrP121-231 M129T and mPrP120-230 M129T in terms of oxidative-induced aggregation was investigated by MCO. Protein samples (44 µM) buffered in 5 mM sodium acetate (pH 5.0) were incubated at 37 °C in the presence of Cu^0 for different time periods (1-90 min. for hPrP121-231 M129T and 1-180 min. for mPrP120-230 M129T). Following a separation of precipitation and high molecular weight aggregates by centrifugation, the remaining concentration of the soluble PrP was determined as a marker for the rate of conversion and aggregation (Fig. 31). The results indicated that hPrP121-231 M129T displayed a significant resistance towards oxidative aggregation (Fig. 31A). hPrP121-231 M129T aggregated at a lower rate than the *wild type* protein resulting in an increased half life of approx. 3-fold ($T_{1/2} = 8$ min.) at the applied conditions compared to that of wild type hPrP121-231 ($T_{1/2} = 3$ min.), indicating a significant impact of Met 129 to the oxidative aggregation of human PrP. Compared to the aggregation rate of v-hPrP121-231 ($T_{1/2} = 24$ min.), the aggregation rate of hPrP121-231 M129T was obviously increased by approx. 3fold. Moreover, MCO of hPrP121-231 M129T was accompanied by formation of only marginal precipitation. In the absence of Cu⁰ (control), no aggregation has been observed. The monomeric PrP molecules remained stable and soluble up to 60 and 90 min. for wild type hPrP121-231 and hPrP121-231 M129T, respectively. Surprisingly, no difference in the aggregation rate of mPrP120-230 M129T and its wild type form was observed (Fig. 31B). The corresponding half life times at the applied conditions were determined to be $T_{1/2} = 15$ min. for both proteins. In the absence of Cu⁰, no aggregation has been observed. The monomeric PrP molecules remained stable and soluble up to 180 min. for both mPrP120-230 M129T and wild type mPrP120-230 PrP.



Fig. 31: Time-resolved monitoring of the *in vitro* aggregation of (A) recombinant *wild type* hPrP121-231, hPrP121-231 M129T, and *v*-hPrP121-231, as well as (B) *wild type* mPrP120-230 and mPrP120-231 M129T induced by MCO. Protein aliquots (44 μ M) buffered at pH 5.0 were incubated in the presence and in the absence (control) of Cu⁰ at 37 °C for the indicated time periods. After removal of the copper pellet, the protein samples were centrifuged and the remaining PrP concentrations in the supernatants were determined. Error bars represent the standard deviations.

The aggregates of hPrP121-231 M129T and mPrP120-230 M129T formed by MCO and isolated from the supernatants by centrifugation were used for structural characterization. Non-reducing SDS-PAGE analysis showed the persistence of the dominating monomeric PrP bands up to 60 min. of incubation for both hPrP121-231 M129T (Fig. 32A) and mPrP120-230 M129T (Fig. 32C). Within the first 10 min. of MCO, additional bands appeared characterized by molecular weights of approx. 26 kDa and 39 kDa and more than 116 kDa. The 26 kDa and 39 kDa protein bands are attributed to the dimeric and trimeric forms of the variant proteins. Again the dimer displayed a high resistance against the reducing conditions of the SDS-PAGE as well as against heat denaturation (Fig. 32B and D). However, the trimer and the high molecular weight aggregates dissociated into the monomeric PrP (13 kDa), indicating a covalent cross-linking mechanism rather than intermolecular disulfide bonds for dimer stabilization. The latter is suggested for the trimeric form and the high molecular weight aggregates of the variant proteins. Moreover, under reducing conditions additional band appeared characterized by a molecular weight of approx. 15 kDa, which is attributed to the alternative conformations and/or denaturation states of monomeric PrP molecules.

Monitoring the MCO-induced secondary structure changes of hPrP121-231 M129T and mPrP120-230 M129T on the pathway of aggregation revealed that all proteins exhibited a typical α -helical secondary structure characterized by two pronounced minima at 208 nm and 222 nm before aggregation. Within the first minute of MCO the CD spectra of the *wild type* hPrP121-231 significantly changed resulting in a flattened β -sheet curve characterized by specific minimum around 215 nm (Fig. 33A). Conversely, within the first minute of MCO the CD signal corresponding to α -helical fold of hPrP121-231 M129T did not change, however the intensity was slightly decreased (Fig. 33B). After 5 min. of MCO, no CD spectra have been observed for both *wild type* and variant human PrP. Interestingly, in the course of mPrP120-230 M129T

aggregation (Fig. 34A), the dichroic signal corresponding to α -helices at 208 nm and 222 nm did not change up to 10 min. of MCO, as it was also observed for the *wild type* protein (Fig. 34B). Contributions of a β -sheet specific signal were not revealed, confirming the resistance of mouse PrP towards MCO-induced aggregation. After 20 min. of MCO, almost no CD signal has been detected for both proteins.



Fig. 32: Non-reducing (A/C) and reducing (B/D) SDS-PAGE analysis (15%) of hPrP121-231 M129T (A/B) and mPrP120-230 M129T (C/D) aggregates formed by MCO. The aggregated proteins were pellet down by centrifugation, separated from the supernatant and resuspended in 5 mM sodium acetate pH 5.0. An appropriate amount of the suspension was mixed with sample buffer and incubated at 95 °C for 10 min.. (M): Page ruler unstained protein marker. (C): hPrP121-231 M129T and mPrP120-230 M129T incubated for 60 min. in the absence of copper (control).



Fig. 33: Far-UV CD spectra monitoring the secondary structure change of (A) hPrP121-231 M129T and (B) wild type hPrP121-231 on the pathway of MCO. Aliquots (44 μM) buffered at pH 5.0 were incubated with Cu⁰ at 37 °C for different time periods. After removal of the copper metal, the samples were centrifuged and the supernatants were analyzed by CD spectroscopy. All measurements were performed at 20 °C using 0.1 mm path length cuvette and 100 nm/min. scan speed.

DLS analysis at the beginning of MCO-induced aggregation confirmed the monodisperse behaviour of the variant prion proteins in the reaction buffer, predominantly consisting of molecules characterized by hydrodynamic radii of 1.90±0.1 nm and 1.86±0.26 nm for hPrP121-231 M129T and the mPrP120-230 M129T, respectively. These radii were close to the theoretical values calculated from the molecular masses (1.78 nm for both hPrP121-231 M129T and mPrP120-230 M129T), assuming a globular shape of the molecules.

During MCO, both hPrP121-231 M129T and mPrP120-231 M129T aggregated without formation of soluble oligomeric intermediates. Like their *wild type* proteins, heterogenic high molecular weight aggregates of more than 100 nm were present in solution that immediately disappeared after centrifugation followed by appearance of the monomeric peaks.

(A)

(B)



Fig. 34: Far-UV CD spectra monitoring the secondary structure change of (A) mPrP120-230 M129T and (B) wild type mPrP120-230 on the pathway of MCO. Aliquots (44 μM) buffered at pH 5.0 were incubated with Cu⁰ at 37 °C for different time periods. After removal of the copper metal, the samples were centrifuged and the supernatants were analyzed by CD spectroscopy. All measurements were performed at 20 °C using 0.1 mm path length cuvette and 100 nm/min. scan speed.

3.4 Effect of β-cyclodextrin on the oxidative induced conversion of the C-terminal domain of mouse and human prion proteins.

β-cyclodextrin (β-CD) has been successfully used by the pharmaceutical industry with respect to its complex-forming ability (162). This ability is due to the structural orientation of the glucopyranose units, which generate a hydrophobic cavity that can facilitate the encapsulation of hydrophobic moieties. Recently, a non-cytotoxic concentration of β-CD has been reported to remove the infectious PrP^{Sc} isoform in scrapie infected neuroblastoma (ScN2a) cell cultures (138). In addition, β-CD has the ability to reduce the toxic effect of β-amyloid protein (residues 1-40) associated with Alzheimer's disease in cell cultures (139). Therefore, the impact of β-CD on the *in vitro* oxidative-induced conversion of PrP was investigated.

3.4.1 Structural conversion induced by metal catalyzed oxidation (MCO)

Protein samples of *wild type* mPrP120-230 and hPrP121-231 (44 μ M) buffered in 5 mM sodium acetate (pH 5.0) were incubated in the presence and absence of Cu⁰ at 37 °C for different time periods (from 1-180 min. for mPrP120-230 and from 1-90 min. for hPrP121-231), partly supplemented with β -CD. The ratio of PrP/ β -CD was adjusted to be 1 to 10. Following a separation of precipitation and high molecular weight aggregates by centrifugation, the remaining concentration of soluble PrP in the supernatant was determined as a marker for the rate of conversion and aggregation. The results showed that in the presence of β -CD both mPrP120-230 (Fig. 35A) and hPrP121-231 (Fig. 35B) displayed a significant enhanced stability against oxidative-induced aggregation by MCO. The corresponding half life times of both proteins in the presence of β -CD ($T_{1/2}^{\beta}$) increased by approx. 3-fold (from $T_{1/2} = 15$ min. to $T_{1/2}^{\beta} = 45$ min. for mPrP120-231 and from $T_{1/2} = 3$ min. to $T_{1/2}^{\beta} = 10$ min. for hPrP121-231). In the absence of Cu⁰, no aggregation has been observed. The monomeric PrP molecules remained stable and soluble up to 180 and 90 min. for mPrP120-230 and hPrP121-231, respectively. During MCO of hPrP121-231 marginal precipitation has been observed, whereas the solution of mPrP120-230 was almost clear during the entire incubation period.



Fig. 35: Time-resolved monitoring of the effect of β -CD on the *in vitro* aggregation of the recombinant C-terminal domain of (A) mPrP120-230 and (B) hPrP121-231 induced by MCO. Protein aliquots (44µM) buffered at pH 5.0 were incubated in the presence and in the absence (control) of Cu⁰ at 37 °C for the indicated time periods. The ratio of PrP/ β -CD was adjusted to be 1 to 10. After removal of copper pellets, the protein samples were centrifuged and the remaining PrP concentrations in the supernatants were determined. Error bars represent the standard deviations.

The change in the secondary structure of the mPrP120-230 and hPrP121-231 induced by MCO in the presence as well as in the absence of β -CD was monitored by far-UV CD spectroscopy. At the beginning all proteins exhibited a mainly α -helical fold also in the presence of β -CD, characterized by two distinct minima at 208 nm and 222 nm. Since the isolated protein aggregates are insoluble, no CD spectra have been obtained. The soluble fractions of mPrP120-230 showed in the presence of β -CD a significant enhancement in the α -helical content. The CD spectra typical for α -helical structures (minima at 208 and 222 nm) did not change and persisted up to 30 min. of MCO. After 60 min. of incubation the typical α -helical signal significantly changed resulting in a weak flattened curve characterized by a specific single minimum at approx. 200 nm, a characteristic for random coil structures (Fig. 36A). However, in the absence of β -CD, the α -helical signal of the soluble fraction of mPrP120-230 persisted only up to 10 min. of incubation (Fig. 36B). The intensity of the CD spectra of mPrP120-230 was markedly decreased after 60 min. of MCO in the presence of β -CD as well as after 20 min. of MCO in the absence of β -CD. A β -sheet specific signal was not observed.

In contrast, the α -helix CD signal of the soluble hPrP121-231 fraction persisted up to 1 min. of MCO in the presence of β -CD. Within the first 5 min. of MCO, both the shape and the intensity of the CD spectra significantly decreased resulting in a flattened curve, which is assigned to a mixture of α -helix and β sheet structures (Fig. 37A). After 10 min. of incubation, no CD spectra have been detected. However, in the absence of β -CD, particularly within the first minute of MCO, the typical α -helical signal transformed into a flattened β -sheet curve characterized by a specific minimum at 215 nm (Fig. 37B), indicating a slight stabilization effect of β -CD in terms of $\alpha \rightarrow \beta$ structural conversion of human PrP induced by MCO.



Fig. 36: Far-UV CD spectra monitoring the secondary structure change of mPrP120-230 (A) in the presence and (B) in the absence of β -CD on the pathway of MCO-induced aggregation. Aliquots (44 μ M) buffered at pH 5.0 were incubated with Cu⁰ at 37 °C for different time periods. The ratio of PrP/ β -CD was adjusted to be 1 to 10. After removal of the copper metal, the samples were centrifuged and the supernatants were analyzed by CD spectroscopy. All measurements were performed at 20 °C using 0.1 mm path length cuvette and 100 nm/min. scan speed.



Fig. 37: Far-UV CD spectra monitoring the secondary structure change of hPrP121-231 (A) in the presence and (B) in the absence of β -CD on the pathway of MCO. Aliquots (44 μ M) buffered at pH 5.0 were incubated with Cu⁰ at 37 °C for different time periods. The ratio of PrP/ β -CD was adjusted to be 1 to 10. After removal of the copper metal, the samples were centrifuged and the supernatants were analyzed by CD spectroscopy. All measurements were performed at 20 °C using 0.1 mm path length cuvette and 100 nm/min. scan speed.

DLS analysis revealed that in the presence of β -CD the monodisperse behaviour of the prion proteins is not affected. However the hydrodynamic radii of both proteins significantly increased by approx. 1.5-fold (from 1.98±0.1 to 2.4±0.31 nm for mPrP120-230 and from 1.94±0.09 to 2.81±0.38 nm for hPrP121-231). In the course of MCO, hPrP121-231 aggregated without formation of soluble oligomeric intermediates. Only high molecular weight heterogenic aggregates of more than 1 μ M were formed in solution during the whole incubation period. These aggregates immediately disappeared from the reaction mixture after centrifugation, resulting in appearance of the monomeric peak. In contrast, within the first minute of incubation mPrP120-230 formed a soluble oligomer characterized by a hydrodynamic radius of 4.57 ± 0.55 nm (O^{120}_{A}) that persisted in solution up to 20 min. of MCO. Subsequently, a second soluble oligomer with a hydrodynamic radius of 28.03 ± 6.28 nm (O^{120}_{B}) appeared within the first 30 min. of MCO. O_{B}^{120} persisted in the reaction mixture up to 60 min. of MCO. Investigating the secondary structure of these oligomers by CD spectroscopy revealed that these molecules consist of a mixture of α -helices and random coil structures (Fig. 36A).

3.4.2 Characterization of β-CD binding to prion proteins

To analyze the molecular basis of the inhibitory effect of β -CD on the oxidativeinduced *in vitro* aggregation of PrP and to obtain first structural insight into the formed complex, recombinant mPrP120-230 and hPrP121-231 was investigated in the presence and in the absence of β -CD using small-angle X-ray scattering (SAXS) techniques. The ratio of PrP/ β -CD was again adjusted to be 1 to 10. In the absence of β -CD the radius of gyration (R_g) of the monomeric PrP molecules was determined to be 1.83±0.02 nm and 1.80±0.02 nm for mPrP120-230 and hPrP121-231, respectively. The determined R_g and the associated molecular masses (13 kDa) of the proteins were in good agreement with those that have been obtained from DLS measurements (1.98±0.1 nm for mPrP120-230 and 1.94±0.09 nm for hPrP121-231). In the presence of β -CD, the recorded SAXS curves significantly deviate from the samples in the absence of β -CD (Fig. 38), indicating differences in the overall shape and in the size of the molecules. The corresponding R_g of the recombinant proteins slightly decreased, resulting in values of 1.66±0.01 nm for mPrP120-231 and 1.66±0.03 nm for hPrP121-231. This effect could indicate that the structure of both proteins is more compact in the presence of β -CD. However, also a co-existence of free unbound β -CD together with the prion proteins in solution is possible, which would result in a reduced average R_g value.



Fig. 38: Comparison of small-angle X-ray scattering curves of the C-terminal domain of human PrP in the presence as well as in the absence of β -CD using protein concentration of 2.9 mg/ml buffered in 10 mM Tris-HCl pH 8.0. The ratio of PrP/ β -CD was adjusted to be 1 to 10. Data were recorded using MAR345 image plate detector at a sample-detector distance of 2.7 m and a wavelength of $\lambda = 0.15$ nm covering the range of momentum transfer (*s*) from 0.08 to 5 nm⁻¹.

Table 2: R_g values of both mPrP120-230 and hPrP121-231 resulted from SAXS measurements in the presence and in the absence of β -CD

Protein	R _g (nm)
mPrP120-230	1.83±0.02
mPrP120-230 (+ β-CD)	1.66±0.01
hPrP121-231	1.80±0.02
hPrP121-231 (+ β-CD)	1.66±0.03

Since the SAXS technique is not appropriate to determine, whether a complex is formed in solution, the binding affinity of β -CD to the recombinant hPrP121-231 was analyzed by surface plasmon resonance (SPR). Two related sugars, α cyclodextrin (α -CD) and cellobiose have been included in this investigation to analyze the binding specificity of different sugars to PrP. The recombinant protein (1 μ M) buffered in 5 mM sodium acetate (pH 5.0) was immobilized on the surface of a CM5 sensor chip by binding to the activated layer of the dextran matrix. The free binding sites of dextran that were not occupied with proteins have been blocked with ethanolamine. Analysis of the SPR data revealed that hPrP121-231 did not specifically bind β -CD (Fig. 39). The dissociation constant (K_D) of β -CD was determined to be 19 mM for hPrP121-231. Similarly, Cellobiose showed non specific binding to the recombinant protein (K_D = 16 Mm), whereas no affinity for α -CD to hPrP121-231has been observed.



Fig. 39: Binding of β -CD to the immobilized recombinant C-terminal domain of hPrP121-231. Protein (1 μ M) buffered in 5 mM sodium acetate pH 5.0 was immobilized on the surface of CM5 sensor chip by binding to the activated layer of dextran matrix using a flow rate of 20 μ l/min.. The unoccupied free binding sites of dextran were saturated with 1 M ethanolamine for 15 min. using a flow rate of 10 μ l/min..

3.5 Summary and comparison of the obtained results

The results obtained for the oxidative induced aggregation of the recombinant C-terminal domain and specific mutants of mouse and human prion proteins by MCO as well as by UV radiation are summarized in Table 3. The mutant vhPrP121-231 showed the highest stability against MCO-induced aggregation, which is accompanied by formation of soluble oligomeric intermediates dominated by α -helical fold. In contrast, hPrP121-231 was the most labile protein characterized by rapid aggregation and $\alpha \rightarrow \beta$ structural conversion. At pH 7.4 hPrP121-231 was the most resistant protein against oxidative damage induced by UV radiation, only soluble oligomeric intermediates characterized by a β -sheet dominated fold were formed. *Wild-type* mPrP120-230 and hPrP121-231 share about 90% overall sequence identity. However, in terms of residues sensitive to oxidation, hPrP121-231 possesses one additional His compared to mPrP120-231, while Trp is completely absent. In the course of MCO mPrP120-230 exhibited increased stability against oxidative-induced aggregation than hPrP121-231. The aggregation of mPrP120-230 was accompanied by the persistence of the α -helical fold in the soluble fraction. Conversely, for UV irradiation at pH 7.4, mPrP120-230 was characterized by a rapid aggregation rate compared to hPrP121-231, which was accompanied by formation of β oligometic intermediates. The presence of β -CD increased the stability of both mPrP120-230 and hPrP121-231 against MCO-induced aggregation by a factor of approx. 3. While mPrP120-231 formed soluble oligomeric intermediates in the presence of β -CD, predominantly consisting of a mixture of α -helix and random coil structure, hPrP121-231 was directed into large heterogeneous aggregates.

Table 3: Summary of the results obtained for oxidative-induced aggregation of hPrP121-231 and mPrP120-230 prion proteins as well as their mutants.

Prion	<i>wild type</i> hPrP121-231	hPrP121-231 M129T	v-hPrP121-231	<i>wild type</i> mPrP120-230	mPrP120-230 M129T		
Number of Met residues	7	6	2	7	6		
Number of His residues	4	4	4	3	3		
Number of Trp residues	-	-	-	1	1		
Number of Tyr residues	11	11	11	11	11		
T _{1/2} MCO	3 min.	8 min.	24 min.	15 min.	15 min.		
$\begin{array}{c} T_{1/2} \stackrel{UV \text{ agg } (\textbf{a})}{(\text{pH 5.0})} \end{array}$	47 min.	-	-	10 min.	-		
T _{1/2} ^{UV trans (b)} (pH 5.0)	3.2 min.	-	-	3.6 min.	-		
T _{1/2} ^{UV agg} (pH 7.4)	80% remained at the end of irradiation time	-	-	45 min.	-		
$\begin{array}{c} T_{1/2}^{UV \text{ trans}} \\ \text{(pH 7.4)} \end{array}$	182 min.	-	-	7.8 min.	-		
T _{1/2} ^{MCO β-CD}	10 min.	-	-	45 min.	-		
CD MCO	β-sheet	α-helix	α-helix	α-helix	α-helix		
CD ^{UV pH 5.0}	random coil	-	-	random coil	-		
CD ^{UV pH 7.4}	β-sheet	-	-	β-sheet	-		
CD ^{MCO β-CD}	mixture of α- helix and β- sheet	-	-	α-helix and random coil	-		
$R_{\rm H}^{0 {\rm min.}}$ (nm)	1.94±0.09	1.90±0.1	1.69±0.48	1.98±0.1	1.86±0.26		
R _H ^{MCO} (nm)	heterogenic high MW aggregates > 100	heterogenic high MW aggregates > 100	Sol. olig. ^(c) 17.3±2.68	heterogenic high MW aggregates > 100	heterogenic high MW aggregates > 100		
$R_{\rm H}^{~~{\rm UV}{\rm pH}5.0}~({\rm nm})$	covalently cross-linked high MW aggregates	-	-	covalently cross-linked high MW aggregates	-		
$R_{\rm H}^{~\rm UV~pH~7.4}$ (nm)	sol. olig. 8.31±0.35	-	-	sol. olig. 13.89±0.19 27.73±5.29	-		
$R_{\rm H}^{\rm MCO\beta-CD}$ (nm)	heterogenic aggregates > 1 µM	-	-	sol. olig 4.75±0.55 28.03±6.25	-		
R _g (nm) of monomer	1.80±0.02	-	-	1.83±0.02	-		
$R_g^{\beta-CD}(nm)$	1.66±0.03	-	-	1.66±0.01	-		

^(a)agg., aggregation; ^(b)trans., transmission; ^(c)sol. olig., soluble oligomer.
4. Discussion

4.1 Motivation

The aim of this study was the identification of the key amino acids within the surface exposed methionine (Met) residues that have a significant contribution to the oxidative conversion and aggregation process of prion proteins. Histidine (His) residues were also reported to be susceptible to oxidation and are suggested to participate in the transformation process by which PrP^{C} is converted to the infectious PrP^{Sc} isoform (116, 123). However, Met is reported to be the most sensitive amino acid towards oxidation by ROS (116, 129). Oxidation of Met residues to Met-sulfoxides has a significant impact on the function, structure, assembly, and solubility of proteins (110, 111). If Met residues are impeded in the hydrophobic core of the protein molecule, they may be less or not susceptible to oxidation (119). In terms of neurodegeneration oxidized Met residues were detected in PrP^{Sc} molecules deposited in the brain (163) as well as in senile plaques consisting of amyloid- β peptide (A β) associated with Alzheimer's disease (128). PrP is considered to be an excellent target for oxidation with respect to the high number of Met residues, particularly the surface accessible ones. Most of the surface exposed Met residues of the PrP molecule are localized in the folded C-terminal domain. Moreover, the structural differences between PrP^C and PrP^{Sc} are only restricted to the folded C-terminal part of the protein, which adopts an α -helical structure in PrP^C and possesses a multimeric β -sheet structure in PrP^{Sc} (71, 129). The formation of β -sheet enriched oligomers (116) as well as of extensive aggregation of the recombinant PrP followed by precipitation (122, 129) was already attributed to the oxidation of Met residues. However, the direct correlation of the $PrP^{C} \rightarrow PrP^{Sc}$ structural conversion to a site-specific Met oxidation was not established so far. To investigate the role of the surface exposed Met residues in the oxidative induced conversion process of PrP, the C-terminal domain of mouse (mPrP120-230) and human (hPrP121-231) PrP have been cloned and recombinantly expressed.

The detailed mechanism of the autocatalytic conversion of PrP^C into the infectious PrPSc isoform is still unknown. Oxidative stress has been implicated in the prion pathogenesis (145, 146). Consequently, several in vitro oxidation systems such as H_2O_2 (122), sodium periodate (129), and metal-induced oxidation (122, 164) were applied to study the oxidative aggregation behaviour of PrP. In this study, two different methods have been used. A de novo cell free conversion assay has been established in our group to study the oxidative aggregation of PrP induced by metal catalysed oxidation (MCO). This assay mimics the physiological increase of the cellular oxidative stress (116). On the other hand, growing evidence for a connective link between cellular oxidative stress and the pathological conversion of prion protein (164) prompted us to systematically investigate the impact of UVB radiation (302 nm) on PrP at pH 7.4 and pH 5.0. The general mechanisms of UV-induced protein damage are already well established and summarized in several reviews (154, 165, 166). Oxidation of a protein structure can be directly mediated via photoionization processes subsequent to the absorption of the incident light by protein side chains. Moreover, additional indirect oxidative damage is frequently induced by free oxygen radicals and singlet oxygen $({}^{1}O_{2})$ molecules, which are formed as a result of electron and energy transfer reactions of excited state species, referred photosensitization mechanisms. Protein cross-linking, nonspecific to as formation of carbonyl groups, and ring-opening reactions as well as cleavage of covalent bonds are reported to be the usual chemical consequences of a huge variety of radical reactions primarily proceeding at the side chains of the protein structures.

An initial requirement for direct photo-oxidation is the presence of suitable chromophores. At a wavelength of 302 nm, chromophoric properties can be assigned only to the aromatic structures of Trp ($\epsilon_{302}=317 \text{ cm}^{-1} \text{ M}^{-1}$) and, to a lesser extent, of Tyr ($\epsilon_{302}=41 \text{ cm}^{-1} \text{ M}^{-1}$) and His ($\epsilon_{302}=8 \text{ cm}^{-1} \text{ M}^{-1}$) residues in purified proteins (167). Because additional exogenous chromophores were not

present, the structural changes are suggested to be a consequence of complex intramolecular radical reactions primarily depending on the amount of the chromophoric residues and on the number and localization of residues highly susceptible to oxidation, including Trp, Tyr, Phe, His, Met, and Cys residues (153, 168, 169). Owing to its intriguing properties, including unusual hydrophilicity as well as intrahelical salt bridges, helix H1 spanning residues 143 to 153 (in human PrP) has recently received attention as a candidate segment mediating PrP conversion (170, 171). Almost two-thirds of all chromophoric amino acids and half of all Met and His residues that are present in hPrP121-231 and mPrP120-230 are located within or next to helix H1 (Fig.40). Therefore, significant UV light absorption and subsequent radical reactions take place in this part of the PrP structure, characterizing this segment as the predominant target for photo-oxidation. Taking into account that the UVB light penetrates the skin up to the upper dermis (172), a biological impact of UV-light-induced PrP conversion cannot be ruled out. Even if the affected tissues are of course not the primary sites of prion pathology, they contain peripheral nerves and muscle fibres that have already been shown to be involved in the pathways by which infectious prions invade a host and spread through the organism (173). Consequently, a potential contribution of photo-oxidation induced by UV light to the formation of infectious PrP seeds that may propagate the disease along neuronal pathways has at least to be carefully considered, as long as the pathogenic events that promote the onset of sporadic forms of TSE are not identified.



Fig. 40: Sequence comparison of hPrP90-230 and mPrP89-231. Identical amino acids are highlighted in black, homologous residues in grey. Amino acids that exhibit significant chromophoric properties at a wavelength of 302 nm are shown in red. Tryptophan residues, which represent the primary positions of singlet oxygen generation within the protein structure, are additionally marked with asterisks. Amino acids that are supposed to act as primary targets for oxidative damage, e.g., histidine and methionine, are in blue. The locations of the secondary structure elements derived from the 3D models of human (PDB code 1QM0) and murine (PDB code 1XYX) prion proteins are schematically indicated above the sequences.

To investigate the impact of Met residues on the oxidative damage of PrP induced by MCO and the subsequent $\alpha \rightarrow \beta$ structural conversion, the all surface exposed Met residues (M129, M134, M154, M166, and M213) in the globular C-terminal domain of human PrP have been substituted with amino acids that are less susceptible to oxidation, such as serine (Ser) or threonine (Thr). Two additional Met residues, M205 and M206, are present in a hydrophobic cluster within the prion proteins and are conserved in all mammalian species (71). Moreover, these residues represent a part of the hydrophobic core of helix III that stabilizes its structural integrity. Substitution of Met residues at position 205 and 206 with hydrophilic amino acids such as serine and arginine has been reported to prevent the *in vivo* folding of the recombinant mutant PrP (129, 174). Therefore, only the entire surface exposed Met residues in the folded C-terminal domain of human prion protein hPrP121-230 excluding M205 and M206 have been replaced with Ser by site directed mutagenesis. Although Ser is shorter and chemically different from Met, it retains the helical propensity as well as the polarity of the sulfoxidized Met-residues (175, 176). The detailed correlation of the proposed effects to the oxidation of a specific Met residue was planned to be achieved by systematic stepwise replacement of the surface exposed Met residues of PrP. In this study the replacement started with residue Met 129 of human and mouse PrP by Thr, since Met 129 is the site of polymorphism in several species. This polymorphism affects both susceptibility to the disease and the onset of clinical symptoms, particularly for the acquired and sporadic form of human prion diseases (93, 96). Most of the vCJD as well as 80% of the sCJD cases possess Met-homozygotes (Met/Met) at position 129 (174), whereas individuals with either heterozygotes Val or Met are characterized by the resistance to the sporadic and the acquired form of prion diseases (129).

4.2 Impact of Met and His residues on the oxidative-induced aggregation of prion proteins

Prion diseases have been reported to be associated with metal-induced oxidative stress that provokes conversion and significant aggregation of the protein (123, 125, 177). Recent studies suggested that the oxidative damage of PrP is mainly mediated by Met and His residues (116, 122, 124). This theory was clearly confirmed within this study. In general, the rate of oxidative induced aggregation strongly depends on the amount of the amino acids Met and His. It was revealed that the prion proteins were structurally affected depending on (i) the applied conversion system, (ii) the pH value of the reaction buffers, and (iii) the sequence of the particular PrP construct. Two pathways of structural conversion and aggregation have been observed, finally resulting in the formation of soluble β -sheeted PrP oligomers as well as in a complete denaturation and precipitation of covalently cross-linked prion proteins.

The results of MCO-induced conversion and aggregation of the recombinant Cterminal domain of mPrP120-230 and hPrP121-231 were compared with the already investigated prion proteins mPrP89-230 and hPrP90-231 that additionally comprise a part of the unstructured N-terminal domain. Both C-

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terminal domains aggregated at significantly higher rate than mPrP89-230 and hPrP90-231. Compared to hPrP90-231 ($T_{1/2} = 30 \text{ min.}$) and mPrP89-230 ($T_{1/2} = 30 \text{ min.}$) 180 min.), the half lives of hPrP121-231 ($T_{1/2} = 3$ min.) and mPrP120-230 ($T_{1/2}$ = 15 min.) were decreased by approx. 10-fold and 12-fold, respectively. This indicates that at least for the applied assay, the N-terminal domain provides a protective effect towards oxidative-induced aggregation of PrP by MCO. This protective effect could be established via oxidation of the surface exposed Met (109/112) and His (96/111) residues in the unstructured N-terminal domain of PrP by ROS generated in the MCO assay and subsequently decreasing the oxidation of the accessible Met/His residues in the folded C-terminal domain of PrP. These results were consistent with that recently published by Nadal et al.(125). Hydroxyl radicals generated by Cu(II) coordinated to the octarepeat region (residues 58-91) of mouse PrP oxidized only His 96/111 and Met 109/112 residues close to the site of copper binding. In contrast, Met and His residues that are localized in the folded C-terminal domain were not affected. Consequently, these results also suggested that PrP(121-230) is more susceptible to oxidation than PrP(90-230). The increased aggregation rate of hPrP90-231 compared to mPrP89-230 can be ascribed to the presence of two additional Met residues at positions 109 and 112 in the human PrP sequence (Fig. 40). Along with Met, His is one of the most sensitive amino acids in terms of oxidation. The C-terminal domains hPrP121-230 and mPrP120-230 contain 7 Met residues as well as 4 and 3 His residues, respectively. Therefore, the reduced His content of mPrP120-230 explains its lower aggregation rate compared to hPrP121-231. The half life time of mPrP120-230 increased by a factor of 5 compared to that of hPrP121-231.

In this study, the structural damage of PrP molecules by UV radiation was assigned to contributions of both direct and indirect protein oxidation including photoionization mechanisms and ROS, respectively. However, the extent of the respective mechanism significantly differed depending on the applied pH value. If identical conditions have been applied during UV irradiation of mouse and human PrP, only the reaction rate is affected by the species. Two different pH values were applied during UV irradiation of the proteins, the physiological pH of 7.4 as well as pH 5.0, which represents the acidified conditions within endocytotic vesicles and lysosomes. Even if the exact subcellular structure of PrP^{Sc} formation is not clearly determined so far, previous studies have mentioned the importance of the late endocytic lysosomal compartment of infected cells for the manifestation of neurodegenerative diseases (178). At pH 5.0 significant contributions of ROS to the UV-light-induced structural damage were detected in this study in addition to the direct photo-oxidation mechanism. ROS are powerful oxidants that can not only oxidize the side chains of specific amino acids, particularly Trp, His, Tyr, Met, and Cys residues, but also can oxidize the back bone of the polypeptide chain that leads to protein fragmentation (117, 154, 166, 167). Therefore, the generation of ROS is supposed to enhance the oxidative damage of the PrP molecules. Consequently, the significant increase of the aggregation rate of mPrP120-230 compared to hPrP121-231 at pH 5.0 can be ascribed to the presence of additional Trp at position 144 in the mouse PrP sequence, which represents the primary position of ${}^{1}O_{2}$ generation within the protein structure. At pH 7.4, only contributions of a direct photo-oxidation process are indicated, without involving ROS. Consequently, the outstanding low aggregation rate of hPrP121-231 at pH 7.4 is proposed to be directly linked to the absence of Trp residues, the major chromophores at the applied wavelength (179). The presence of an additional Trp (W144) and Tyr (Y154) residue in the sequence of mPrP120-231 obviously increased the aggregation rate. This effect is supported by the additional Met residue at position 137, which has previously been implicated as species barrier amino acid, identified between mouse and human PrP aggregation (122, 180). The strong protective effect of ascorbic acid on the structural integrity of mPrP120-230 at pH 5.0 was largely attributed to its function as a competitive

quencher of the incident UV light rather than to a radical scavenging activity, because ascorbic acid strongly absorbs UV radiation (181). This is confirmed by a completely blocked transmission of the laser beam through the cuvette containing a mixture of mPrP120-230 and ascorbic acid even at the beginning of the irradiation experiments.

Following replacement of the surface exposed Met residues in the folded Cterminal domain of human PrP with Ser residues, the refolding time of vhPrP121-231 after recombinant expression was increased by a factor of 2.5 compared to that of the *wild type* form. However, investigating the secondary structure of v-hPrP121-231 by CD spectroscopy revealed a highly similar, but not identical secondary structure compared to that of the *wild type* protein. Serine, threonine, and to a lesser extent, aspartic and glutamic acid as well as their amides have been reported to affect the helical structure of proteins due to their tendency to access the surface of proteins (182). Consequently, the slight decrease in the α-helical content of v-hPrP121-231 revealed by CD spectroscopy compared to the *wild type* form can be assigned to the polarity of the Ser residues. The high stability of v-hPrP121-231 towards the oxidative-induced aggregation by MCO is directly related to the mutated surface exposed Met residues. The halfe life of the variant protein ($T_{1/2} = 24$ min.) was increased by approx. 8-fold compared to that of the *wild type* protein ($T_{1/2} = 3$ min.). Our results are comparable with that of Wolschner et al. (129). This group showed that the replacement of the entire Met residues of recombinant full length PrP (residues 23-231) by the non oxidizable Met-analogue norleucine (Nle) exhibited significant resistance against oxidation and subsequent conversion by periodate. The persistent ability of v-hPrP121-231 to aggregate following oxidation even after mutation of all surface exposed Met residues reflects the importance of the other sensitive amino acids such as His and Tyr for the conversion process of PrP, which cannot be neglected. As a result it can be concluded that v-hPrP121-231 represents a reliable model that confirms the proposed important role of the surface exposed Met residues in the oxidative damage of PrP.

The presence of Met 129 polymorphism in human PrP has been linked to the late onset of sCJD compared to patients exhibiting valine at position 129 of the PrP sequence by decreasing the conversion rate mediated by ROS under oxidative stress (126, 183). Conversely, Met 129 has been mentioned to be located in a specific region of PrP that mediates its transformation into the infectious PrPSc isoform (122, 163). In this study it was shown that the individual substitution of Met 129 with Thr in the C-terminal domain of human PrP (hPrP121-231 M129T) resulted in a significant gain of stability of the variant protein against oxidative-induced aggregation by MCO. The half life of hPrP121-231 M129T ($T_{1/2} = 8$ min.) was 3 times higher than that of the *wild* type form ($T_{1/2} = 3$ min.). The increased aggregation rate of hPrP121-231 M129T compared to that of v-hPrP121-231 is clearly correlated to the presence of four additional surface exposed Met residues (M134, M154, M166, and M213) found in the sequence of hPrP121-231 M129T. These results lead to the conclusion that Met 129 represents one of the hot spots involved in the sporadic conversion of cellular PrP^C. Although mouse and human PrP share about 90% sequence identity, no difference in the aggregation behaviour of mPrP120-230 M129T and its wild type mPrP120-230 has been observed. One possible explanation could be that mouse PrP is more resistant toward individual Met substitution oxidation due to the low His content of mPrP120-230 (3 His) than hPrP121-231 (4 His) that reduces the susceptibility to oxidative-induced aggregation by MCO.

4.3 Structural consequences of oxidative-induced aggregation of PrP by MCO and UV radiation

The aggregates of mouse and human (*wild type* and variant) PrP formed during oxidative induced conversion have been characterized to identify the type of interactions as well as the structural changes that occur during the aggregation process. Previous studies reported that the reduction of the disulfide bridge induces the $PrP^{C} \rightarrow PrP^{Sc}$ conversion (58, 78, 184). In contrast, PrP^{Sc} has been reported to contain an intact intramolecular disulfide bond (185). Non-reducing SDS-PAGE of hPrP121-231 aggregates formed by MCO confirmed an increase in the intensity of the dimeric state together with the formation of high molecular weight aggregates with incubation time. Under reducing conditions the dimer and the high molecular weight aggregates were consistently dissociated into the monomeric form of PrP. Consequently, the results of this study strengthen the theory that a molecular rearrangement of the disulfide bridge from an intramolecular to an intermolecular state takes place during the structural conversion (92).

Dityrosine has been detected in a wide variety of oxidatively modified proteins such as α-synuclein (186) and oxyhemoglobin (187) resulting in an increase of their stability. The C-terminal domain of both mouse and human PrP contain 11 Tyr residues. Since the observed *v*-hPrP121-231 dimer did not dissociate under reducing conditions, it is suggested that the *v*-hPrP121-231 aggregates are stabilized via covalent cross-linking interactions by dityrosine. Most likely the decreased number of Met residues in *v*-hPrP121-231 resulted in an enhancement of the oxidative damage of other amino acids sensitive to oxidation, such as His and Tyr. The dimer formed by both hPrP121-231 M129T and mPrP120-230 M129T is supposed to be stabilized by a similar pathway. Covalently cross linked aggregates characterized by high molecular weights (>150 kDa) have also been detected after UV irradiation of mPrP120-230 and hPrP121-231 at pH 5.0.

The formation of these aggregates was attributed to the enhanced oxidative damage of protein by ROS in addition to direct photo-oxidation.

The conversion of the recombinant prion protein in the absence of PrP^{Sc} in a cell free conversion system is closely correlated with the sporadic form of prion diseases rather than with the acquired form (60). However, the *in vitro* conversion of the recombinant PrP did not result in infectious molecules so far. Depending on the experimental conditions as well as on the cofactors supplemented in the conversion reactions, an increase in proteinase K (PK) resistance or β -sheet secondary structures was observed (45). Here it is shown that hPrP121-231 aggregates formed by MCO possessed a significantly increased resistance to PK compared to the monomeric form, which is completely sensitive to PK digestion. Moreover, CD analysis of the soluble fraction of hPrP121-231 revealed an increase in β -sheet conformation. Consequently, the MCO induced oxidative conversion of recombinant PrP mimics the sporadic conversion of human PrP in the applied assay.

Partially unfolded structures are believed to represent monomeric precursor states of amyloidogenic proteins that initiate the oligomerization process (16). Recently, intermediate states were indeed detected on the pathway of folding and misfolding of prion proteins, confirming a three-state model (188-190). Following this emerging theory the folding pathways that progressed during MCO and photo-oxidation of PrP are proposed to be strongly determined by the population and the stability of the associated intermediate states. Specific conversion and stepwise formation of the detected soluble oligomers is favoured only if the extent of structural damage of prion proteins independent of the applied system closely resembles the partially unfolded state of the dedicated precursor. Otherwise the introduced destabilization leads to the completely unfolded state of PrP, as previously reported for PrP conversion in the presence of denaturants (190). We show that MCO of mPrP120-230 and hPrP121-231 at pH 5.0 resulted in complete denaturation without formation of soluble

oligomeric intermediates. This finding can be attributed to the absence of the aforementioned protective effect of the unstructured N-terminal domain encompasses Met (109/112) and His (96/111) residues. This results in an enhanced oxidative damage of the surface exposed Met and His residues in the folded C-terminal domain of both proteins by ROS generated during the MCO assay. However, the detected β -sheet CD spectroscopy signal in the soluble fraction of hPrP121-231 gives a slight indication for the presence of small amounts of soluble β -oligomers. These oligomers could not be detected by DLS due to their low concentrations.

At pH 5.0 the structural damage of PrP by UV radiation was found to be enhanced in addition to the direct photo-oxidation mechanism due to the contribution of ROS. These reactive species are able to oxidize specific amino acid residues in the peptide chain of PrP molecule like Trp, His, Tyr, Met, and Cys (154, 166, 167). In contrast, all His residues are positively charged at slightly acidic pH values due to protonation, which completely abolished the UV absorption at a wavelength of 302 nm and therefore prevented direct photooxidation reactions at His residues. Moreover, an increased conformational mobility of the α -helical structure of PrP^C leading to significant structural rearrangements was attributed to the protonation of His residues (191, 192, 146), whereas recent studies demonstrated an enhanced stability of the corresponding intermediate states at acidic pH values (188-190). The degree of each stabilizing and destabilizing contribution potentially affecting the UV-light-induced aggregation of PrP remains to be elucidated. However, the extremely fast denaturation and precipitation of mPrP120-230 and hPrP121-231 at pH 5.0 without formation of soluble oligomeric intermediates can be explained in this context by a combination of enhanced oxidative damage and decreased conformational stability of the PrPs. For mPrP89-230 and hPrP90-231, the stabilization of the intermediate states and other potential stabilization mechanisms are supposed to prevail over the enhanced oxidative damage,

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because a pathway switch to specific PrP conversion was observed although the amount of chromophoric and oxidation-sensitive residues is increased compared to the proteins comprising only the C-terminal domain. The identification of the oxidation products is required to understand the molecular mechanisms of UV-induced PrP conversion in detail.

The formation of soluble β -oligomeric intermediate of hPrP121-230 (R_H = 8.31±0.35 nm) and mPrP120-230 (R_H = 13.89±0.19 nm and 27.51±4.49 nm) at pH 7.4 can be assigned to the only contribution of the direct photo oxidation and subsequent decrease of the structural damage of both proteins, which is closely resembles the stabilization state required to initiate the oligomerization process. One additional factor is the absence of Trp residue in the sequence of hPrP121-231, a major chromophore at the applied wavelength of 302 nm (171). The presence of one Trp in the sequence of mPrP120-230 results in an increase of the oligomerization rate.

For UV irradiation of hPrP90-231 and mPrP89-230 at pH 7.4, a pathway switch from specific PrP conversion to protein denaturation was observed, mainly attributed to the presence of a further Trp residue at positions 99 and 89, respectively. Consequently, it can be suggested that the degree of structural damage exceeded the level required for stabilization of the intermediate states associated with oligomerization, resulting in a rapid denaturation of the protein. These data conclude that photo-oxidized PrPs are able to oligomerize into three spherical species, with associated hydrodynamic radii of $R_H \sim 7.5$, ~14, and ~27.5 nm. A sequential formation is indicated. Regarding size, the oligomers characterized in this study share a remarkably high degree of identity with oligomeric species formed after structural destabilization of prion proteins by heat (57), denaturants (109), and metal induced oxidation (116). Consequently, PrP misfolding seems to be linked to well-defined pathways resulting in oligomerization and/or fibrillization, if the required partially unfolded state is

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formed, largely independent of the triggering method. Otherwise the structural impairment results in complete denaturation of the protein.

The formation of a soluble α -oligomeric *v*-hPrP121-231 intermediate at pH 5.0 and subsequent inhibition of $\alpha \rightarrow \beta$ structural conversion can directly be assigned to the absence of surface accessible Met residues as well as to the reduced oxidatie damage of the *v*-hPrP121-231 by MCO. Conversely, the aggregation of hPrP121-231 M129T and mPrP120-230 M129T induced by MCO at pH 5.0 without formation of soluble oligomeric intermediates can be related to the enhanced oxidative damage due to presence of four additional Met residues in the sequence of both proteins. Moreover, the inhibition of $\alpha \rightarrow \beta$ transition for hPrP121-231 M129T revealed by CD spectroscopy confirmed the significant impact of Met 129 to the oxidative-induced conversion of human PrP by MCO.

4.4. β-cyclodextrin decreases the MCO-induced aggregation rate of PrP by complexation of Cu²⁺

On searching for an effective therapy against TSEs, β -cyclodextrin (β -CD) gained attention in the field of anti-prion compounds. β -CD was reported to clear the infectious PrP^{Sc} isoform from scrapie infected neuroblastoma (ScN2a) cell cultures (138). Moreover, β -CD has the ability to reduce the neurotoxic effects of the amyloid- β (A β) protein (residues 1-40) associated with Alzheimer's disease in cell cultures (139). An NMR study showed that β -CD interacts with A β (1-40) via encapsulation of the Phe residues at position 19 and 20 in its hydrophobic cavity (193). So far the influence of β -CD on the *in vitro* aggregation behaviour of PrP was not investigated. Therefore, the effect of β -CD on the oxidative damage as well as on the structural conversion of the C-terminal domain of both mouse and human PrP induced by MCO was analyzed. The presence of β -CD did not affect the native conformation of PrP. However, the aggregation rate of both mPrP120-230 and hPrP121-231 was significantly reduced by a factor of 3. This effect was attributed to a decreased rate of $\alpha \rightarrow \beta$

transition, which implies two possible mechanisms of protein stabilization (i) direct interaction or (ii) reduction of the MCO-induced oxidation. A compact structure of PrP in the presence of β -CD observed by SAXS measurements initially supported the first mechanism. However, SPR data unambiguously confirmed that no significant binding affinity of β -CD was detected to hPrP121-231. While α -cyclodextrin (α -CD) and cellobiose were used to determine the binding specificity of β -CD to human PrP, no specific binding has also been observed as well.

It is well established that β -CD binds divalent metal ions like iron, cadmium, manganese, calcium, magnesium, and also copper (194). During MCO assay free Cu²⁺-ions are formed in solution, which mediate the generation of ROS by Fenton's reaction. Complexation of Cu^{2+} -ions in a redox-inactive state by β -CD disable this mechanism, resulting in a significantly reduced amount of oxidative protein damage. Since a direct interaction was clearly ruled out by SPR data, this proposed mechanism explains the stabilization effect of β -CD. It has been reported that copper ions facilitate the refolding of the partially denatured PrP^{Sc} (195). Copper as well as metal chelations were reported to play a significant role in altering the protease cleavage pattern of PrP^{Sc} isoform (196), suggesting that metal ions may direct PrP into a certain conformation that promote $PrP^{C} \rightarrow PrP^{Sc}$ conversion. Moreover, the injection of scrapie infected mice with the copper chelator D-(-)-penicillamine (D-PEN) delayed the onset of prion disease by about 11 days (197). B-CD was mentioned to be able to pass the blood brain barrier (BBB) up to 4 mM (138). Taking all together, it can be proposed that the clearance of copper by β -CD from scrapie infected brain may reduce the rate of PrP^{Sc} formation and can cause subsequent prolongation of the incubation time of the prion disease.

For mPrP120-230 the decrease in the oxidative damage resulted in a significant change in the aggregation pathway. Stable and soluble oligomeric intermediates are formed by MCO in the presence of β -CD, whereas mPrP120-230 was

directed into denaturation and precipitation if β -CD is absent. A comparable pathway shift was not observed for hPrP121-231, which contains one additional His residue that increases the susceptibility to oxidative damage.

4.5. Conclusions

Within this study, the direct link between oxidative stress and PrP conversion depending on different specific pathways was analyzed and proven. Two independent pathways were observed: (i) complete unfolding of the protein structure associated with rapid precipitation and (ii) specific structural conversion into distinct β -soluble oligomers. The choice of the pathway was directly attributed to the chromophoric properties of PrP species and the susceptibility to oxidation. Replacement of all surface exposed Met residues of hPrP121-231 enhanced the resistance of the variant protein towards oxidativeinduced aggregation by MCO. The initial detailed investigation about the contribution of the individual Met residues assigned a significant impact to Met 129. These results strongly supported the hypothesis that oxidative modification of the surface exposed Met residues represents the initial event for the sporadic $PrP^{C} \rightarrow PrP^{Sc}$ conversion. The inhibitory effect of β -CD on the oxidative inducedaggregation of mouse and human PrP is rather due to the chelation of copper ions generated by MCO than to a direct interaction with PrP. Although a close correlation between amyloidogenesis and neurotoxicity has been reported several times in the past (198-200), the soluble oligomers detected in this study pave the way in terms of the ongoing search to understand the the molecular mechanism of neurotoxicity in TSEs. The highest specific infectivity in TSEinfected hamster brains was recently correlated to fractions solely containing small spherical oligomers (198). Therefore, the investigations performed in terms of the summarized thesis provide new insights to understand the mechanism of prion conversion as well as to develop new lead structures for TSEs drugs.

6. References

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7. Hazardous Materials (Gefahrstoffe)

Compound (Verbindung) Acrylamide (Acrylamid)	Symbols of hazardous materials (Gefahrstoffsymbole) T	R-statements (R-Sätze) 45-46-20/21- 25-36/38-43- 48/23/24/25-62	S-statements (S-Sätze) 53-45
Ampicillin	Xn	36/37/38-42/43	22-24-26-37
APS	O, Xn	8-22- 36/37/38/- 42/43	22-24-26-37
DMSO	Xi	36/38	26
DTT	Xn, Xi	22-36/38	36/37/39
EDTA-disodium	Xn	22	
Acetic acid (Essigsäure)	С	10-35	23.2-26-45
Ethanol	F	11	7-16
EtBr	T+	22-26- 36/37/38-40	26-28.2-36/37- 45
HCL	С	34-37	26-36/37/39-45
Imidazole	F, Xn, N	11-38-48/20- 51/53-62-65-67	9-16-29-33- 36/37/-61-62
Methanol	Т	61	26-36/37-39-45
NaOH	F, T	11-23/24/25- 39/23/24/25	7-16-36/37-45
Ni-NTA Agarose	0, C	8-35	8-27-39-45
NaCl	F, Xi	11-36-67	7-16-24/25-26
2-Propanol	Т	24/25-34	28.6-45

SDS	С	34-37	26-36/37/39-45
TEMED	Xn	22-36/38	22-24/25
Tetracyclin	Xi	36/37/38	26-36
Tris	F, C	11-20/21/22-35	3-16-26-29-
			36/37/39-45

7.1 Symbols of hazardous materials (Gefahrstoffsymbole)

- Е Explosive (Explosionsgefährlich) С Caustic (ätzend) F+ Extremely flammable (hochentzündlich) Irritant (reizend) Xi Oxidizing (brandfördernd) 0 Highly flammable (leichtentzündlich) F Toxic (giftig) Т Very toxic (sehr giftig) T+ Harmful (gesundheitsschädlich) Xn
- N Hazardous to the environment (umweltgefährlich)

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	C, Schulz B, et al. (2009) UV-light-induced conversion and		
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Erklärung über frühere Promotionsversuche

Hiermit erkläre ich, dass vorher keine weiteren Promotionsversuche unternommen worden sind, oder an einer anderen Stelle vorgelegt wurden.

Hamburg, den

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-Unterschrift-

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, dass die vorliegende Dissertationsschrift selbstständig und allein von mir unter den angegebenen Hilfsmitteln angefertigt wurde.

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