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The recombinant murine prion protein and its carboxy-terminal domain
biophysical and structural characterization

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The recombinant murine prion protein and its carboxy-terminal domain: Biophysical and structural characterization

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presented by
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Meinen Eltern und
meiner Schwester Isabel
Publications on which this thesis is based:


Reviews and further publications:


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Abstract

Transmissible spongiform encephalopathies (TSE) such as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and the Creutzfeldt-Jakob disease (CJD) in humans are caused by an unique infectious agent, the prion. According to the "protein-only" hypothesis, the prion is believed to consist mostly, if not entirely, of an abnormal form, PrPSc, of the benign, host-encoded cellular prion protein, PrPC. PrPC and PrPSc seem to be identical in their covalent structures, but differ in their three-dimensional structures. While PrPC is a monomer and predominantly consists of α-helical secondary structure, PrPSc is an insoluble oligomer with an increased β-sheet content when compared with PrPC.

A prerequisite for elucidating the molecular mechanisms underlying the conversion of PrPC into PrPSc is the knowledge of the folding, the thermodynamic stability and the three-dimensional structures of PrPC and PrPSc. Because of the low solubility of PrPSc and the low yields of PrPC obtained after purification from its natural source such studies have been fraught with difficulties in the past.

In the first part of this thesis, an efficient system for the functional secretory expression of an N-terminally truncated segment of the mouse prion protein in the periplasm of Escherichia coli was developed. This segment comprises the C-terminal 111 residues of mouse PrPC and was designated mPrP(121–231). The exact size of this domain was deduced from the resistance of residues 121–231 against degradation by periplasmic E. coli proteases. mPrP(121–231) is a soluble protein and could be purified in large quantities from periplasmic E. coli extracts by conventional chromatographic techniques. First biochemical and biophysical studies of purified, soluble mPrP(121–231) revealed that the protein is a monomer which is rich in α-helical secondary structure. Therefore, the recombinant protein has the same characteristics as natural PrPC. Unfolding and refolding experiments on this protein demonstrated that mPrP(121–231) folds cooperative and reversible, and represents an autonomously folding domain.

In the second part of this thesis, the first expression and purification system for the recombinant, full-length mouse prion protein, mPrP(23–231) was established. Circular dichroism and fluorescence studies on mPrP(23–231) in comparison with mPrP(121–231) indicated that the amino-terminal
polypeptide segment 23–120 does not adopt a defined three-dimensional structure.

In collaboration with R. Riek, Dr. G. Wider and Dr. M. Billeter in the group of Prof. Dr. Wüthrich the three-dimensional structures of \( m\text{PrP}(121-231) \) and \( m\text{PrP}(23-231) \) were determined by nuclear magnetic resonance (NMR) spectroscopy in solution. For this purpose, the production of \(^{15}\text{N}-\) and \(^{13}\text{C}-\) labeled protein samples was established. In contrast to previous theoretical predictions of the structure of PrPC, the NMR structure of \( m\text{PrP}(121-231) \) has a novel folding motif containing three \( \alpha \)-helices and a two-stranded, antiparallel \( \beta \)-sheet. The structural analysis of \( m\text{PrP}(121-231) \) allowed the identification of possible sites in PrPC which may be important for the species barrier of TSE transmission and predictions on the effects of inherited amino acid exchanges in human PrP on the stability of PrPC. The NMR-studies on \( m\text{PrP}(23-231) \) showed that the globular fold of the C-terminal domain \( m\text{PrP}(121-231) \) is retained in the complete mouse prion protein and that the N-terminal polypeptide segment 23–120 is indeed flexibly disordered.

Denaturant-induced equilibrium transitions of \( m\text{PrP}(23-231) \) and \( m\text{PrP}(121-231) \) demonstrated that folding of both proteins is fully reversible. The thermodynamic stability of both proteins decreases with lowered pH. Specifically, \( m\text{PrP}(121-231) \) forms an acid-induced equilibrium unfolding intermediate with increased \( \beta \)-sheet content at pH 4.0. This intermediate is populated to 0.2% in the absence of denaturants. As it shares structural features with PrP\text{Sc}, the intermediate may resemble a monomeric precursor of PrP\text{Sc}, which may also be formed at acidic pH \textit{in vivo} during endocytosis of PrPC. Unfolding studies on \( m\text{PrP}(23-231) \) also indicated that \( m\text{PrP}(23-231) \) has a slightly lower thermodynamic stability at pH 7.0 compared to \( m\text{PrP}(121-231) \), which may arise from interactions between residues of the N- and C-terminal part of \( m\text{PrP}(23-231) \) in the unfolded state. This could also explain why the acid-induced equilibrium unfolding intermediate for \( m\text{PrP}(121-231) \) is not significantly populated in the full-length prion protein.

Overall, the results obtained in this thesis should contribute to a better understanding of prion propagation and provide the basis for the rational design of future \textit{in vitro} and \textit{in vivo} experiments on prion proteins and prion diseases.
Zusammenfassung

Übertragbare spongiforme Enzephalopathien (TSE) wie Scrapie in Schafen, die bovine spongiforme Enzephalopathie (BSE) in Rindern und die Creutzfeldt-Jakob Erkrankung im Menschen werden durch einen neuartigen Erreger, das Prion, hervorgerufen. Entsprechend der "Nur-Eiweiss"-Hypothese wird angenommen, dass das Prion weitestgehend, wenn nicht sogar ausschliesslich aus einer abnormalen Form, PrPSc, des gutartigen, wirtskodierten zellulären Prionproteins, PrPC, besteht. PrPC und PrPSc scheinen in ihrer kovalenten Struktur identisch zu sein, sich jedoch in ihrer dreidimensionalen Struktur voneinander zu unterscheiden. Im Gegensatz zu PrPC, das ein Monomer ist und hauptsächlich aus α-Helices besteht, stellt PrPSc ein unlösliches Oligomer dar, das im Vergleich zu PrPC einen erhöhten β-Faltblattanteil aufweist.

Eine der wichtigsten Vorraussetzungen für die Aufklärung der molekularen Mechanismen, die der Umwandlung von PrPC in PrPSc zugrundeliegen, ist die Kenntnis des Faltungsverhaltens, der thermodynamischen Stabilität und der dreidimensionalen Struktur von PrPC und PrPSc. Aufgrund der geringen Löslichkeit von PrPSc einerseits und der geringen Verfügbarkeit von natürlichem PrPC andererseits waren physikalische und strukturelle Untersuchungen an PrPC bis zum Beginn dieser Arbeit grösstenteils unmöglich.

Im ersten Teil dieser Arbeit wurde deshalb ein effizientes System für die funktionelle, sekretorische Expression eines N-terminal verkürzten Segments des Mausprionproteins entwickelt. Dieses Segment umfasst die C-terminalen 111 Reste des gesamten, zellulären Mausprionproteins und wurde als mPrP(121–231) bezeichnet. Die exakte Größe dieser Domäne wurde aus der Beobachtung abgeleitet, dass die Reste 121–231 vor dem Abbau durch periplasmatische E. coli Proteasen geschützt sind. mPrP(121–231) ist ein lösliches Protein, das in grossen Mengen aus periplasmatischen E. coli Extrakan mittels konventioneller Methoden gereinigt werden kann. Erste biochemische und biophysikalische Studien ergaben, dass das rekombinante Protein ein Monomer und reich an α-helikaler Sekundärstruktur ist und somit die selben Charakteristiken aufweist wie natürliches PrPC. Entfaltungs- und Rückfaltungsexperimente zeigten, dass mPrP(121–231) reversibel und kooperativ faltet und eine autonome Faltungseinheit bildet.

Im zweiten Teil dieser Arbeit wurde eine Expressions- und Reinigungsmethode für das rekombinante Vollängenprionprotein der Maus,
mPrP(23–231), etabliert. Zirkulardichroismus- und Fluoreszenzmessungen deuten darauf hin, dass das amino-terminale Polypeptidsegment 23–120 im Vergleich zu mPrP(121–231) keine definierte dreidimensionale Struktur annimmt.

In Kollaboration mit R. Riek, Dr. G. Wider und Dr. M. Billeter in der Gruppe von Prof. Dr. Wüthrich wurden die dreidimensionalen Strukturen von mPrP(121–231) und mPrP(23–231) mittels kernmagnetischer Resonanzspektroskopie (NMR) in Lösung bestimmt. Zu diesem Zweck wurden $^{15}$N- und $^{13}$C-markierte Proteinproben hergestellt. Im Gegensatz zu früheren theoretischen Strukturvorhersagen für PrP^C weist mPrP(121–231) ein neues Falungsmotif auf, das aus drei $\alpha$-Helices und einem zweistängigen, antiparallelen $\beta$-Faltblatt besteht. Die strukturelle Analyse von mPrP(121–231) erlaubte sowohl die Identifizierung von möglichen Stellen in PrP^C, die für die Speziesbarriere bei TSE-Übertragungen wichtig sein könnten, als auch Vorhersagen über die Auswirkung von vererblichen Aminosaureaustauschen im menschlichen Prionprotein auf die Stabilität von PrP^C. Die NMR-Struktur von mPrP(23–231) zeigte, dass die globuläre Faltung der C-terminalen Domäne im kompletten Mausprionprotein erhalten bleibt, und dass das N-terminale Polypeptidsegment 23–120 in Lösung tatsächlich flexibel und ungeordnet ist.

Anhand von denaturierungsmittelinduzierten Gleichgewichtsübergängen wurde gezeigt, dass mPrP(23–231) and mPrP(121–231) reversibel falten, und dass die thermodynamische Stabilität beider Proteine mit verringertem pH-Wert abnimmt. mPrP(121–231) bildet bei pH 4.0 ein säure-induziertes Gleichgewichtsentfaltungsintermediat mit einem erhöhten $\beta$-Faltblattanteil. Dieses Intermediat ist auch in Abwesenheit von Denaturierungsmitteln zu 0.2% populiert. Da es strukturelle Ähnlichkeiten zu PrP$^\Sc$ aufweist, könnte es einen monomeren Vorläufer von PrP$^\Sc$ darstellen, der auch in vivo während der Endozytose von PrP$^C$ bei saurem pH auftreten könnte. Wie die Entfaltungsstudien ferner zeigen, besitzt mPrP(23–231) eine geringfügig niedrigere thermodynamische Stabilität als mPrP(121–231), die möglicherweise auf Wechselwirkungen zwischen Resten des N- und C-terminalen Teils von mPrP(23–231) im entfalteten Zustand zurückzuführen ist. Dies könnte ebenfalls erklären, warum das säureinduzierte Gleichgewichtsintermediat für mPrP(23–231) nicht so signifikant populiert wird.

1. Introduction

Prions are a novel class of pathogens that cause a series of fatal neurodegenerative diseases in animals and humans (Prusiner, 1989; Prusiner, 1991). The most common form is scrapie found in sheep and goats, which was first discovered in Great Britain 250 years ago. Infected animals lose their coordinations and, in some cases, they feel an intense itch, which led them to scrape off their wool, hence the name "scrapie". During the last years, a new prion disease, designated as bovine spongiform encephalopathy (BSE) or mad cow disease, has moved into the center of public interest. Since the disease was diagnosed in 1986 (Wells et al., 1987), more than 160,000 cattle have died of BSE in Great Britain (Anderson et al., 1996). The source of the epidemic was found in food supplements, presumably derived from scrapie-contaminated sheep dry food and later from cattle offal (Wilesmith et al., 1992). It is, however, also possible that BSE originated as a sporadic disease in cattle and was then spread by contaminated cattle offal (Weissmann, 1996). Since the use of animal-derived feed supplements was banned in 1989, the epidemic in Great Britain has reached its peak in 1992 with 36,681 cases for this year (Skegg, 1996) and thereafter it has strongly declined to 120 cases in March 1997 (Weissmann and Aguzzi, 1997).

Prion diseases are also known in humans and are unique in that they appear as sporadic, inherited or infectious illnesses (for review see Prusiner, 1995; Prusiner 1997; Weissmann et al., 1996) (Tab. 1). 10–15% of all human prion diseases are inherited and comprise the familial Creutzfeldt-Jakob disease (CJD), the Gerstmann-Sträussler-Scheinker syndrome (GSS) and the recently discovered fatal familial insomnia (FFI) (Goldfarb et al., 1992).
## Introduction

<table>
<thead>
<tr>
<th>Disease</th>
<th>Typical symptoms</th>
<th>Route of acquisition</th>
<th>Distribution</th>
<th>Span of overt illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuru (strongly declined since 1958)</td>
<td>Loss of coordination, often followed by dementia</td>
<td>Infection (probably through cannibalism)</td>
<td>Known only in the highlands of Papua New Guinea</td>
<td>Three months to one year</td>
</tr>
<tr>
<td>Creutzfeldt-Jakob disease (CJD)</td>
<td>Dementia, followed by loss of coordination, although sometimes the sequence is reversed</td>
<td>Sporadic occurrence, no mutation in the gene coding for PrP in 85–90% of all cases</td>
<td>Sporadic form: 1 person per million worldwide</td>
<td>Typically about one year; range: one month to more than 10 years</td>
</tr>
<tr>
<td></td>
<td>Typical age of onset: 55–70 years</td>
<td>Inheritance of a mutation in the gene coding for PrP</td>
<td>Inherited form: some 100 families have been identified worldwide</td>
<td>Inherited form: about 80 cases have been identified</td>
</tr>
<tr>
<td>New variant Creutzfeldt-Jakob disease (vCJD)</td>
<td>Behavioral changes, ataxia, dysesthesias</td>
<td>Possibly by transmission of BSE to humans</td>
<td>20 cases have been identified</td>
<td>Typically about one to two years</td>
</tr>
<tr>
<td></td>
<td>Typical age of onset: 19–39 years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gerstmann-Sträussler-Scheinker disease (GSS)</td>
<td>Loss of coordination, often followed by dementia</td>
<td>Inheritance of a mutation in the PrP gene</td>
<td>Some 50 extended families have been identified</td>
<td>Typically about two to six years</td>
</tr>
<tr>
<td>Fatal familial insomnia (FFI)</td>
<td>Trouble sleeping and disturbance of autonomic nervous system, followed by insomnia and dementia</td>
<td>Inheritance of a mutation in the PrP gene</td>
<td>Nine extended families have been identified</td>
<td>Typically about one year</td>
</tr>
</tbody>
</table>

Table 1: Human prion diseases (cf. Prusiner, 1995; Weissmann and Aguzzi, 1997).
All these inherited diseases are fatal and have been associated with mutations in the prion-protein gene (PRNP) (Prusiner, 1993; Parchi and Gambetti, 1995). Acquired prion diseases include Kuru (Gajdusek and Zigas, 1959; Hadlow, 1959), which was spread amongst the Fore tribe of Papua New Guinea, and iatrogenic Creutzfeldt-Jakob disease (CJD), which has apparently been transmitted e.g. by corneal transplantation, use of contaminated surgical instruments or injection of human growth hormone (see DeArmond and Prusiner, 1996).

However, most cases of human prion diseases occur as sporadic CJD, striking one person in a million per year worldwide, with a typical onset of disease at an age between 55 and 70 years (Weissmann and Aguzzi, 1997). Recently, a new form of human prion disease was observed in Great Britain, affecting also young people with a pathology strongly reminiscent of BSE (Collinge et al., 1996). It is likely that this disease, which is designated as new variant CJD (vCJD), is transmitted to humans from cattle through infected beef (Chazot et al., 1996; Will et al., 1996).

1.1 Nature of the prion agent and the "protein-only" hypothesis

The nature of the transmissible agent of prion-diseases, the prion, is hitherto unique (Weissmann, 1996; Prusiner, 1996; Horwich and Weissman, 1997). The infectious agent differs from any known bacteria, virus or viroid. Early investigations already suggested that the prion might be devoid of any nucleic acid, as it is extremely resistant against e.g. ultraviolet and ionizing radiation or chemical treatments that modify or destroy nucleic acids (Alper et al., 1967; Diener et al., 1982; Gabizon et al., 1987; Gabizon et al., 1988). Experiments to
prove the participation of a nucleic acid in the replication process and survival of the agent have so far been unsuccessful whereas procedures which normally denature or degrade proteins reduced infectivity (Gabizon and Prusiner, 1990). The first purification of the prion agent by Prusiner and colleagues, who obtained a preparation which essentially consisted of a single protein, indicated that the prion is a self-propagating infectious protein (Prusiner et al., 1982). In 1985, the group of C. Weissmann discovered by cloning the corresponding gene that the predominant protein contained in prion preparations is a host-encoded protein (Oesch et al., 1995). Because of these findings the unusual agent was termed "prion", derived from "proteinous infectious particle" (Prusiner, 1982), and the "protein-only" hypothesis first described in 1967 (Alper, 1967; Griffith, 1967) was re-established by Prusiner in 1982. Despite many well justified concerns (Chesebro, 1998), this hypothesis is to date the most widely accepted proposal to explain the nature of the infectious agent. The "protein-only" hypothesis proposes that PrP\textsuperscript{Sc}, the "scrapie" form of the prion protein, is identical in its covalent structure with its cellular isoform, designated as PrP\textsuperscript{C}, but differs from PrP\textsuperscript{Sc} in its three-dimensional structure and is capable to propagate by imposing its fold on PrP\textsuperscript{C}.

The "protein-only" hypothesis is supported by findings such as that PrP knockout mice are resistant to scrapie, that transgenic mice overexpressing PrP\textsuperscript{C} are especially susceptible to scrapie (Büeler et al., 1993), and that inherited human prion diseases are linked with point mutations in the gene coding for the prion protein (Prusiner, 1993; Parchi and Gambetti, 1995). However, the crucial experiment to prove the "protein-only" hypothesis, i.e. the conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Sc} \textit{in vitro} by inoculation with catalytic amounts of PrP\textsuperscript{Sc} has so far been unsuccessful. In this context, an important recent finding
was that transmission of human prions to transgenic mice expressing human PrP is not possible. Mice expressing a chimeric human/mouse PrP are susceptible to human prions (Telling et al., 1995), indicating that besides PrP<sub>C</sub> and PrP<sub>Sc</sub> a third component participates in the formation of PrP<sub>Sc</sub>. This component was assumed to be a species-specific host protein and designated protein "X" (Telling et al., 1995).

### 1.2 Properties of PrP<sub>C</sub> and PrP<sub>Sc</sub>

The mammalian prion protein is a glycoprotein consisting of 209 amino acids (residues 23 to 231, numbering according to the human prion protein (Westaway et al., 1987)) (Fig. 1). It includes five tandem repeats of 8 amino acids near the N-terminus ("octarepeats") and a long hydrophobic segment in the middle of the protein (residues 118–133) that is most conserved in the known prion protein sequences (Stahl et al., 1990; Come et al., 1993).

**Figure 1:** Schematic presentation of the posttranslational modifications of the cellular prion protein (amino acids 23–231) and predicted secondary structure elements (helix 1 to 4). The five octapeptide repetitions within the segment 51–91 are depicted as hatched rectangles. Posttranslational modifications of the natural PrP<sub>C</sub> are the two glycosylations at Asn-181 and Asn-197, the single disulfide bridge between Cys-179 and Cys-214, and the glycosyl-inositol-phospholipid anchor at the C-terminal Ser-231.
Overall, the known primary structures of mammalian prion proteins are strikingly similar and pairs of sequences are more than 90% identical (Schätzl et al., 1995). Furthermore, the mature form of the prion protein possesses a single disulfide bridge between residues 179 and 214, two N-glycosylation sites at Asn-181 and Asn-197 (Bolton et al., 1985; Meyer et al., 1986; Endo et al., 1989) and is attached to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor at its carboxy-terminal serine 231 (Stahl et al., 1987).

The glycoprotein PrPc is predominantly localized on the surface of neurons but also present in many other tissues. Studies with PrP-knock out mice suggest that PrPc seems to be necessary for normal synaptic function (Collinge et al., 1994), long-term survival of Purkinje neurons (Sakaguchi et al., 1996) and the regulation of circadian rhythms and sleep (Tobler et al., 1996). However, observations with knock out mice derived from other strains led partially to controversial results (Lledo et al., 1996). Despite the nearly normal phenotypes of PrP-deficient mice, it cannot be excluded that PrPc might have important functions in wild type mice, since knockout mice could possibly adapt to the lack of PrP during early development (Aguzzi and Weissmann, 1997).

Very recently, it was found that neuronal membrane preparations devoid of PrP have a strongly decreased copper content, indicating that PrP might bind Cu2+ ions in vivo, but the function of copper binding is still unclear (Homshaw et al., 1995; Brown et al., 1997).

Both PrPc and PrPSc are encoded by the same chromosomal gene (Basler et al., 1986) but differ in many physical properties. (i) Although PrPSc most likely possesses the same covalent structure as PrPc (Stahl and Prusiner, 1990), it differs from PrPc in its tertiary structure. Fourier-transform infrared (FTIR) and circular dichroism (CD) spectroscopy demonstrated that
PrPC has a high α-helical content and no β-sheet, whereas PrPSc is rich in β-sheet (Pan et al., 1993; Gasset et al., 1993; Caughey et al., 1991). (ii) While PrPSc is unsoluble in detergents, PrPC is soluble even in nondenaturing detergents (Meyer et al., 1986). (iii) PrPC is readily degraded by proteases, while PrPSc can only be partially hydrolysed to form an N-terminally truncated fragment of 27 to 30 kDa, designated as PrP27–30, under the same conditions (Oesch et al., 1985; Meyer et al., 1986). The N-terminus of PrP27–30 is frayed and ranges from residues 73–90 (Weissmann et al., 1996). (iv) PrPC is monomeric, whereas PrPSc forms oligomeric aggregates. (v) PrPC is localized on the cell surface, while PrPSc is found primarily in endosomes of scrapie-infected cells (Taraboulos et al., 1990).

1.3 Models of prion propagation

Two different prion-propagation models are currently discussed for the conformational conversion of PrPC into PrPSc: the catalytic or template-assistance model (Prusiner, 1991) (Fig. 2) and the nucleation-polymerization model (Jarret and Lansbury, 1993) (Fig. 3).

Figure 2: The template-assistance model.
In the template-assistance model, the rate-limiting step is supposed to be an initial irreversible conversion of monomeric PrP\text{C} into monomeric PrP\text{Sc} (Prusiner, 1991) (Fig. 2). Further formation of PrP\text{Sc} may then be autocatalyzed by PrP\text{Sc} with PrP\text{Sc} acting as a template.

The alternative nucleation-polymization model (Jarret and Lansbury, 1993) proposes a fast equilibrium between PrP\text{C} and a monomeric precursor of PrP\text{Sc} which is capable to form an oligomer of critical size that acts as nucleus for further irreversible incorporation of PrP\text{Sc} monomers (Fig. 3). In the absence of a stable nucleus the conversion between PrP\text{C} and PrP\text{Sc} is assumed to be reversible and PrP\text{C} the thermodynamically favored form. If nuclei of PrP\text{Sc} are present, however, the PrP\text{Sc} oligomers will grow further and pull PrP\text{Sc} monomers from equilibrium.

Both prion propagation models have been shown to be kinetically indistinguishable (Eigen, 1996), but the template-assistance model appears to be less likely. The most important difference between both models is that the template-assistance model requires formation of a PrP\text{C}/PrP\text{Sc} heterooligomer and thus a direct contact between the cellular prion protein and its infectious form, which is not necessary in the nucleation-polymization model.
1.4 Considerations on the principal difficulties of proving the "protein-only" hypothesis

Although the "protein-only" hypothesis is now accepted by many scientists in TSE research, there are still considerable and justified concerns (Chesebro, 1998). Most importantly, the crucial experiment to prove the "protein-only" hypothesis, i.e. the conversion of PrPC into infectious PrPSc in vitro by applying the appropriate reaction conditions or by inoculation of a PrPC preparation with catalytic amounts of PrPSc, has so far been unsuccessful. On one hand, a failure of these experiments may be explained by the absence of a so far unknown host factor protein "X". On the other hand, if generation of infectivity from PrPC in vitro indeed requires inoculation with catalytic amounts of PrPSc "nuclei", it is extremely difficult to prove that the concentration of infectious units has increased. The reason for this dilemma is that the only available assay for infectivity, namely the injection of the infectious agent into the brain of mice and subsequent measurement of the incubation time is extremely insensitive towards the concentration of the infectious agent. Specifically, concentration differences between two samples with the prion agent can only be identified reliably when the concentrations differ by two to three orders of magnitude (Prusiner, 1982; Brandner et al., 1996). Consequently, an in vitro conversion experiment triggered by catalytic amounts of PrPSc must work so well that a 1000-fold amplification of PrPSc occurs. Consequently, the maximal concentration of PrPSc that can be used in such an experiment is 1/1000 of the concentration of PrPC. This is in contrast with all in vitro experiments reported so far in which proteinase K-resistant PrP could be generated, as these experiments required approximately equimolar concentrations of the inoculum PrPSc (Kocisko et al., 1994; Bessen et al., 1995; DebBurman et al., 1997). Moreover, if one assumes that the conversion
of PrPc to PrPSc is not quantitative under in vitro conditions, the maximal inoculum/PrPc ratio becomes even lower (e.g. 1/1,000,000 for an efficiency of 0.1%). Therefore, the in vitro conversion experiments performed so far may not have worked just because the reactions are too slow due to the minute amounts of inoculum that can be used and possibly also due to nonoptimal conditions in vitro.

Another important concern against the "protein-only" hypothesis comes from the fact that, similar to many viral diseases, there are many different strains of the scrapie agent, especially in sheep and mice, which maintain their phenotypic properties even after passage through several different hosts (Collinge et al., 1996; Weissmann and Aguzzi, 1997). This occurrence of different prion strains might however be explained by the nucleation-polymerization model, according to which different scrapie strains could be caused by slightly different arrangements of the PrPSc-subunits in the infectious PrPSc-oligomer, so that a given subunit arrangement in a certain prion strain determines the subunit arrangement of the newly formed PrPSc oligomers.

1.5 Biosynthesis and proteolytic degradation of PrPc and PrPSc

The prion protein from all species is synthesized with an N-terminal signal sequence that targets the protein to the endoplasmic reticulum (ER), where the signal sequence is cleaved off (Hope et al., 1988) (Fig. 4). In addition, the C-terminal residues of the polypeptide are replaced in the ER by the GPI anchor which becomes covalently linked to Ser-231 (Stahl et al., 1990), and Asn-181 and Asn-197 become glycosylated (Bolton et al., 1985; Meyer et al. 1986;
Endo et al., 1989). PrP is then transported to the Golgi apparatus where the N-linked carbohydrates are modified. Finally, the protein is translocated to the surface of the plasma membrane where PrPC stays anchored via the GPI anchor which inserts into the membrane.

**Figure 4**: Biosynthesis of PrPC and PrPSc (Weissmann et al., 1996). Maturation of the PrP precursor involves cleavage of the signal sequence, removal of 23 carboxy-terminal amino acids, attachment of a GPI anchor at Ser-231, and glycosylation (CHO) at Asn-181 and Asn-197. Mature PrPC is anchored to the outer surface of the membrane and sensitive to proteinase K. PrPSc is partially resistant to proteinase K and yields PrP27–30 after digestion. PrP27–30 has a frayed amino-terminus, ranging from 73 to 90.

PrPC also appears to be subject to endocytosis and possibly even recycling (Caughey et al., 1991a; Borchelt et al., 1992; Shyng et al., 1993). A critical aspect regarding the mechanism of prion propagation in vivo is the cellular compartment where the transition from PrPC into PrPSc takes place. Pulse-chase experiments in scrapie-infected neuroblastoma cells suggest that PrPC is converted into PrPSc either on the plasma membrane or during the following internalization (Caughey and Raymond, 1991). Like other GPI-anchored proteins, PrPC appears to re-enter the cell through a subcellular
compartment, bounded by cholesterol-rich, detergent-insoluble membranes, which are obviously derived from caveolae and might be early endosomes (so-called caveolae-like domains; Tarabolous et al., 1995). Whether PrPSc is formed on the plasma membrane or during the endocytotic pathway is unknown (Borchelt et al., 1992; Tarabolous et al., 1995; Caughey and Raymond, 1991; Caughey et al., 1991a). PrPSc, however, is found to accumulate in late endosomes or lysosomes (Laszlo et al., 1992; Tarabolous et al., 1990; Tarabolous et al., 1992; McKinley et al., 1991), where the pH varies between pH 4.0 and 6.0 (Arnold et al., 1996). PrPSc may then be trimmed at the N-terminus by endosomal proteases to form PrP27–30 in cultured cells (Caughey et al., 1991a; Tarabolous et al., 1992).

The frayed ends of PrP27–30 are located in the segment comprising residues 73–90 (Weissmann et al., 1996). PrP27–30 can also be obtained in vitro by digestion of PrPSc with proteinase K. Therefore, residues 90–231 constitute the proteinase-resistant core of PrPSc (Meyer et al., 1986). Intriguingly, infectivity is retained in PrP27–30. Within the framework of the "protein-only" hypothesis, this finding implies that residues 90–231 also contain all the information required for the ability of PrPC to be converted to PrPSc and the ability of PrPSc to impose its fold on PrPC.

1.6 Molecular models of PrPC and PrPSc

In 1994, a computational analysis of the known amino acid sequences of mammalian prion proteins was published which predicted four putative α-helices for the carboxy-terminal segment of PrPC comprising residues 108–218, whereas predictions of regular secondary structure elements failed for the N-terminal region (Gasset et al., 1992; Huang et al., 1994). Based on this
result and genetic data, a three-dimensional model for the segment 108–218 of PrP<sup>C</sup> was developed in which the four putative helices form a four-helix bundle domain (Huang et al., 1994) (Fig. 5).

![Figure 5: Hypothetical structures of PrP<sup>C</sup> (left side) and PrP<sup>Sc</sup> (right side) (Huang et al., 1996).](image)

The four-helix-bundle model was subsequently also used to predict the three-dimensional structure of a PrP<sup>Sc</sup> subunit. Based on the experimentally proof, demonstrating an increased β-sheet content of PrP<sup>Sc</sup> (Pan et al., 1993; Caughey et al., 1991) a model was proposed in which the first two α-helices of the PrP<sup>C</sup> model adopt a four-stranded β-sheet structure in PrP<sup>Sc</sup> and the last two helices are still present in PrP<sup>Sc</sup> (Huang et al., 1996) (Fig. 5).
2. Aim of this work

A prerequisite for understanding the molecular mechanisms of prion diseases and the processes underlying the conformational changes that occur during the production of \( \text{PrP}^{\text{Sc}} \) is the knowledge of folding, thermodynamic stability and the three-dimensional structure of \( \text{PrP}^{\text{C}} \). Folding and structural studies of \( \text{PrP}^{\text{C}} \) in solution had, however, been hampered by its low solubility in the absence of detergents and the low yields of \( \text{PrP}^{\text{C}} \) obtained after purification from its natural source.

In the first part of this work, an *Escherichia coli* expression system and a purification procedure should be established to obtain a C-terminal segment of the mouse prion protein, comprising the four putative \( \alpha \)-helices, in large quantities and as a soluble protein in order to create the basis for the determination of its three-dimensional structure, for biophysical studies on its folding and stability and to verify the structural prediction for \( \text{PrP}^{\text{C}} \).

The aim of the second part of this work was the expression in *E. coli* and purification of the mature, full-length mouse prion protein comprising residues 23–231, the determination of its three-dimensional structure and the comparison of its structural and biophysical properties with those of its C-terminal segment.
3. Results

3.1 Folding, thermodynamic stability and three-dimensional structure of the carboxy-terminal segment of the mouse prion protein, mPrP(121–231)

3.1.1 Identification of the carboxy-terminal domain of the murine prion protein

In order to produce the proposed four-helix-bundle domain of PrPc, comprising amino acids 108–218, two different fragments of the murine prion protein with residues 95–231 and 107–231 were chosen for recombinant expression in the periplasm of *Escherichia coli* (amino acid numbering according to the human prion protein with deletion of codon 55 (Westaway et al., 1987)). The secretory expression in the periplasm was used to achieve formation of the single disulfide bond of PrP during expression *in vivo*. For this purpose the genes coding for both fragments were fused to the gene of the bacterial OmpA signal sequence, which should direct the transport of the recombinant proteins to the oxidizing environment of the periplasm of *E. coli*. For both fragments, expression under control of the T7 promotor yielded large amounts of soluble protein in the periplasmic fraction. However, both fragments were N-terminally degraded at three different sites after residues 112, 118 and 120, as shown by Edman sequencing. Consequently, the segment 121–231 which proved to be resistant to further degradation by periplasmic proteases was directly fused to the OmpA signal sequence and a soluble protein with homogeneous N-terminus starting with residue 121 was obtained and termed mPrP(121–231).

3.1.2 Expression and purification of mPrP(121–231)

The expression plasmids for mPrP(121–231) and the fragments mPrP(95–231) and mPrP(107–231) were constructed as follows: The three corresponding genes were amplified by polymerase chain reaction (PCR) from a mouse PrP c-DNA template (Fischer et al., 1996), digested with Nroul and BamHI and cloned into the secretory T7 expression plasmid pRBI-PDI-T7 (Strobl et al., 1995) behind the OmpA signal sequence via the Stul and BamHI restriction sites. The resulting plasmid for the expression of mPrP(121–231) was designated pPrP-C.

When the plasmid pPrP-C was used for expression, a modified form of mPrP(121–231) with an about 1 kDa higher apparent molecular mass, as judged by SDS-PAGE, was coproduced to about 10% compared to correct mPrP(121–231). N-terminal sequencing and mass spectrometry showed that this modified form had the correct N-terminus but an elongation at the C-terminus, presumably caused by the natural codons of Arg-228 and Arg-229 at the 3’-end of the mouse prion protein gene. These codons are the rarest Arg-codons in E. coli and were assumed to cause a frameshift during translation of mPrP(121–231), since a stop codon was present in one of the other reading frames nine codons after the first natural stop codon. The measured mass of the elongated protein (14,107 Da) was in good agreement with this assumption (assumed C-terminal modification:
Replacement of the rare Arg codons (AGA) by the most frequent Arg codon (CGT) in strongly expressed *E. coli* genes (Zhang et al., 1991; Chen and Inouye, 1994) prevented expression of the modified form of mPrP(121–231). In addition, a two-fold higher yield of mPrP(121–231) could be achieved. The new expression plasmid for mPrP(121–231) with the exchanged Arg codons was termed pPrP-C(RR) (Fig. 6).

**Figure 6:** Secretory expression plasmid pPrP-C(RR). T7 p/lac o: T7 promoter/lac operator region; OmpA/mPrP(121–231): gene coding for mPrP(121–231) fused to the OmpA leader peptide; f1-IG: intergenic region of phage f1; bla: β-lactamase; ori: colE1-origin of replication; lac I: lac repressor gene.
For purification of mPrP(121–231), the host strain BL21(DE3) transformed with pPrP-C or pPrP-C(RR) was used and induced cells were grown overnight at 26 °C in LB medium. The cells were harvested by centrifugation, and mPrP(121–231) was purified from the periplasmic extracts to homogeneity by anion exchange chromatography, hydrophobic chromatography and gel filtration in the absence of any detergents (Fig. 7). The quantitative formation of the single disulfide bridge was verified by HPLC analysis before and after reduction of mPrP(121–231) with dithiothreitol. Typically, 10 mg per liter of homogeneous recombinant mPrP(121–231) were obtained by this procedure.

Figure 7: Purification of recombinant mPrP(121–231) from periplasmic extracts of E. coli BL21(DE3)/pPrP-C. A Coomassie-stained SDS-15% (w/v) polyacrylamide gel is shown. Lanes: S, molecular mass standard; 1, periplasmic extract of non-induced cells; 2, periplasmic extract of induced cells; 3, pooled fractions after anion exchange chromatography; 4, purified mPrP(121–231) after chromatography on Phenyl Sepharose and gel filtration.

3.1.3 Biochemical characteristics of mPrP(121–231)

The molecular mass of mPrP(121–231) was verified by mass spectrometry (calculated mass for the oxidized protein: 13,334.8 Da; measured: 13,334.0 Da). mPrP(121–231) was proved to be stable against aggregation in a pH-range between 1.0 and 8.5 and soluble up to concentrations of 1 mM in distilled water.

Figure 8: Analytical gel filtration of mPrP(121–231). Gel filtration was performed at room temperature on a Superdex 75 HR size exclusion (30 x 1) in 50 mM sodium phosphate, pH 7.0, and a flow rate of 0.2 ml/min. Proteins used as molecular mass standards were bovine serum albumin (66 kDa), hen egg albumin (45 kDa), equine myoglobin (18 kDa) and hen egg lysozyme (14 kDa). This mixture of proteins (dashed line) and 50 μl of mPrP(121–231) with a concentration of 0.1 mM (solid line) were applied to the column.
The oligomerization state of mPrP(121–231) was investigated by analytical gel filtration on a Superdex 75 HR column (Fig. 8). In comparison to a mixture of standard proteins with known molecular mass, a molecular mass of about 13 kDa could be determined for mPrP(121–231), independent of protein concentration, which is in full agreement with the monomeric state of the protein in solution.


3.1.4 Spectroscopic characteristics of mPrP(121–231)

Absorption
The extinction coefficient of unfolded mPrP(121–231) was calculated from the amino acid sequence as described by Gill and von Hippel. A specific absorbance \((A_{280\text{nm}}, 1\text{mg/ml}, 1\text{cm})\) of 1.55 for native, oxidized mPrP(121–231) was determined through comparison of the absorbance of native and denatured, oxidized mPrP(121–231) at 280 nm (Fig. 9).

Circular dichroism spectroscopy
Circular dichroism-spectra of mPrP(121–231) were recorded in the far- and near-UV region between pH 1.0 and 7.0 (Fig. 10). The far-UV circular dichroism spectra of mPrP(121–231) revealed two minima at 208 nm and 222 nm and a mean residue ellipticity of -15,600 deg cm\(^2\) dmol\(^{-1}\) at 222 nm and pH 7.0, which is typical for proteins with a high α-helical content.
Figure 9: (A) Absorbance spectra of native (−) and denatured, oxidized (−−) mPrP(121–231) (B) Absorbance difference spectrum. The absorbance was recorded at 25 °C and protein concentrations of 48 μM in 100 mM sodium phosphate, pH 7.0 with and without GdmCl using a 1 cm cuvette.

Figure 10: (A) Far-UV CD spectra between pH 1.0 and 7.0. (B) Near-UV CD spectra between pH 1.0 and 7.0.
The shapes of all spectra between pH 3.0 and 7.0 are very similar, showing that the structure does not change significantly with decreasing pH. However, at pH 1.0 and 2.0 the mean residue ellipticity is less negative and only one minimum at 215 nm is visible, demonstrating that a rearrangement to β-sheet-like structure might occur at pH values below 3.0. In addition, characteristic near-UV circular dichroism spectra of mPrP(121–231) were obtained between pH 1.0 and 7.0. The shapes of these spectra are also very similar, except for the spectra at pH 1.0 and 2.0, which indicate lower mean residue ellipticities for mPrP(121–231), possibly indicating significantly different tertiary structures at these pHs.

**Fluorescence spectroscopy**

At an excitation wavelength of 280 nm, the fluorescence emission spectrum of native mPrP(121–231) exhibits an emission maximum at 340 nm, which corresponds to the single tryptophan of the C-terminal domain and a maximum at 305 nm with lower intensity corresponding to the 11 tyrosine residues of the protein (Fig. 11). Reference spectra of tryptophan-containing native proteins show generally maxima between 320 nm and 335 nm, whereas free tryptophans in aqueous solution exhibit emission maxima of 348 nm (Schmid, 1997). The relatively high emission maximum of 340 nm thus indicated that the single tryptophan is likely to be solvent exposed, which proved to be consistent with the three-dimensional structure of mPrP(121–231) (see chapter 3.1.7).


3.1.5 Autonomous folding of mPrP(121–231)

To investigate whether mPrP(121–231) represents an autonomous folding unit, its guanidinium chloride (GdmCl-) dependent unfolding/refolding transition was recorded at pH 7.0 using its far-UV CD signal at 222 nm as a measure of native structure. The unfolding/refolding transition of mPrP(121–231) is cooperative and completely reversible. Reversibility was also confirmed through identical far-UV CD spectra of native and refolded mPrP(121–231) at pH 7.0. Analysis of the transition according to a two-state model of folding revealed a free energy of folding of -21.8 kJ/mol and a midpoint of transition of 2.53 M GdmCl. The cooperativity of folding (m-value)
was calculated to 8.6 (± 0.5) kJ mol⁻¹ M⁻¹ GdmCl and is in the range expected for a 13.3 kDa protein (Myers et al., 1995).


3.1.6 Identification of an acid-induced folding intermediate of mPrP(121–231)

The pH-dependence of the folding of mPrP(121–231) was measured with urea as denaturant and followed by the far-UV circular dichroism signal at 222 nm (Fig. 12). Evaluation of the transitions revealed apparent two-state mechanisms of folding at pH 6.0 and 7.0 with similar cooperativities, transition midpoints and free energies of folding. When the pH decreased further, the unfolding transitions are shifted to lower urea concentrations. Thus, acidic pH destabilizes the C-terminal domain of mPrP. Importantly, an equilibrium unfolding intermediate was observed at pH 4.0 and pH 4.5 became evident by the plateau phase in the transitions (Fig. 12). Analysis of these data according to a three-state model of folding revealed that the intermediate is populated to 0.2% in the absence of urea and enriched to 95% in the presence of 3.5 M urea at pH 4.0. This provided the possibility to characterize the intermediate of mPrP(121–231) spectroscopically. In contrast to the far-UV CD spectrum of native mPrP(121–231) at pH 4.0, which exhibits a high α-helical content with minima at 208 nm and 222 nm and a mean residue ellipticity of -13,600 deg cm² dmol⁻¹, the far-UV CD spectrum of the intermediate has a minimum at 215 nm with a mean residue ellipticity of -7000 deg cm² dmol⁻¹ (Fig. 13), indicating an increased content of β-sheet structure and loss of α-helical structure.
Figure 12: Urea-dependent unfolding/refolding transitions of mPrP(121–231) between pH 4.0 and 7.0 at 22 °C. Closed symbols correspond to the unfolding and open symbols to the refolding transitions.

Figure 13: Far-UV circular dichroism spectra of native mPrP(121–231) at pH 4.0 in the absence of urea (solid line), the equilibrium unfolding intermediate at pH 4.0 and 3.5 M urea (dotted line) and the acid denatured state (dashed line) of mPrP(121–231) at pH 2.0 and 3.5 M urea at 22 °C.
3.1.7 The three-dimensional NMR structure of the C-terminal domain mPrP(121−231)

The three-dimensional structure of mPrP(121−231) was determined in solution by NMR (nuclear magnetic resonance) spectroscopy. For this purpose, uniformly $^{15}$N-labeled, $^{15}$N/$^{13}$C-doubly labeled and 10% $^{15}$N/$^{13}$C-biosynthetically-directed labeled mPrP(121−231) had to be produced. *E. coli* BL21(DE3)/pPrP-C(RR) were grown in minimal medium containing $^{15}$N(NH$_4$)$_2$SO$_4$ as the sole nitrogen source and $[^{13}$C$_6]$-glucose, or/and unlabeled glucose as the sole carbon source. The NMR-measurements were performed at pH 4.5 and 20 °C at a protein concentration of 0.8 mM either in 90% H$_2$O/10% ²H$_2$O or in 99% ²H$_2$O.

The recording and evaluation of the NMR spectra and the structure calculations were performed by R. Riek, Dr. G. Wider and PD Dr. M. Billeter in the group of Prof. Dr. K. Wüthrich. The sequence-specific polypeptide backbone assignments for mPrP(121−231) were obtained using 3D $^{15}$N-resolved [$^1$H,$^1$H]-NOE spectroscopy (NOESY) for observation of sequential NOE connectivities (Wüthrich, 1986) and triple resonance experiments for identification of intraresidual and sequential heteronuclear scalar coupling connectivities (Cavanagh *et al.*, 1996). The side-chain signals were assigned from 3D through bond correlation NMR experiments. Stereospecific assignments for all isopropyl methyls of Val and Leu were obtained from 2D [$^{13}$C,$^1$H]-COSY experiments using the $^{15}$N/10% $^{13}$C-labeled sample (Neri *et al.*, 1989). Distance constraints for the calculation of the 3D structure were

derived from 3D $^{13}$C- or $^{15}$N-resolved $[^1H,^1H]$-NOESY spectra recorded with a mixing time of 50 ms.

The NMR structure was calculated with the program DYANA (Güntert et al., 1997) using 1592 NOE distance constraints and 229 dihedral angle constraints. The 20 best DYANA conformers have an average residual target function value of $1.02 \pm 0.48 \text{ Å}$. These conformers were energy minimized in a water shell using the AMBER all-atom force field (Cornell et al., 1995) as implemented in the program OPAL (Luginbühl et al., 1996). For residues 124–166 and 172–226 the RMSD (root mean square distance) to the mean structure of these 20 conformers was determined to 0.8 Å for the N, C$\alpha$ and C$'$ atoms, and 1.2 Å for all heavy atoms.

The NMR structure of mPrP(121–231) contains three $\alpha$-helices comprising residues 144–154, 175–193 and 200–219, respectively, a short, two-stranded antiparallel $\beta$-sheet spanning residues 128–131 and 161–164 and a short helical segment comprising residues 222–226 (Fig. 14A). The first turn of the second helix and the last turn of the third helix are linked by the disulfide bridge, which is highly shielded from the solvent in the core of the protein. These two longest helices, which are arranged as twisted V-shape, form the scaffold of the protein, onto which the short $\beta$-sheet and the first helix are anchored. mPrP(121–231) possesses a hydrophobic core of 20 tightly packed amino acids, most of which belong to helix 2 and 3.

Figure 14B shows that the structure prediction of Huang et al. was entirely wrong for the segment 121–178, but the positions of the last two helices were predicted with resonable accuracy. A detailed discussion of the implications of the three-dimensional structure of mPrP(121–231) for the transition to PrP$^{Sc}$, the species barrier of prion diseases and inherited human prion diseases is presented in the discussion.
Figure 14A: Ribbon diagram of the three-dimensional structure of the C-terminal domain of the mouse prion protein. mPrP(121–231) contains three α-helices, a short, two-stranded, antiparallel β-sheet and a short helical turn near the C-terminus. The figure was prepared with the program MOLMOL (Koradi et al., 1996).

Figure 14B: Regular secondary structure (A) in the NMR structure of mPrP(121–231) and (B) in the predicted four-helix bundle structure of PrPc (Huang et al., 1994).
3.1.8 Identification of a possible binding site

Within the framework of the "protein-only" hypothesis and according to the template-assistance model for the formation of PrP\textsuperscript{Sc}, PrP\textsubscript{C} is believed to be bound by PrP\textsuperscript{Sc} at a specific binding site, whereby PrP\textsuperscript{Sc} imposes its three-dimensional structure upon PrP\textsubscript{C}. To identify such a possible binding site of PrP\textsubscript{C}, a peptide screening assay was performed using a peptide library. This library consisted of a gridded array of peptides comprising 99 polypeptides of 13 amino acids, shifted by two amino acids and covering the entire mature mouse PrP sequence. The N-termini of the peptides were acetylated to eliminate the potential influence of charged groups, and the C-termini were covalently coupled to the filter.

The binding affinity of mPrP(121–231) to the mouse prion protein peptides were analysed by incubation of the filter with biotinylated mPrP(121–231) at room temperature, transfer of bound mPrP(121–231) via electroblotting to PVDF-membranes and specific staining of transferred mPrP(121–231) using a streptavidin-alkaline phosphatase conjugate and chromogenic alkaline phosphatase substrates.

Spots corresponding to the region of the first \(\alpha\)-helix (spots 55 to 60) gave the strongest signals after blotting for 90 minutes and colorimetric staining, indicating that this region might be a possible binding site of mPrP(121–231) for PrP\textsuperscript{Sc} (Fig. 15).
**Results**

Figure 15: Peptide spot binding assay. (A) Peptide spots to which mPrP(121–231) have a high binding affinity are indicated (blotting time: 90 min). In the control experiment which was performed without mPrP(121–231), none of the spots showed a chromogenic reaction. (B) Sequences of the spots to which mPrP(121–231) shows a binding affinity. Amino acids corresponding to the first α-helix are shown in bold letters. Biotinylation of mPrP(121–231): Two molar equivalents of D-biotinoyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim, Switzerland; in DMF) were added to 1 mg/ml mPrP(121–231) in 50 mM boric acid/NaOH, pH 8.6 and incubated for 1 h. The mixture was applied to a PD10 column preequilibrated with PBS-buffer (100 mM sodium phosphate, pH 7.0, 100 mM sodium chloride), and eluted with the same buffer. Peptide binding assay: The peptide library (Jerini Bio Tools, Berlin) was first washed with 10 ml methanol and three times with 10 ml TBS-buffer (137 mM NaCl, 2.7 mM KCl, 50 mM Tris/HCl, pH 7.0) and incubated overnight in 10 ml membrane blocking reagent (Cambridge Research Biochemicals, Northwich, UK). At the next day, the membrane was washed three times with 10 ml TBS containing 0.05% (v/v) Tween 20 (Serva, Heidelberg, Germany). Eventually, the membrane was incubated with biotinylated mPrP(121–231) with a final concentration of 1 μM in 10 ml TBS-buffer (137 mM NaCl, 2.7 mM KCl, 50 mM Tris/HCl, pH 7.0) and incubated overnight in 10 ml membrane blocking reagent. The membrane was washed three times with 10 ml TBS/Tween and incubated with 20 μl streptavidin-alkaline phosphatase conjugate in 10 ml blocking buffer. After washing twice with 10 ml TBS/Tween and twice with CBS-buffer (137 mM NaCl, 2.7 mM KCl, 10 mM sodium citrate, pH 7.0), the chromogenic reaction was performed in 20 ml CBS-buffer, 120 μl thiazoly blue (MTT, Sigma, Germany; 5% (w/v) in 70% DMF) and 80 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Biomol, Hamburg, Germany; 6% (w/v) in DMF), and 50 μl 1 M MgCl₂. The reaction was stopped by immersing the filter in distilled water. The polyacrylamide filter with the peptide library was regenerated by the following washing procedure: 2 x 5 min in distilled water, 2 x DMF in a sonification until the decoloration was complete, 1 x 10 min 8 M urea, 1% (w/v) SDS, 0.5% mercaptoethanol, adjusted to pH 7.0 with acetic acid, 3 x 5 min in distilled water, 3 x 5 min in 70% (v/v) ethanol. The filter was then air-dried and stored at -20 °C.
Weaker signals under the same conditions were observed for the octapeptide region (spots 17 to 19) and for several regions of the second $\alpha$-helix (spots 78, 79, 81, 83 and 84). A slightly different result was, however, obtained, if the peptide library was directly colorimetrically stained. The strongest signals were then observed for spots 61 to 64 (Tab. 2), including the first $\alpha$-helix, but also residues between the first $\alpha$-helix and the second strand of the $\beta$-sheet.

Table 2: Sequences of spots 61 to 64. Amino acids corresponding to the first $\alpha$-helix are shown in bold letters.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>DWEDRYRENNMYR</td>
</tr>
<tr>
<td>62</td>
<td>EDRYYRENNMYRYP</td>
</tr>
<tr>
<td>63</td>
<td>RYYRENNMYRYPNQ</td>
</tr>
<tr>
<td>64</td>
<td>YRENNMYRPNQVY</td>
</tr>
</tbody>
</table>

Since the peptides, whose sequences correspond to the first $\alpha$-helix, show the highest binding affinities to $mPrP(121-231)$, the peptide, $mPrP(144-158)$ with the sequence (AcN)-D-W-E-D-R-Y-Y-R-E-N-M-Y-R-Y-P-(COOH) was synthesized by Jerini Bio Tools (Berlin) for subsequent binding studies.

Figure 16 shows the far-UV CD spectra of $mPrP(144-158)$ in 20 mM sodium phosphate, pH 7.0, and in 20 mM sodium acetate, pH 4.5, respectively, in the presence of increasing concentrations of 2,2,2-trifluoroethanol (TFE). The spectra of $mPrP(144-158)$ in aqueous solution show two minima at 208 nm and 222 nm with low mean residue ellipticities, which is consistent with the peptide adopting only partially $\alpha$-helical secondary structure. However, $mPrP(144-158)$ shows strongly increased helicity with increasing concentrations of trifluoroethanol independent of pH (Fig. 16), demonstrating the intrinsic tendency of the segment 144–158 to adopt an $\alpha$-helical conformation despite its unusual amino acid composition.
Results

Figure 16: Far-UV CD spectra of mPrP(144–158) in (A) 20 mM sodium phosphate, pH 7.0, (solid line), 20 mM sodium phosphate, pH 7.0/20% (v/v) TFE (dashed line) and 20 mM sodium phosphate, pH 7.0/50% (v/v) TFE (dotted line) and in (B) 20 mM sodium acetate, pH 4.5, (solid line), 20 mM sodium acetate, pH 4.5/20% (v/v) TFE (dashed line) and 20 mM sodium acetate, pH 4.5/50% (v/v) TFE (dotted line). The spectra were recorded on a Jasco J710 CD spectropolarimeter in 0.2 mm cuvettes at 22 °C at peptide concentrations of 0.5 mg/ml, and corrected for the buffers. The concentration of the peptide was determined by its specific extinction coefficient which was calculated from its amino acid composition. (MW= 2312 Da, \(A_{280\text{nm}, 1\text{mg/ml}, 1\text{cm}}= 10.8\)).

In order to investigate affinity between mPrP(121–231) and the peptide mPrP(144–158) in solution, fluorescence difference spectroscopy was tried, assuming that binding of the peptide might result in a fluorescence change in the complex compared to the sum of the fluorescence spectra of the isolated components. However, mixing of mPrP(121–231) and mPrP(144–158) at an 1:1 ratio (3 µM each) did not result in a significant fluorescence change (Fig. 17). Therefore, the fluorescence does either not change upon peptide binding or the affinity is rather low with \(K_D\approx 10 \mu M\).
Results

Figure 17: Fluorescence emission addition spectrum of single spectra of mPrP(121-231) and mPrP(144-158) (solid line) and of a spectrum of a sample containing mPrP(121-231) and mPrP(144-158) in equimolar amounts (dashed line). The spectra were recorded with protein and peptide concentration of 3 μM each. The samples were incubated for 1 h at 22 °C in 100 mM sodium phosphate, pH 7.0. The spectra were corrected for the buffers (excitation at 280 nm).

3.1.9 Identification of a possible structural relationship between PrP<sup>C</sup> and signal peptidases

It was found that long-term incubation of the recombinant, murine prion protein mPrP(23-231) at high protein concentrations (~1 mM) and pH 4.5 resulted in slow degradation of the amino-terminal polypeptide segment (23–120), while the carboxy-terminal domain was not affected. In addition, expression of the fragments 95–231 and 107–231 in the periplasm of E. coli was accompanied by amino-terminal degradation and purified mPrP(121–231) was found to be susceptible to proteolytic cleavage of the peptide bond Tyr226–Asp227 under the conditions used for NMR structure determination (0.8 mM mPrP(121–231),
pH 4.5). The minute proteolytic activity found in the preparations of mPrP(121–231) and mPrP(23–231) might result either from protease impurities or might be caused by a hydrolytic activity which is intrinsically present in mPrPC.

Therefore, a systematic sequence comparison of the mouse prion protein with sequences of proteases from different families was performed, which revealed a striking and surprising sequence similarity between mPrP(121–231) and the catalytic domains of monomeric bacterial signal peptidases as well as with the catalytic subunits of eukaryotic signal peptidases (Glockshuber et al., 1998). The strongest homology was found for the catalytic subunit of rat signal peptidase with 23% sequence identity and 41% similarity.

A relation between PrPC and signal peptidases is further supported by the fact that the residues Tyr-128 and His-177, which align with the presumed active-site residues of E. coli signal peptidases (Ser-90 and Lys-146) are in close proximity in the three-dimensional structure of mPrP(121–231). Moreover, the hydroxyl group of Tyr-128 can be brought into hydrogen bond distance with the Nδ1 nitrogen of His-177 bond after rotation of Tyr-128 about χ1 and His-177 about χ1 and χ2. Further inspection of the structure of mPrP(121–231) and modelling studies revealed that each of the side chains of Asn-174 and Asn-173 can principally form an additional hydrogen bond to His-177, resulting in a modeled hydrogen bond network Tyr-128, His-177 and Asn-174 (or Asn-173) reminiscent of that of thiol proteases (Cys-His-Asn) (Polgar, 1990).

In a first approach to demonstrate an intrinsic hydrolytic activity of mPrP(121–231), the presumed nucleophilic residue Tyr-128 was replaced by phenylalanine and the variant was produced in the periplasm of E. coli. To compare the hydrolytic activity of preparations of the wild-type protein with the
activity of the preparation of the Tyr128Phe variant, the following chromogenic substrates for proteases of different specificity such as trypsin, chymotrypsin and elastase-like protease specificities were tested (Fig. 18): N-Succinyl-Ala-Ala-Pro-Leu-p-Nitroanilide, N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide, N-p-Tosyl-Gly-Pro-Lys-p-Nitroanilide, N-p-Tosyl-Ala-Ala-Ala-p-Nitroanilide and Universal Protease Substrate™.

However, no significant protease activity could be detected with any of these substrates, and the very small activities observed after incubation of the substrates with mPrP(121–231) and its variant for three days in some cases even resulted in a higher hydrolytic activity for the Tyr128Phe variant compared to the wild type sequence. Thus, the hydrolytic activity observed in the preparations of mPrP(121–231) and mPrP(23–231) is most likely result from impurities with other proteases. In addition, pre-β-lactamase was used as a substrate. This however leaves the possibilities that PrPC could have a very finite but narrow specificity for an unknown substrate or is hydrolytically active only in conjunction with other proteins.

In a second approach, the possibility that the previously observed N-terminal truncation of the segments mPrP(95–231) and mPrP(107–231) in vivo occurs by an autocatalytic activity should be investigated. Therefore, the Tyr128Phe mutation was introduced into mPrP(95–231) (Fig. 19). Expression in the periplasm of E. coli yielded high quantities of the variant and the corresponding wild-type protein. Edman sequencing, however, revealed that the variant was N-terminally degraded at exactly the same site (after residue 112) as found for the wild-type protein. This result indicates that the N-terminal degradation in the periplasm of E. coli of fragments longer than the C-terminal domain is presumably not caused by an autocatalytic activity of PrP, but possibly by periplasmic E. coli proteases.
The protease activities of mPrP(121–231) and mPrP(121–231)Y128F were tested with the following protease substrates: 1) N-Succinyl-Ala-Ala-Pro-Leu-p-Nitroanilide (Sigma, Deutschland), 2) N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (Sigma, Deutschland), 3) N-p-Tosyl-Gly-Pro-Lys-p-Nitroanilide (Sigma, Deutschland), 4) N-p-Tosyl-Ala-Ala-Ala-p-Nitroanilide (Sigma, Deutschland), 5) Universal Protease Substrate\textsuperscript{TM} (Boehringer Mannheim, Deutschland). The assays were performed in 100 mM Tris/HCl, pH 7.5, at room temperature in a volume of 0.5 ml and protein concentrations of 0.1 mg/ml. Reactions with the substrates 1–5 were started by addition of 25 µl protease substrate to a final concentration of 0.5 mM; reaction 6 was started by addition of 25 µl of the substrate (stock solution: 4 mg/ml). After incubation for 3 days, substrate hydrolysis was followed by an increase in absorbance at 405 nm for substrates 1–5 and at 574 nm for substrate 6, using samples without protein for baseline subtraction. No substrate hydrolysis could be observed in the absence of mPrP(121–231) and mPrP(121–231)Y128F.

Protease activity assays with pre-β-lactamase as substrate were performed in 10 mM Tris/HCl, pH 8.0, with and without 1% Triton X100 at protein concentrations of 0.1 mg/ml and analysed for cleavage of pre-β-lactamase by SDS-PAGE after an incubation time of 3 days at 37 °C. Site-directed mutagenesis and purification of mPrP(121–231)Y128F: Site-directed mutagenesis was carried out according to Kunkel using uridinylated single-stranded DNA of the plasmid pPrP-C(RR) from phage infection (Vieira and Messing, 1987) of the E. coli strain CJ236 (Mutagene phagemid kit, Bio Rad) and the following mutagenesis primer: Y128F: 5'-GGG CCT GCTCATGGCTGAGCCCAGCATGAAGCCACCAAG GCC-3'. The mutation was identified by restriction analysis and verified by dideoxynucleotide sequencing. mPrP(121–231)Y128F was expressed and purified like wild type mPrP(121–231).
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Figure 19: Expression test of mPrP(95-231) and mPrP(95-231)Y128F. A Coomassie-stained SDS-15% (w/v) polyacrylamide gel is shown. Lanes: S, molecular mass standard; 1, purified mPrP(121-231); 2, periplasmic extract of induced cells harboring the plasmid mPrP(95-231)Y128F; 3, periplasmic extract of non-induced cells harboring the plasmid mPrP(95-231)Y128F; 4, periplasmic extract of induced cells harboring the plasmid mPrP(95-231); 5, periplasmic extract of non-induced cells harboring the plasmid mPrP(95-231). Cells of E. coli BL21(DE3) (Studier and Moffat, 1986) containing the corresponding plasmid were grown at 37 °C in 400 ml LB medium containing ampicillin (100 μg/ml) until an A550 of 0.8 to 1.0 was reached. After addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM, the cells were grown overnight at 26 °C, harvested by centrifugation and suspended in 5 ml 50 ml Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mg/ml Polymyxin B. After stirring for one hour at 0 °C, the suspensions were centrifuged at 39,000g and the supernatants applied to an SDS-15% (w/v) polyacrylamide gel. The amino-termini of the periplasmically expressed mouse prion protein fragments (mPrP(95-231) and mPrP(95-231)Y128F) were determined by Edman sequencing after applying the periplasmic extracts to an SDS-15% (w/v) polyacrylamide gel and blotting the prion protein fragments onto a PVDF membrane. Site-directed mutagenesis of mPrP(95-231)Y128F: Site-directed mutagenesis was carried out according to Kunkel using uridylinated single-stranded DNA of the plasmid pHPrP(95-231) (Hornemann and Glockshuber, 1996) from phage infection (Vieira and Messing, 1987) of the E. coli strain CJ236 (Mutagenex phagemid kit, Bio Rad) and the following mutagenesis primer: Y128F: 5'-GGG CCT GCT CAT GGCTGAGCCCAG CATGAAGCCACCAAGGCC-3'. The mutation was identified by restriction analysis and verified by dideoxynucleotide sequencing.

3.2 Folding, thermodynamic stability and three-dimensional structure of mPrP(23–231)

3.2.1 Expression and purification of mPrP(23–231)

To obtain an efficient expression system for the production of the recombinant, full-length prion protein (residues 23–231) of the mouse with intact disulfide bridge in E. coli, it was first tried to express the protein in the periplasm of E. coli via the OmpA signal sequence. Therefore, the gene of mPrP(23–231) was amplified by polymerase chain reaction from a mouse c-DNA template (Fischer et al., 1996) and cloned via the restriction sites NruI and BamHI in the expression plasmid pRBI-PDI-T7 (Strobl et al., 1995). Although this expression system yielded high amounts of soluble mPrP(121–231) in the periplasm of E. coli, this approach failed for the production of mPrP(23–231), as mPrP(23–231) could neither be detected in the periplasmic fraction, nor in the insoluble fraction of cell extracts. Similar observations have recently been reported for the attempt to express hamster PrP(23–231) in the periplasm of E. coli (Mehlhorn et al., 1996). Therefore, mPrP(23–231) had to be expressed in the cytoplasm of E. coli. The amplified gene of mPrP(23–231) was digested with Ndel and BamHI and cloned in the expression plasmid pRBI-PDI-T7t-Ndel (Hermanns and Glockshuber, unpublished), which had been cut with the same enzymes (Fig. 20). The expression plasmid pRBI-PDI-T7t-Ndel represents a derivative of the plasmid pRBI-PDI-T7 (Strobl et al., 1995), additionally containing a T7-terminator and a single Ndel restriction site at the start codon. The rare, natural codons of Arg-25 at the 5'-end and of Arg-228 and Arg-229 at the 3'-end of the mouse prion protein gene were replaced by the most frequent Arg codon in strongly expressed E. coli genes to prevent expression of modified forms of mPrP(23–231) (see also chapter 3.1.2). In the
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resulting plasmid, pPrP(23–231), the PrP gene is under the control of the T7-promotor/lac operator. The recombinant protein expressed with this plasmid contains like mPrP(121–231) an additional Ser at its carboxy-terminus and according to the N-end rule in bacteria (Tobias et al., 1991) an additional Ser at the amino-terminus of mPrP(23–231) to minimize proteolytic degradation in the cytoplasm.

![Diagram of plasmid pPrP(23-231)]

Figure 20: Cytoplasmic expression plasmid pPrP(23-231). T7 p/lac o: T7 promotor/lac operator region; mPrP(23–231): gene coding for mPrP(23–231); f1-IG: intergenic region of phage f1; bla: β-lactamase; ori: colE1-origin of replication; lac I: lac repressor gene.

E. coli cells BL21(DE3) harboring this plasmid produced large amounts of reduced recombinant mPrP(23–231). Because formation of disulfide bridges is an oxidation process which does not occur in the reducing environment of the cytoplasm of E. coli the protein accumulated in insoluble inclusion bodies (Fig. 21). The inclusion bodies were solubilized in 8 M urea
and \( m\text{PrP}(23\text{--}231) \) was enriched by cationic exchange chromatography on SP-Sepharose in the presence of 8 M urea.

![Figure 21: Purification of \( m\text{PrP}(23\text{--}231) \). A Coomassie-stained 15% polyacryl-amide/SDS gel is shown. Lanes: S, molecular mass standard; 1, solubilized inclusion bodies prepared from \( E. \) coli BL21(DE3)/p\text{PrP}(23\text{--}231); 2, refolded and purified recombinant \( m\text{PrP}(23\text{--}231) \); 3, purified \( m\text{PrP}(121\text{--}231) \).](image)

In a first attempt, \( m\text{PrP}(23\text{--}231) \) was oxidized by the standard method with a mixture of oxidized and reduced glutathione. This method, however, failed and the single disulfide bridge was then formed in 8 M urea and pH 8.7 by air oxidation with 1 \( \mu \text{M} \) \( \text{Cu}^{2+} \) as a catalyst, using a relatively low protein concentration of 100 \( \mu \text{M} \) to avoid formation of intermolecular disulfide bridges. The usage of this method was possible, because \( m\text{PrP}(23\text{--}231) \) contains only one disulfide bridge, so that no incorrect disulfide bridges could be formed. After a further purification step by cation exchange chromatography, the denaturant was removed by extensive dialysis against water. Oxidized \( m\text{PrP}(23\text{--}231) \) was then purified by cationic exchange chromatography on
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CM52 cellulose under native conditions at pH 7.0. To prevent proteolytic degradation due to protease impurities, protease inhibitors were added for long-time storage. Typically, about 100 mg of pure mPrP(23–231) were obtained from 10 l bacterial culture in rich medium by this method.


3.2.2 Biochemical characteristics of mPrP(23–231)

mPrP(23–231) possesses a molecular mass of 23,113 kDa as verified by electrospray mass spectrometry (calculated mass: 23,107 kDa), is soluble up to concentrations of at least 1.5 mM and shows no aggregation between pH 1.0 and 8.5.


3.2.3 Spectroscopic characteristics of mPrP(23–231)

Absorption

The specific absorbance ($\text{A}_{280\text{nm}}$, $1\text{mg/ml}$, $1\text{cm}$) = 2.7 of native, oxidized mPrP(23–231) was determined as described for mPrP(121–231).

Circular dichroism spectroscopy

To follow the contents of secondary and tertiary structure at various pH, the far- and near-UV spectra of mPrP(23–231) were recorded between pH 1.0 and 7.0. mPrP(23–231) like mPrP(121–231) possesses typical $\alpha$-helical far-UV CD spectra, with minima at 208 nm and 222 nm (Fig. 22A).
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The mean residue ellipticities of mPrP(23–231) are however less negative than those of mPrP(121–231), indicating that the percentage of residues in regular secondary structures is higher in mPrP(121–231) than in the full-length protein. The same trend was observed for the near-UV CD spectra (Fig. 22B). The shapes are also very similar, but less negative mean residue ellipticities were obtained for mPrP(23–231) than for mPrP(121–231). According to the far- and near-UV CD spectra of mPrP(121–231), the structure of mPrP(23–231) at pH 1.0 and pH 2.0 varies from that at higher pH values and indicate a conformational change at pH 1.0 and 2.0 which may be accompanied by partial unfolding.
Fluorescence properties of mPrP(23–231)

Compared to mPrP(121–231) which only possesses a single tryptophan (W145) mPrP(23–231) has seven additional tryptophans in the N-terminal segment 23–231 (W31, W57, W65, W73, W81, W89 and W99). Therefore, the fluorescence spectrum of mPrP(23–231) is dominated by these additional tryptophan residues in the N-terminal segment (Fig. 23).

![Fluorescence spectrum of mPrP(23–231) at 22 °C and pH 7.0.](image)

**Figure 23:** Fluorescence spectrum of mPrP(23–231) at 22 °C and pH 7.0.

As expected, mPrP(23–231) has a much stronger fluorescence emission maximum of 345 nm, indicating that most or all of the seven tryptophan residues in the segment 23–120 are solvent exposed and not part of a hydrophobic environment (Fig. 23), as buried tryptophans in proteins show maxima between 320 and 335 nm and free tryptophan in solution have maxima at 348 nm (Schmid, 1997).
3.2.4 Urea-induced unfolding and refolding of mPrP(23-231) at various pH

Equilibrium unfolding/refolding transitions of mPrP(23-231) were measured between pH 3.7 and pH 7.0 by monitoring the far-UV circular dichroism signal at 222 nm as a function of the urea concentration (Fig. 24).

Figure 24: Urea-induced unfolding/refolding transitions of mPrP(23-231) between pH 3.7 and 7.0. The closed symbols correspond to the unfolding and the open symbols to the refolding transitions of mPrP(23-231). The measurements were performed at 22 °C and constant ionic strength of 88 mM. Buffers were the same as those used for measuring the far- and near-UV CD spectra of mPrP(23-231) (see chapter 3.2.3), additionally containing different concentrations of urea. Native or in 8 M urea denatured mPrP(23-231) was diluted 1:11 with buffer (final concentration of mPrP(23-231): 13–26 μM) and incubated for 36 hours. The mean residue ellipticity at 222 nm was then recorded for 2 min in 0.1 cm cuvettes and averaged. Data analysis. The original CD data were analyzed according to the two-state model of folding, using a six-parameter fit as described by Santaro and Bolen.
As in mPrP(121–231), the midpoints of the transitions shift to lower urea concentrations with decreasing pH, indicating that acidic pH also destabilizes mPrP\textsuperscript{C}. A comparison of the cooperativities of mPrP(121–231) and mPrP(23–231) shows significantly lower cooperativities for mPrP(23–231) than expected from those of mPrP(121–231) (Tab. 3).

<table>
<thead>
<tr>
<th>pH-value</th>
<th>ΔG\textsubscript{NU} (kJ/mol)</th>
<th>m\textsubscript{NU} (kJ/mol M)</th>
<th>urea\textsubscript{1/2} (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPrP(23–231) pH 7.0</td>
<td>-22.28 (±1.47)</td>
<td>3.71 (±0.27)</td>
<td>6.01</td>
</tr>
<tr>
<td>mPrP(23–231) pH 5.0</td>
<td>-18.53 (±1.48)</td>
<td>4.01 (±0.32)</td>
<td>4.62</td>
</tr>
<tr>
<td>mPrP(23–231) pH 4.5</td>
<td>-16.52 (±0.89)</td>
<td>4.19 (±0.21)</td>
<td>3.94</td>
</tr>
<tr>
<td>mPrP(23–231) pH 4.0</td>
<td>-11.44 (±1.04)</td>
<td>3.67 (±0.27)</td>
<td>3.11</td>
</tr>
<tr>
<td>mPrP(23–231) pH 3.7</td>
<td>-8.32 (±0.69)</td>
<td>3.58 (±0.18)</td>
<td>2.32</td>
</tr>
<tr>
<td>mPrP(121–231) pH 7.0</td>
<td>-28.60 (±0.95)</td>
<td>4.73 (±0.16)</td>
<td>6.05</td>
</tr>
</tbody>
</table>

Table 3: Thermodynamic parameters of the fitted unfolding transitions of mPrP(23–231) between pH 3.7 and 7.0 in comparison to those of mPrP(121–231) at pH 7.0. ΔG\textsubscript{NU} is the free energy difference between the native and the unfolded protein at zero molar denaturant, m\textsubscript{NU} is the corresponding cooperativity of the transition and urea\textsubscript{1/2} indicates the urea concentration at the respective transition midpoint.

In contrast to the transitions of mPrP(121–231), which demonstrated the presence of an equilibrium unfolding intermediate under acidic conditions, the analysis of the unfolding transitions of mPrP(23–231) reveals that the presence of an unfolding intermediate of the complete, recombinant prion protein is not evident from a plateau phase in the transition at pH 4.0.
However, the significantly reduced cooperativity of the transitions suggests a deviation from the two-state model of folding in mPrP(23–231).

The far-UV CD spectrum of mPrP(23–231), which was recorded under the conditions used to induce the unfolding intermediate of mPrP(121–231), revealed a mixed spectrum of native and unfolded mPrP(23–231) (Fig. 25). This indicates that mPrP(23–231) may not adopt the same alternative conformation as mPrP(121–231) at acidic pH, where mPrP(121–231) shows the spectral characteristics reminiscent of a β-sheet protein.

Figure 25: Far-UV circular dichroism spectra of mPrP(23–231) in the presence of 3.5 M urea at pH 7.0 (---), pH 4.0 (— — —) and pH 2.0 (— — —) and of the equilibrium unfolding intermediate of mPrP(121–231) (——) at pH 4.0. The spectra were recorded at 22 °C on a Jasco J710 CD spectropolarimeter at protein concentrations of 0.3–0.6 mg/ml using a 0.02 cm cuvette. Buffers were the same as those used for measuring the unfolding/refolding equilibria. Spectra were corrected for the buffers.
3.2.5 NMR-studies of mPrP(23–231)

To determine the NMR structure of mPrP(23–231), the recombinant protein was expressed in E. coli BL21(DE3) with uniform $^{15}$N-labeling and purified as described for non-labeled mPrP(23–231) (final yield: 2.7 mg/liter bacterial culture). The protein was even more soluble as mPrP(121–231), so that the measurements were carried out at protein concentrations of 1.2 mM at pH 4.5 and 20 °C in a solvent of 90% H$_2$O/10% D$_2$O containing 0.01 mM EDTA, 0.01 mM PMSF, 0.1 μM Pepstatin and the protease inhibitor cocktail COMPLETE™ (Boehringer Mannheim). The addition of several protease inhibitors was necessary to obtain a stable preparation of mPrP(23–231), because the N-terminal segment 23–118 proved to be particularly susceptible to degradation by impurities of cytoplasmic E. coli proteases.

Figure 26: The structure of the full-length mouse prion protein consists of the folded C terminal domain, residues 126–231, and a flexibly-disordered and extended N-terminal tail (residues 23–125).
The NMR studies revealed that the globular three-dimensional structure of the C-terminal domain is preserved in the intact protein, and that the N-terminal polypeptide segment 23–125 is extended and flexibly disordered in solution (Fig. 26). This result is on the one hand based on the nearly complete sequence-specific resonance assignment of residues 121–231 in intact mPrP(23–231) for the C-terminal domain mPrP(121–231) which proved to be essentially indicated to the sequence-specific assignments for the backbone $^{15}$N, $^1$H$^N$ and $^1$H$^\alpha$ atoms. The assignments for mPrP(23–231) were obtained from 2D $^{[15]N,^1H}$-COSY and 3D $^{15}$N-resolved $[^1H,^1H]$-NOESY.

The practically unchanged $^{15}$N and $^1$H$^N$ chemical shifts of mPrP(121–231) in mPrP(23–231) indicate that the three-dimensional structure of mPrP(121–231) is entirely preserved in the intact protein. This finding is supported by the fact that sequential and medium-range NOE connectivities for the three helices and the single turn near the C-terminus comprising residues 222–226 are also present in mPrP(23–231). mPrP(23–231) also contains the antiparallel $\beta$-sheet observed for mPrP(121–231), as identified by chemical shifts as well as by a long-range NOE.

The linewidth in heteronuclear $^1$H-$^{15}$N correlation spectra and $^{15}$N($[^1H]$)-NOEs in a $[^{15}N,^1H]$-COSY-relayed $^{15}$N($[^1H]$)-NOE experiment showed that the well-structured residues 126–231 have rotational correlation times of several ns, which is typical for globular proteins of this size, while the correlation times for all residues of segment 23–125 are shorter than 1 ns, which is characteristic for a flexible random coil-like polypeptide chain.

4. Discussion

Within the framework of the "protein-only" hypothesis, the molecular event underlying in the propagation of prions and the pathogenesis of prion diseases seems to be a profound conformational change in PrPC as it is converted into PrPSc (Caughey et al., 1991; Pan et al., 1993). To understand the molecular mechanisms that lead to the conversion of PrPC into PrPSc the knowledge of folding, the thermodynamic stability and the three-dimensional structure of PrPC and its isoform PrPSc are of central interest. Because of the low amounts of PrPC obtained after purification from its natural source and the insolubility of PrPSc, such investigations had been severely hampered in the past. The bacterial expression systems developed in this thesis allowed the production of high amounts of pure and soluble PrPC and opened the door for biophysical and structural studies on PrPC.

The primary structure of mammalian prion proteins is highly conserved between different species, and pairs of sequences are generally more than 90% identical (Schätzl et al., 1995; Billeter et al., 1997). As a sequence identity higher than 25% with a protein of known structure is generally sufficient for a prediction of the overall fold of a new protein, all mammalian prion proteins are likely to possess essentially the same three-dimensional structures. Therefore, the biophysical and structural studies on the mouse prion protein, which are presented in this work should also be representative for structure-function relationships in all other mammalian prion proteins.
Identification of the carboxy-terminal domain, \textit{mPrP(121–231)}

Several approaches to investigate the structure of PrP\textsuperscript{C} and its isoform PrP\textsuperscript{Sc} had been explored until 1996, e. g. structural investigations with PrP peptides of several length (Zhang et al, 1995) and theoretical model predictions for the tertiary structures of PrP\textsuperscript{C} and PrP\textsuperscript{Sc} (Huang et al., 1994; Huang et al., 1996). The models predicted a four-helix-bundle domain of the segment 108–218 of PrP\textsuperscript{C}, whereas reasonable secondary structure predictions failed for the N-terminal region 23–107 (Huang et al., 1994). In order to probe the validity of this model, an efficient bacterial expression system for the presumed four-helix-bundle domain 108–218 of the mouse prion protein should be developed. Production of two different fragments comprising residues 95–231 and 107–231 of the mouse prion protein in the periplasm of \textit{E. coli} led to the identification of an intrinsically stable folding unit, which comprises the C-terminal 111 residues of the complete prion protein and was designated \textit{mPrP(121–231)}. The exact domain borders of \textit{mPrP(121–231)} were deduced from the observation that the expression of the fragments was accompanied by amino-terminally degradation within the predicted first $\alpha$-helix (residues 109 to 122) of PrP\textsuperscript{C}. This finding was surprising because proteolytic cleavage of folded proteins generally occurs at domain borders or within exposed loop regions rather than within regular secondary structure elements (Price and Johnson, 1993) and gave a first hint that the proposed three-dimensional structure for the PrP\textsuperscript{C} segment 108–218 (Huang et al., 1994) was wrong.

In addition, an efficient expression and purification method was established for the production of the intact, full-length prion protein of the mouse (residues 23–231) in \textit{E. coli}. 
Biophysical studies on the recombinant full-length prion protein and its carboxy-terminal domain

The first circular dichroism studies on mPrP(121–231) and mPrP(23–231) revealed that both proteins are rich in α-helical secondary structure elements and possess a defined tertiary structure. The CD spectra of mPrP(23–231) in comparison with those of its carboxy-terminal domain indicated further that the structure of the C-terminal domain is retained in full-length mPrP(23–231) and that the N-terminal polypeptide segment is not significantly involved in tertiary structure formation. This was also confirmed by the florescence spectra of the proteins, which are showing that most of the seven tryptophan residues of the region 23–120 are solvent exposed and not part of a defined structure.

As the spectroscopic data of mPrP(23–231) and mPrP(121–231) are in full agreement with those of PrPc purified from its natural source (Pan et al., 1993), it can be concluded that the three-dimensional structure of recombinant mPrP(23–231) is identical with that of natural PrPc and that the posttranslational modifications as glycosylation at Asn-181 and Asn-197 and fusion with the GPI-anchor have a most minor influence of the folding and solubility of PrPc.

Denaturant-induced unfolding and refolding experiments showed that mPrP(121–231) unfolds highly cooperative and reversible and represents an isolated domain with deduced tertiary structure and a high intrinsic stability. Furthermore, refolding of chemically denatured mPrP(121–231) yields a molecule indistinguishable from the native, recombinant protein. Within the framework of the "protein-only" hypothesis the reversibility of mPrP(121–231) folding was an important result under the assumption that the complete, natural PrPc molecule with its additional posttranslational modifications also folds reversible. If PrPc and PrPSc do possess identical covalent structures
(Stahl and Prusiner, 1991), both isoforms of the prion protein should adopt identical conformation in the presence of high concentrations of urea or GdmCl, so that after refolding in vitro exclusively folded PrPc would be obtained, independent of whether the experiments were started with PrPc or PrPSc. This explains, why all experiments to recover infectivity after solubilisation of PrPSc with high concentrations of urea or guanidinium chloride have so far failed (Prusiner et al., 1993).

Overall, the physico-chemical data demonstrate that the C-terminal domain, mPrP(121–231), possesses a defined three-dimensional structure and that this fold is retained in the complete, cellular prion protein, while the N-terminal polypeptide segment 23–120 seems to be nearly unstructured. The lack of defined structure within the segment 23–120 was not entirely unexpected, because the amino-terminal region of PrPc (residues 51–91) contains five octapeptide repeats (Westaway et al., 1987) and a high content of glycine residues. These findings were confirmed by the determination of the three-dimensional structures of mPrP(121–231) and mPrP(23–231) in solution (Riek et al., 1996; Riek et al., 1997; Riek et al., submitted).

The three-dimensional NMR solution structure of the C-terminal domain of the mouse prion protein, mPrP(121–231)

The three-dimensional structures of recombinant murine mPrP(23–231) and its carboxy-terminal domain mPrP(121–231) were determined in collaboration with the group of Prof. Dr. Wüthrich. The C-terminal domain mPrP(121–231) is the only segment of mPrPc which adopts a defined three-dimensional structure consisting of three α-helices spanning residues 144–154, 175–193
and 200–219, respectively, a two-stranded antiparallel β-sheet spanning residues 128–131 and 161–164 and a short helical turn near the C-terminus.

The structure shows that mPrP(121–231) has an uneven distribution of negatively and positively charged surface residues. The dipolar character of the domain may contribute to the orientation of mPrP^C relative to the cell membrane. The positively charged side of the protein would then be orientated to the cell membrane, whereas the negatively charged side, on which the two glycosylation sites and the single tryptophan are located, would be exposed to the solvent. Another remarkable feature of the structure of mPrP(121–231) is the fact, that the first α-helix is relatively isolated from the rest of the structure. This helix is predominantly hydrophilic and does not show amphipathic character.

A systematic search of the Brookhaven data bank (Bernstein et al., 1977) with the program DALI (Holm et al., 1994) showed that mPrP(121–231) has a so far unknown protein fold and no proteins with a similar fold could be identified. Additionally, the orientation of the three helices is clearly different from the proposed four-helix-bundle model for PrP^C, and the occurrence of the β-sheet was not predicted at all. Regarding the Fourier-transform infrared data on PrP^Sc which indicate an increased β-sheet content in PrP^Sc when compared with PrP^C (Pan et al., 1993), one can speculate that the short, antiparallel β-sheet might be a nucleation site for the conformational conversion to the β-sheet-rich PrP^Sc form, which could also involve helix 1 and the loops connecting helix 1 with the β-strands. In this context, it is interesting to note that the polymorphism at codon 129 in human PrP (methionine or valine), where homozygosity has been linked to predisposition to sporadic CJD (Palmer et al., 1991), lies within the first strand of β-sheet.
Structural aspects of the localization of residues that have been related with inherited human prion diseases and the species barrier

The knowledge of the three-dimensional structure of mPrP(121–231) allows the localization of residues that have been proposed to be important for the species barrier of prion disease transmission and that have been associated with inherited human prion diseases. Within the framework of the "protein-only" hypothesis, the probability of transmission of TSEs between different species decreases with differences in primary structure between their prion proteins. Therefore, mapping of the amino acid replacements onto the three-dimensional structure of the prion protein domain mPrP(121–231) should provide a better understanding of possible roles of individual exchanges for the transmission of prion diseases between different species.

A comparison of 23 mammalian prion protein sequences shows 18 positions with relevant amino acid replacements (Billeter et al., 1997). These amino acid substitutions can be classified into four groups because of their locations in the three-dimensional structure of mPrP(121–231). Three of these regions constitute potential surface recognition sites, which may represent possible binding sites of PrPc for PrPSc from other species or for protein "X", a hypothetical species-specific cofactor that might be involved in the PrPc into PrPSc conversion (Telling et al., 1995; Kaneko et al., 1997).

The murine mPrP(121–231) is highly homologous (94% sequence identity) to the corresponding domain in the human prion protein, which contains 8 out of a total of 11 TSE-related amino acid replacements that have been linked with inherited prion diseases (Prusiner, 1991) (Fig. 27). These eight exchange sites in hPrP(121–231) are identical in wild type human and mouse PrP, and so are the residues that form direct contacts with these amino
acids in the structure. Therefore, \textit{mPrP}(121–231) constitutes a good model system to analyze likely impacts of individual ones of these disease-related amino acid exchanges on the structure and stability of human PrP\textsuperscript{C}.

\textbf{Figure 27}: Ribbon diagram of the NMR structure of \textit{mPrP}(121–231) and location of the point mutation sites that have been related to inherited human prion diseases. Mutation sites are D178N(V129), T183A, E200K, R208H and V210I for the Creutzfeldt-Jakob disease, V180I, F198S and Q217R for the Gerstmann-Sträussler-Scheinker syndrome and D178N(M129) for the fatal familial insomnia.

The mutation sites within the polypeptide segment 121–231 are located within or sequentially adjacent to helix 2 and 3, but none of them is directly located in the relatively isolated first helix. No clusters of amino acid
replacements which are linked to the human TSE phenotypes could be observed in the three-dimensional structure of mPrP(121–231), which excludes the possibility that there are separate disease-specific subdomains in hPrPC that would be responsible for the development of these disease phenotypes.

A previously proposed hypothesis suggested that all 11 amino acid substitutions in the cellular human PrP might destabilize the three-dimensional structure of mPrP(121–231) and thereby facilitate the conversion into PrPSc (Huang et al., 1994).

Inspection of the three-dimensional structure of mPrP(121–231) and recent studies of these mutant proteins produced in E. coli revealed that some of the amino acid replacements lead to a reduced stability, whereas others have no or only a slight influence on the stability of mPrP(121–231) (Riek et al., 1998; Liemann and Glockshuber, submitted). Thus, for example, the mutation Asp178Asn removes the salt bridge Asp178–Arg164, which involves two residues that are highly conserved in the prion proteins of mammalian species (Schätzl et al., 1995; Billeter et al., 1997). Furthermore, the mutation Thr183Ala eliminates two hydrogen bonds that establish a side chain-mediated link between helix 2 and the β-sheet (Thr183OH′–O′Cys179 and Tyr162HN–O′Thr183) and the mutation Glu217Arg eliminates a hydrogen bond with the carbonyl oxygen Ala133 and introduces a positive charge into the otherwise uncharged region. The replacement Phe198Ser causes a major disruption of side chain packing, which leads to a reduced stability of the mutant protein. Only minor effects on protein stability are expected from structural analysis of the remaining point mutations. For example, for the replacements of Val by Ile in either of the positions 180 or 210 there is enough
space to accommodate the somewhat larger Ile side chains without major rearrangement of other side chains.

These considerations from inspection of the three-dimensional structure in addition with the biochemical and biophysical studies of the amino acid replacements in mPrP(121–231) led to the conclusion that destabilization of PrPc cannot be the only general mechanism underlying the facilitated formation of the infectious scrapie agent in inherited human prion diseases (Riek et al., 1998; Liemann and Glockshuber, submitted). Therefore, other mechanisms for the development of inherited TSEs caused by point mutations have to be taken into consideration. These include altered glycosylation patterns, increased stabilities of the variant PrPSc forms, and accelerated kinetics of PrPSc formation from variant proteins.

The three-dimensional NMR solution structure of the full-length, mouse prion protein, mPrP(23–231)

The NMR studies on the murine full-length prion protein revealed that the globular three-dimensional structure of the C-terminal domain, mPrP(121–231) is retained in intact mPrPc and that the N-terminal polypeptide segment 23–125, which is extended and flexible disordered in solution, does not significantly influence its three-dimensional fold (Riek et al., 1997). Similar results were subsequently reported for the full-length prion protein from Syrian hamster and its fragment 90–231 (James et al., 1997; Donne et al., 1997).

The presence of a flexible-ordered N-terminal tail is an important result with regard of the conversion of PrPc into PrPSc, because the segment 90–120 is protected against proteinase K digestion in PrPSc, indicating that residues 90–120 become structured in PrPSc. This view is supported by the
identification of PrP\textsubscript{C}\textsuperscript{-} and PrP\textsubscript{Sc}\textsuperscript{-}specific epitopes in the segment 90–120 with recombinant antibody Fab fragments (Peretz \textit{et al.}, 1997). A structural change in the segment 90–120 thus appears to be the minimal requirement for the transition from PrP\textsubscript{C} to PrP\textsubscript{Sc}.

Within the framework of the "protein-only" hypothesis, segment 90–231 of the prion protein appears to be the minimal infectious unit. This is supported, firstly, by experiments with transgenic mice which exclusively expressed an N-terminally truncated PrP variant lacking residues 32 to 80, showing that the segment 81–231 is sufficient for the generation and propagation of the prion agent (Fischer \textit{et al.}, 1996). Secondly, this segment contains most of the amino acid replacement which have been associated with inherited prion diseases (Schätzl \textit{et al.}, 1995; Prusiner, 1993). Thirdly, the polymorphism at residue 129 in human PrP, which appears to influence susceptibility to the Creutzfeldt-Jakob disease, is also located within this segment (Palmer \textit{et al.}, 1991). These findings raise the question whether mPrP(121–231) contains sufficient information for generation and propagation of infectivity. The corresponding experiment with transgenic mice exclusively expressing mPrP(121–231) has been performed by the group of Prof. Dr. Weissmann (Shmerling \textit{et al.}, 1998). Unfortunately, however, these mice spontaneously develop a disease not related to scrapie and die after about 3 months, so that it remains to be established whether mPrP(121–231) is sufficient for susceptibility to scrapie.
Discussion

mPrP(121–231) adopts an alternative conformation under acid conditions

Folding studies of mPrP(121–231) show that the conformational differences between PrPC and PrPSc subunits may not be restricted to the prion protein segment 90–120. These urea-dependent unfolding and refolding studies of mPrP(121–231) at different pH demonstrate the presence of an equilibrium unfolding intermediate, which is populated under acidic conditions. The intermediate shows the spectral characteristics of a pure β-sheet protein and is in rapid equilibrium with native mPrP(121–231), which is in accordance with the nucleation/condensation model for the propagation of the PrPSc oligomer (Jarret and Lansbury, 1993). This model predicts a fast equilibrium between monomeric PrPC and monomeric PrPSc and a rate-limiting step in which PrPSc monomers form an oligomer of critical size, which acts as nucleus, for further irreversible incorporation of PrPSc monomers into the growing PrPSc oligomer. Within the framework of this model, the equilibrium between PrPC and PrPSc should depend on the environment and might also be sensitive to point mutations in PrPC. Thus, a shift from neutral pH to acidic pH could lead to a higher fraction of PrPSc monomers and consequently to an acceleration of PrPSc nucleus formation.

Interestingly, the cellular prion protein appears to be clustered in cholesterol-rich invaginations of the cell surface, in so-called caveolae, where physiological pH is prevalent, while the endosomal lumen is the compartment in which insoluble PrPSc amyloid accumulates in scrapie-infected cells (Arnold et al., 1995; Borchelt et al., 1992) and where the pH varies between 4.0 and 6.0 (Lee et al., 1996). The findings indicate that the propagation of the infectious agent may occur during endocytosis at acidic pH. Since the acid-induced equilibrium unfolding intermediate of mPrP(121–231) may resemble
a precursor of the PrPSc-oligomer, the propagation of the infectious agent could occur by removing the acid-induced intermediate from the equilibrium by irreversible incorporation into the PrPSc-oligomer, triggering formation of PrPSc to re-establish the equilibrium.

physiological pH:

\[ N \leftrightarrow U \]

endosomal pH:

\[ N \leftrightarrow I \leftrightarrow U \]

Importantly, acid pH has also been demonstrated to promote the formation of amyloid fibrils of another amyloidogenic protein, transthyretin, which represents the main β-sheet amyloid deposit of familial amyloidotic polyneuropathy (FAP) (Lai et al., 1996).

An equilibrium unfolding intermediate was also observed for the recombinant fragment 90–231 of hamster and human PrP, respectively, in the presence of guanidinium chloride (Zhang et al., 1997; Swietnicki et al., 1997). The data of mPrP(121–231), however, show that the formation of the equilibrium unfolding intermediate is an intrinsic property of the C-terminal domain mPrP(121–231) and is independent of an additional folding process within the segment 90–120. The pH-dependence of folding pathway and stability of mPrP(121–231) indicates that some of the conformational differences between PrPC and PrPSc may also include the region 121–231. This is in agreement with the finding that the only PrPSc-specific antibody that
is presently available exclusively recognizes epitopes of the C-terminal domain of the prion protein (Korth et al., 1997).

\[ mPrP(23-231) \] does not form an equilibrium unfolding intermediate under acidic conditions in significant amounts

Unfolding and refolding studies on \( mPrP(23-231) \) in comparison with those on \( mPrP(121-231) \) revealed that the full-length prion protein is reproducibly slightly less stable than its carboxy-terminal domain and that it does not form the intermediate in significant amounts. The lower stability of the full-length prion protein might be explained by an interaction between the N-terminal polypeptide segment with the segment 121–231 forming residual structure in the unfolded state, which should lower the difference in accessible surface area between the unfolded and folded state of a protein (Myers et al., 1995). A similar interaction may also prevent the formation of the intermediate of \( mPrP(23-231) \). Interestingly, such an interaction has recently been proposed from NMR data for the native hamster full-length prion protein (Prusiner, 1997; Donne et al., 1997). The studies on this protein indicate a transient interaction between the flexibly-disordered N-terminal polypeptide and the second helix of the C-terminal domain (Prusiner, 1997; Donne et al., 1997). This interaction could principally strongly lower the conformational entropy of the N-terminal segment (Ladumer and Fersht, 1997; Nagi and Regan, 1997), but the explanation that the stability of \( mPrP(121-231) \) is reduced by an interaction between residues from the N- and C-terminal part of the protein in the unfolded state seems to be most likely. Indeed, a comparison of the transitions of \( mPrP(121-231) \) and \( mPrP(23-231) \) in urea shows that the somewhat lower free energy of folding of the full-length prion protein (−22.3 kJ/mol compared to
-28.6 kJ/mol for mPrP(121–231)) exclusively results from a reduced cooperativity of the truncation, while the transition midpoints are identical.

The observation that the complete, intact murine prion protein does not populate an acid-induced unfolding intermediate in significant quantities and that N-terminally truncated segments of the prion protein are present in normal and prion-infected brains (Chen et al., 1995) as well as in scrapie-infected cells (Rogers et al., 1993) might be explained by the following mechanism of prion formation and propagation as a modification of the nucleation-polymerization model (Jarret and Lansbury, 1993).

The formation of N-terminal truncated segments of the full-length prion protein may occur by proteolyses in endosomes or lysosomes (Caughey and Raymond, 1991; Caughey et al., 1991; McKinley et al., 1991a; Borchelt et al., 1992). These N-terminal truncated fragments may then form the acid-induced equilibrium unfolding intermediate which may be able to form a nucleus by an oligomerisation process. After this hypothetical nucleus has reached a critical size, further truncated PrP as well as full-length prion protein molecules may become incorporated into the growing oligomeric PrPSc. Incorporation of full-length PrP may hereby occur by an interaction of the nucleus with the N-terminal region of the full-length prion protein and switching of this towards the nucleus, whereby a conformational change from the cellular form of the prion protein into the infectious scrapie form may be facilitated.

This is supported by the finding that insoluble PrPSc amyloid accumulates in the endosomal lumen (Arnold et al., 1995; Borchelt et al., 1992) and that the intermediates of mPrP(121–231) and hPrP(90–231) are exclusively formed at acidic pH which is present in these compartments (Lee et al., 1996). Thus, truncation and conversion of PrPC into the infectious scrapie form may well occur during endocytosis at acidic pH.
Conclusions

In conclusion, the physico-chemical and structural studies on the murine full-length prion protein and its C-terminal domain, mPrP(121–231), carried out in this work yielded the first structural information on the cellular prion protein. It was demonstrated that mPrP(121–231) constitutes an autonomous folding unit and represents the only segment of mPrPC with a defined three-dimensional structure. An important result from the three-dimensional structure of mPrPC was that the segment 90–120, which is flexibly disordered in PrPC and becomes protease-resistant in PrPSc, must undergo a significant conformational change during conversion from PrPC into PrPSc. Although the exact molecular mechanism of PrPSc formation remains to be elucidated, the identification of a folding intermediate which is only formed after truncation of the N-terminal residues 23–89 of PrPC under acidic conditions, and the intrinsic folding properties of mPrP(121–231) represent a first step in understanding the molecular events underlying the generation of prion diseases and provide a basis for the rational design of future in vitro and in vivo experiments for the long-term goal of developing diagnostics and therapeutics for prion diseases and, eventually, proving or disproving the "protein-only" hypothesis.
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### 6. Appendix

#### 6.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>2D</td>
<td>2-dimensional</td>
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<tr>
<td>3D</td>
<td>3-dimensional</td>
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<tr>
<td>BSE</td>
<td>bovine spongiform encephalopathy</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>COSEY</td>
<td>correlation spectroscopy</td>
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<tr>
<td>FFI</td>
<td>Fatal familial insomnia</td>
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<tr>
<td>GdmCl</td>
<td>guanidinium chloride</td>
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<tr>
<td>GSS</td>
<td>Gerstmann-Sträussler-Scheinker syndrome</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>hPrP</td>
<td>human prion protein</td>
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<tr>
<td>mPrP</td>
<td>mouse prion protein</td>
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<tr>
<td>mPrP(121–231)</td>
<td>fragment of the mouse prion protein comprising residues 121–231</td>
</tr>
<tr>
<td>mPrP(23–231)</td>
<td>complete polypeptide of the mature mouse prion protein</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<tr>
<td>NOESY</td>
<td>NOE spectroscopy</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PrP</td>
<td>prion protein</td>
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<tr>
<td>PrPC</td>
<td>cellular isoform of PrP</td>
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<tr>
<td>PrPSc</td>
<td>scrapie isoform of PrP</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>------------------------------------------------------</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TFE</td>
<td>2,2,2-Trifluoroethanol</td>
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<tr>
<td>TSE</td>
<td>transmissible spongiform encephalopathy</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>vCJD</td>
<td>new variant Creutzfeldt-Jakob disease</td>
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6.2 Publications


LETTERS TO NATURE

NMR structure of the mouse prion protein domain PrP(121-231)

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The 'protein only' hypothesis states that a modified form of normal prion protein triggers infectious neurodegenerative diseases, such as bovine spongiform encephalopathy (BSE), or Creutzfeldt–Jakob disease (CJD) in humans. Prion proteins are thought to exist in two different conformations: the 'benign' PrPc form, and the infectious 'scrapie form', PrPSc. Knowledge of the three-dimensional structure of PrPc is essential for understanding the transition to PrPSc. The nuclear magnetic resonance (NMR) structure of the autonomously folding PrP domain comprising residues 121–231 (ref. 6) contains a two-stranded antiparallel β-sheet and three α-helices. This domain contains most of the point-mutation sites that have been linked, in human PrP, to the occurrence of familial prion diseases. The NMR structure shows that these mutations occur within, or directly adjacent to, regular secondary structures. The presence of a β-sheet in PrP(121–231) is in contrast with model predictions of an all-α structure. The presence of a β-sheet in PrP(121–231) is important for the initiation of the transition from PrPc to PrPSc.

The NMR structure of PrP(121–231) (Fig. 1a and Table 1) contains three α-helices and a two-stranded antiparallel β-sheet. The approximate lengths of the helices are from residues 144 to 154, 179 to 193, and 200 to 217, and the lengths of the β-strands range from residues 128 to 131, and 161 to 164. The first turn of the second helix and the last turn of the third helix are linked by the single disulphide bond in the protein. The twisted V-shaped arrangement of these two longest helices forms the scaffold onto which the short β-sheet and the first helix are anchored. At the present stage of refinement, all regular secondary-structure elements and the connecting loops are well defined (see Table 1 and Fig. 1d, e), with the sole exception of residues 167 to 176, and 203 to 218. The second helix (Val 161), the first, mostly hydrophilic, helix (Tyr 150), the third helix (residues 203, 206, 209, 210, 213 and 214), the β-sheet (Val 161), the first, mostly hydrophobic helix (Tyr 150), and three loop regions (residues 134, 137, 139, 141, 157, 158 and 198). With the exceptions of Ile 139, Ile 184 and Val 203, the residues of the hydrophobic core are invariant in the known mammalian prion protein sequences, whereas PrP(121–231) is otherwise characterized by a markedly uneven distribution of positively and negatively charged residues (Fig. 1b, c).

Mature mouse PrPc is a glycosylated 208-residue protein (codons 23–231, with deletion of codon 55 (ref. 9)) that is attached to the cell surface by means of a glycosyl phosphatidylinositol anchor at its carboxy-terminal Ser 231 (ref. 10). It is stable against degradation in E. coli, folds cooperatively and reversibly at pH 7, has an intraprotein AMBER energy (kcal mol−1), and is soluble at 1 mM concentration in distilled water between pH 4.0 and pH 8.5 (ref. 6). This segment contains six of nine point-mutation sites in mature PrP that have been associated with familial prion diseases (Fig. 3a), as well as glycosylation sites of PrP and its single disulphide bond. Therefore, we chose to use PrP(121–231) for the present NMR structure determination. This choice was also supported by the demonstration that the segment 81–231 of mouse PrP is sufficient for propagation of the prion disease in vivo9, indicating that the C-terminal part of PrP is of special functional importance.

The context of the structure predictions for PrPc (ref. 8), the β-sheet in the NMR structure of PrP(121–231) is an unexpected feature. Evidence for the identification of the β-sheet is shown in Figure 1. The NMR structure of PrP(121–231) was calculated with the program DIANA21. Starting from 103 randomized structures, 20 conformers with the lowest DIANA target function values were energy minimized in a water shell of 6 Å minimal thickness, using the program OPAL (P. LugrinBüh, P. Güntert, M. Billeter and K. Wüthrich, submitted) with the AMBER force field.22

![PrP(121-231) NMR structure](image)

**Table 1.** Parameters characterizing the NMR structure determination of PrP(121–231)

| Extent of assignments (backbone and side chain H, H, backbone H) | 93% |
| Number of distance constraints | 1,368 |
| Number of dihedral angle constraints | 227 |
| Distance constraint violations >0.1 Å (per conformer) | 1.5 ± 1.3 |
| Dihedral angle constraint violations >2.5° (per conformer) | 0.15 ± 0.36 |
| Intra-protein AMBER energy (kcal mol−1) | −5,041 ± 97 |
| R.m.s.d. to the mean for N, Cα and C of residues 125–166 and 177–219 | 1.4 Å |
| R.m.s.d. to the mean for all heavy atoms of residues 125–166 and 177–219 | 2.0 Å |

The NMR structure of PrP(121–231) was calculated with the program DIANA. Starting from 100 randomized structures, the 20 conformers with the lowest DIANA target function values were energy minimized in a water shell of 6 Å minimal thickness, using the program OPAL (P. LugrinBüh, P. Güntert, M. Billeter and K. Wüthrich, submitted) with the AMBER force field.22

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FIG. 1 Globular fold and surface properties of PrP(121–231). a, Ribbon diagram of the structure of the mouse prion protein domain PrP(121–231), indicating the positions of the three helices (yellow) and the antiparallel two-stranded β-sheet (cyan). The connecting loops are displayed in green if their structure is well-defined, and in magenta otherwise. The disulphide bond between Cys 179 and Cys 214 is shown in white. The N-terminal segment of residues 121–124 and the C-terminal segment 220–231 are disordered and not displayed. b, c, Surface of the structure of PrP(121–231). Colours indicate the electrostatic potential, with blue for positive charges, red for negative charges. d, e, Same orientation as a, c. View after 180° rotation about a vertical axis. f, g, h, i, e, View after 180° rotation about a vertical axis. j, k, l, Representation of the precision of the structure determination. k, l, Display of the backbone of PrP(121–231) as a cylindrical rod of variable radius which represents the global displacements among the 20 conformers used to represent the NMR structure (Table 1). l, k, Same orientation as a. Yellow represents residues 125–166 and 177–219, and the disordered loop of residues 167–176 is shown in magenta. m, Superposition of the 20 conformers; colours as in a.

METHODS: For the production of uniformly 15N-labelled and 15N/13C-doubly labelled PrP(121–231), cells of E. coli BL21(DE3) containing the T7-expression plasmid pPrP-C were grown at room temperature in 1 l of minimal medium containing [(15NH₄)₂SO₄ (2 g l⁻¹)] and unlabelled glucose (5 g l⁻¹) or [(15NH₄)₂SO₄ (2 g l⁻¹) and [(13C₆)]glucose (2 g l⁻¹), respectively. After induction with isopropyl (1 d thiogalactoside (final concentration 1 mM) at an absorbance at 550 nm of 0.7, the cells were grown overnight and collected. PrP(121–231) was purified to homogeneity from the periplasmic fraction by anion exchange chromatography on DE52, hydrophobic chromatography on phenyl sepharose and gel filtration on Superdex 200 as described elsewhere. The final yield was 28 mg of 15N-labelled PrP(121–231) and 16 mg of 13C/15N-labelled PrP(121–231), respectively. All NMR spectra were acquired at 20°C either on a Bruker AMX 600 or a Varian Unity+ 750 NMR spectrometer equipped with triple-resonance z-gradient probes. The NMR samples contained 0.8 mM PrP(121–231) in 90% H₂O/10% D₂O at pH 4.5, and no buffer or salts were added. The (1H, 13C and 15N resonances with the backbone were assigned using the uniformly 15N- and 13C/15N-doubly labelled proteins by establishing intra-residual and sequential correlations of the amide 1H and 15N resonances with C', C' and H' signals using three-dimensional (3D) triple resonance experiments and 3D 15N-resolved [1H, 1H]-NOE spectroscopy (NOESY). The side-chain signals were assigned from 3D through bond correlation NMR experiments. Distance constraints for the calculation of the 3D structure were derived from 3D 1H- or 15N-resolved [1H, 1H]-NOESY spectra recorded with a mixing time of 50 ms. Amide proton exchange rates were measured by recording a series of [15N, 1H]-correlation spectroscopy (COSY) experiments immediately after dissolving lyophilized PrP(121–231) in D₂O. The program MOLMOL was used to generate the figure.
proteins with folds similar to PrP(121–231), and the relative orientation of the three helices in PrP(121–231) is clearly different from the proposed four-helix-bundle model.

Mapping onto the three-dimensional structure of PrP(121–231) of sequence variability in mammalian prion proteins, of residues important for the species barrier of prion disease transmission and for predisposition to familial prion diseases, of biochemical properties of the prion protein, and of residues that have been associated with species barrier of prion disease transmission between mice and humans, locations of selected residues in the three-dimensional structure of PrP(121–231).

Fig. 3 Location in the 3D structure of PrP(121–231) of residues involved in sequence variations among mammalian prion proteins, and of residues that have been associated with the species barrier of prion disease transmission and with inherited prion diseases. a, Sequence and regular secondary structure of mouse PrP(121–231). Residues contributing to the hydrophobic core of the domain are underlined; variable residues among mammalian prion proteins are marked with asterisks. Line (1), mutations in human PrP that have been associated with inherited prion diseases (a stop codon at residue 145, which has been reported in addition to these point mutations), is not considered here, nor is the Met232Arg mutation, which is not contained in mature PrP. All of these residues are identical in wild-type human and mouse PrP. The polymorphism at codon 129 in human PrP, where homozygosity appears to increase the susceptibility to sporadic CJD, is marked by italics. Line (2), residues in PrP(121–231) for which experimental evidence has been presented that they contribute to the species barrier of prion disease transmission between mice and humans. b, Locations of selected residues in the three-dimensional structure of PrP(121–231). The backbone is shown in grey and the disulfide bond is shown in yellow. Five residues that may be involved in the species barrier (line (2) in a) are shown in blue. Figure generated using the program MOLMOL.

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COMMUNICATION

 Autonomous and Reversible Folding of a Soluble Amino-terminally Truncated Segment of the Mouse Prion Protein

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Autonomous and Reversible Folding of a Soluble Amino-terminally Truncated Segment of the Mouse Prion Protein

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Prion diseases are assumed to be caused by the infectious isoform, PrP\textsuperscript{*}, of a single cellular surface protein, PrP\textsuperscript{c}. PrP\textsuperscript{*} is an insoluble form of PrP\textsuperscript{c} and is believed to possess a different three-dimensional fold. It may propagate by causing PrP\textsuperscript{c} to adopt its own infectious conformation by an unknown mechanism. Studies on folding and thermodynamic stability of prion proteins are essential for understanding the processes underlying the conversion from PrP\textsuperscript{c} to PrP\textsuperscript{*}, but have so far been hampered by the low solubility of prion proteins in the absence of detergents. Here, we show that the amino-terminally truncated segment of mouse PrP comprising residues 121 to 231 is an autonomous folding unit. It consists predominantly of α-helical secondary structure and is soluble at high concentrations up to 1 mM in distilled water. PrP\textsubscript{(121-231)} undergoes a cooperative and completely reversible unfolding/refolding transition in the presence of guanidinium chloride with a free energy of folding of \(-22 \text{ kJ/mol}\) at pH 7. The intrinsic stability of segment 121-231 is not in accordance with present models of the structure of PrP\textsuperscript{c} and PrP\textsuperscript{*}. PrP\textsubscript{(121-231)} may represent the only part of PrP\textsuperscript{c} with defined three-dimensional structure.

Keywords: prion protein; protein folding; protein stability; proteolytic degradation; periplasmic expression in Escherichia coli

The "protein only" hypothesis (Griffith, 1967) has turned out to be the most suitable working hypothesis for investigating the processes involved in the development and transmission of prion diseases (transmissible spongiform encephalopathies). It proposes that the infectious agent, the prion, is a single protein, termed PrP\textsuperscript{\textbullet}. PrP\textsuperscript{\textbullet} appears to represent a modified form of a natural cellular surface protein of the host, PrP\textsuperscript{c} (Prusiner, 1982). PrP\textsuperscript{\textbullet} is likely to be chemically identical with PrP\textsuperscript{c} (Stahl & Prusiner, 1991), but may possess a different three-dimensional structure (Pan et al., 1993). It may multiply by a PrP\textsuperscript{\textbullet}-catalyzed conformational transition from PrP\textsuperscript{c} to PrP\textsuperscript{\textbullet} (Prusiner, 1991). A large body of evidence including in vitro as well as in vivo experiments has been accumulated in the last few years supporting the protein only hypothesis (Prusiner, 1991; Weissmann, 1994, 1995).

The known sequences of mammalian prion proteins are strikingly similar and pairs of sequences are generally more than 90% identical (Schatz et al., 1995). The mature form of murine PrP\textsuperscript{c} consists of 209 amino acids (Westaway et al., 1987) (corresponding to residues 23 to 231 in hamster PrP) and appears to be necessary for normal synaptic function (Collinge et al., 1994) and long-term survival of Purkinje neurons (Sakaguchi et al., 1996). PrP\textsuperscript{c} has a single disulfide bond between residues 179 and 214, two N-glycosylation sites at residues 181 and 197 and is attached to the cellular surface via a glycosyl phosphatidyl inositol (GPI) anchor at the carboxyl-terminal serine 231 (Stahl & Prusiner, 1991; Figure 1).
Attempts to model the three-dimensional structure of mammalian PrP(23-231) revealed that numerous algorithms failed to predict secondary structures within the amino-terminal part of the protein (residues 23 to 108) including its characteristic, fivefold octapeptide repeat (Huang et al., 1994). However, plausible three-dimensional models for the carboxy-terminal segment of PrP comprising residues 108 to 218 could be elaborated (Huang et al., 1994, 1996; Kazmirski et al., 1995). These models predict that the segment 108-218 in PrPc is a four-helix bundle domain (Figure 1) whose first two helices are rearranged to a four-stranded, anti-parallel β-sheet in PrPsc (Huang et al., 1994; Huang et al., 1996).

A prerequisite for understanding the processes underlying the conversion of PrPc to PrPsc is the knowledge of the thermodynamic stability of PrPc and the reversibility of PrPc folding. To study folding of the predicted four-helix bundle domain 108-218 of PrPc, we recombinantly expressed two segments of mouse PrP comprising residues 95 to 231 and 107 to 231 in Escherichia coli. Both segments were fused to the bacterial OmpA sequence for secretory periplasmic expression to allow formation of the single disulfide bond. Expression yielded large amounts of soluble protein in the periplasmic fraction. However, Edman sequencing revealed that the segments 95-231 and 107-231 were amino-terminally degraded in vivo. All cleavage sites were found within the predicted first helix of PrPc (amino acids 109 to 122) after residues 112, 118, and 120 (Figure 1). This suggested that the segment 121-231, which is resistant to degradation in the periplasm of E. coli, represents an intrinsically stable domain and that residues 108 to 120 are not part of this domain.

When PrP(121-231) was directly fused to the OmpA signal sequence, expression yielded a soluble 13.3 kDa protein with homogeneous aminoterminal. PrP(121-231) was purified to homogeneity in the absence of any detergents by anion exchange chromatography, hydrophobic chromatography and gel filtration (Figure 2). HPLC analysis before and after reduction of purified PrP(121-231) with dithiothreitol revealed that the single disulfide bond was quantitatively formed (data not shown). PrP(121-231) is soluble at high concentrations up to 1 mM in distilled water and stable against aggregation between pH 4 and 8.5. Far-UV circular dichroism (CD) spectra of purified PrP(121-231) revealed two minima at 208 and 222 nm and a mean residue ellipticity of $-15,600$ deg cm$^2$/dmol at 222 nm, which demon-

![Figure 1. Predicted secondary structure elements (helices 1 to 4) and post-translational modifications of the mature, natural mouse prion protein (residues 23 to 231), and attempts to express its amino-terminally truncated segments 93-231 and 107-231 in the periplasm of E. coli. Positions within the predicted helix 1, where proteolytic cleavage of the segments 93-231 and 107-231 occurred in vivo, are marked by vertical arrows. In contrast to segments 93-231 and 107-231, the segment comprising residues 121 to 231 was stable against degradation. The disulfide bond (SS) between cysteine residues 179 and 214, both N-glycosylation sites (CHO) at residues 181 and 197, and the glycosyl-phosphatidyl-inositol anchor (GPI) at the carboxy-terminal residue 231 of natural PrPc are indicated. For efficient cleavage of the OmpA signal sequence by leader peptidase, a serine residue was inserted at the aminoterminal of the recombinant PrP fragments. To avoid a negative charge at the carboxy-terminal residue (Ser231), which is also present in natural PrPc due to the attachment of the GPI anchor, an additional serine residue was inserted before the stop codons, which is also present in unprocessed PrPc. Recombinant PrP (121-231) thus contains an additional serine at the amino and carboxyterminus. The following oligonucleotide primers were used to amplify the carboxy-terminal fragments of the mouse PrP gene by the polymerase chain reaction (PCR) from a mouse c-DNA (short incubation period) template (Fischer et al., 1996). Amino-terminal primers: N1 (95-231), 5'-TGGGCCAACGTGCGAGTACC-CATAATCAGTGGAACAAGC-3', carboxy-terminal primer, 5'-AGGAGGGGAGGGGATCCAAGCTTACTAGCTGGATCTTCTCCCGTCGTAATAG3' polymerase chain reaction (PCR) fragments were digested with NruI and BamHI and cloned into the secretion T7 expression vector pRBI-PDI-T7 (Strobl et al., 1995) which had been cut with Stul and BamHI to remove the genes coding for RBI and DsbA. pRBI-PDI-T7 contains a single Stul site directly at the last codon of the OmpA signal sequence. All cloned genes were verified by dyeideo sequencing. The plasmid for expression of PrP (121-231) was termed pPrP-C. E. coli BL21(DE3) was used as host strain in all expression experiments (Studier & Moffat, 1986). The amino-termini of expressed mouse PrP fragments were determined by Edman sequencing after applying periplasmic extracts to an SDS-15% (w/v) polyacrylamide gel and blotting the PrP fragments onto a PVDF membrane. Bacterial growth and periplasmic extracts were performed as described under Figure 2.  

![Diagram showing predicted secondary structure elements and post-translational modifications of the mature, natural mouse prion protein (residues 23 to 231), and attempts to express its amino-terminally truncated segments 93-231 and 107-231 in the periplasm of E. coli.](image-url)
Figure 2. Purification of recombinant PrP(121-231) from periplasmatic extracts of E. coli BL21(DE3)/pPrP-C. A Coomassie-stained SDS-15% (w/v) polyacrylamide gel is shown. S, molecular mass standard; 1, periplasmatic extract of non-induced cells; 2, periplasmatic extract of induced cells; 3, pooled fractions after anion exchange chromatography on DE52 cellulose; 4, purified PrP(121-231) after chromatography on Phenyl Sepharose and gel filtration on Superdex 200. Cells of E. coli BL21(DE3)/pPrP-C were grown at 25°C in 1 l LB medium containing ampicillin (100 μg/ml) until an A600 of 0.8 to 1.0 was reached. After addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM, the cells were grown overnight at 25°C, harvested by centrifugation and suspended at 0°C in 100 ml 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mg/ml Polymyxin B. After stirring for one hour at 0°C, the suspension was centrifuged at 39,000 g. The supernatant was dialyzed against 5 mM Tris-HCl (pH 8.5) and applied to a DE52 cellulose column (15 ml, Whatman). PrP(121-231) was eluted with a linear NaCl gradient (500 ml) from 0 to 100 mM NaCl in 5 mM Tris-HCl (pH 8.5). Fractions containing PrP(121-231) were combined, mixed with 4 M ammonium sulfate and 0.1 M Mes-NaOH (pH 6.0) to final concentrations of 1.2 M ammonium sulfate and 20 M Mes-NaOH and applied to a phenyl Sepharose column (15 ml, Pharmacia). PrP(121-231) was eluted with a linear gradient (250 ml) from 1.2 to 0 M ammonium sulfate in 20 mM Mes-NaOH (pH 6.0). Fractions containing PrP(121-231) were combined, concentrated to a volume of 6 ml and applied to a Superdex 200 column (320 ml; Pharmacia). PrP(121-231) was eluted with 50 mM sodium phosphate (pH 7.0). Fractions containing pure PrP(121-231) were combined and dialyzed against distilled water. Typically, about 30 mg of pure PrP(121-231) were obtained. The correct amino-terminus of PrP(121-231) was proven by Edman sequencing, and its mass was verified by electrospray mass spectrometry (calculated mass for the oxidized protein, 13,334.8 Da; measured: 13,334.0 Da).

Figure 3. Far-UV circular dichroism spectra of native (continuous line), unfolded (broken line) and refolded (dotted line) PrP(121-231) at pH 7.0 and 22°C (Inset: near-UV CD spectrum of native PrP(121-231)). Spectra were measured at protein concentrations of 0.5 mg/ml in 20 mM sodium phosphate (pH 7.0). The sample of unfolded PrP(121-231) additionally contained 6 M GdmCl. Refolding of PrP(121-231) was achieved by dialysis against 20 mM sodium phosphate (pH 7.0). Spectra were recorded on a Jasco J710 CD spectropolarimeter in 0.2 mm cuvettes for far-UV CD spectra and in 1 cm cuvettes for near-UV CD spectra, and corrected for the buffer. The concentration of PrP(121-231) was determined by its absorbance at 280 nm (A280, mg/ml cm⁻¹ = 1.55; Gill & von Hippel, 1989). The correct amino-terminus of PrP(121-231) was proven by Edman sequencing, and its mass was verified by electrospray mass spectrometry (calculated mass for the oxidized protein, 13,334.8 Da; measured: 13,334.0 Da).

The cooperative and completely reversible unfolding transition (Figure 4). The spectroscopic properties of the refolded protein are identical to those of native PrP(121-231) (Figure 3). Evaluation of the data according to a two-state mechanism of folding (Pace, 1986) yielded a free energy of folding of -21.8 (±1.4) kJ/mol and a midpoint of unfolding at 2.53 M guanidinium chloride (GdmCl). The cooperativity of folding (m-value), which is proportional to the size of the
protein and difference in accessible surface area between the unfolded and folded state (Myers et al., 1995), has a value of 8.6 (± 0.5) kJ mol⁻¹ M⁻¹ GdmCl and is in the range expected for a 13.3 kDa protein (Myers et al., 1995).

We have shown that the amino-terminally truncated segment of the mouse prion protein (residues 121 to 231) is an isolated domain with tertiary structure and high intrinsic stability, whose folding and solubility does not require N-glycosylation at residues 181 and 198. Most importantly, refolding of chemically denatured PrP(121-231) is cooperative and reversible and yields a molecule indistinguishable from the native recombinant protein. We are aware of the fact that the intrinsically stable folding unit PrP(121 to 231) is not in accordance with the proposed three-dimensional structure of the PrP0 segment 108-218 (Huang et al., 1994) as it lacks the first helix (residues 109 to 122) of the predicted four-helix bundle. However, several lines of evidence suggest that the main and possibly the only part of PrP0 with defined three-dimensional structure is represented by residues 121 to 231, and that most of the proposed structural changes linked to the transition from PrP0 to PrPSc may occur within this segment. (1) Amino- or carboxy-terminal secondary structure elements being part of a single, small protein domain generally cannot be deleted without loss of protein stability and cooperativity of folding, since one-domain modules appear to fold in a concerted, cooperative mechanism and not in a hierarchical process (de Prat Gay et al., 1995a,b). (2) It was demonstrated that peptide fragments comprising isolated helices of myohemerythrin, a four-helix bundle protein, spontaneously form helical structures (Dyson et al., 1992). However, a synthetic peptide spanning the first, predicted α-helix (residues 106 to 126) did not exhibit α-helical structure in aqueous solution and appeared to be a mixture of β-sheet and random coil structure (de Gioia et al., 1994). (3) Proteolytic cleavage of a protein domain should occur mainly at exposed loop regions rather than within secondary structure elements (Price & Johnson, 1993). The experimental evidence thus indicates that the fold of PrP(121-231) is incompatible with the structure previously proposed for PrPSc.

It was recently shown that transgenic mice exclusively expressing a PrP variant lacking residues 32 to 80 are still susceptible to infection by mouse PrPSc and capable of propagating the infectious agent, which means that all residues required for the conversion to PrPSc are located within segment 81-231 (Fischer et al., 1996). PrP(121-231) represents 74% of this segment and contains ten of the 13 codons in human PrP, where point mutations are assumed to be associated with inherited prion diseases (Schätzl et al., 1995; Prusiner, 1993). The polymorphism at residue 129 in human PrP, which appears to influence susceptibility to the Creutzfeldt-Jakob disease (Palmer et al., 1991), also lies within PrP(121-231). Therefore, it will be most interesting to see whether transgenic mice exclusively expressing the autonomously folding fragment PrP(121-231) will still be susceptible to scrapie.

The reversibility of PrP(121-231) folding raises principle questions on the folding pathways of PrP0 and PrPSc. When we neglect an autocatalytic mechanism for formation of monomeric PrPSc and simply assume that formation of insoluble, protease-resistant PrPSc-oligomers (PrPSc), is an irreversible process an oligomerization-competent PrPSc monomer may be: (1) by an on-pathway or (2) by an off-pathway folding intermediate of PrP0 (both of which could be molten globule-like; Safar et al., 1994); or (3) the final product of PrP folding.
which would mean that PrP\textsuperscript{C} is a kinetically
trapped folding intermediate of PrP\textsuperscript{Sc}.

\[
\text{unfolded PrP} \rightleftharpoons \text{PrP}\textsuperscript{Sc} \rightleftharpoons \text{PrP}\textsuperscript{C}
\]

(1)

\[
\text{(PrP}\textsuperscript{Sc})\textsubscript{n+1} \rightleftharpoons \text{PrP}\textsuperscript{Sc} \rightleftharpoons \text{unfolded PrP} \rightleftharpoons \text{PrP}\textsuperscript{C}
\]

(PrP\textsuperscript{Sc})\textsubscript{n}

(2)

\[
\text{unfolded PrP} \rightleftharpoons \text{PrP}\textsuperscript{Sc} \rightleftharpoons \text{PrP}\textsuperscript{C}\rightleftharpoons (\text{PrP}\textsuperscript{Sc})\textsubscript{n+1}
\]

(3)

All models predict that the kinetics of formation of PrP\textsuperscript{Sc}-oligomers are strongly dependent on protein concentration, which is consistent with the observation that transgenic mice overexpressing the natural prion protein exhibit a strong increase in susceptibility to infection by prions (Büeler et al., 1993; Fischer et al., 1996). Since examples for protein folding reactions under kinetic control are extremely rare and have so far only been reported for polypeptides with pro-sequences (Baker et al., 1992; Eder et al., 1993a,b), model (3) appears to be most unlikely, and the conformation of recombinant PrP(121-231) may indeed be identical with its three-dimensional fold in the natural PrP\textsuperscript{C} protein.

We believe that the high stability and solubility of PrP(121-231) over a wide range of conditions provides the basis to investigate its kinetics of folding and unfolding, to identify the nature of possible folding intermediates of PrP experimentally (Baldwin, 1996), and to solve the three-dimensional structure of the folded part of PrP\textsuperscript{C}, which is of central importance for understanding the molecular mechanisms underlying the transmission of prion diseases.

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Recombinant full-length murine prion protein, mPrP(23-231): purification and spectroscopic characterization

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Abstract The cellular prion protein of the mouse, mPrPC, consists of 208 amino acids (residues 23-231). It contains a carboxy-terminal domain, mPrP(121-231), which represents an autonomous folding unit with three α-helices and a two-stranded antiparallel β-sheet. We expressed the complete amino acid sequence of the prion protein, mPrP(23-231), in the cytoplasm of Escherichia coli. mPrP(23-231) was solubilized from inclusion bodies by 8 M urea, oxidatively refolded and purified to homogeneity by conventional chromatographic techniques. Comparison of near-UV circular dichroism, fluorescence and one-dimensional 1H-NMR spectra of mPrP(23-231) and mPrP(121-231) shows that the amino-terminal segment 121-230, which includes the five characteristic octapeptide repeats, does not contribute measurably to the manifestation of three-dimensional structure as detected by these techniques, indicating that the residues 121-231 might be the only polypeptide segment of PrPC with a defined three-dimensional structure.

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Key words: Transmissible spongiform encephalopathies (TSEs); Cellular prion protein; Protein conformation; Circular dichroism spectroscopy

1. Introduction

Transmissible spongiform encephalopathies (TSEs) such as the Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle are believed to be caused by a novel class of infectious pathogens, the 'prions' [1-3]. According to the protein-only hypothesis [4-6], the prion consists of an abnormal oligomeric form, PrPsc, of the host-encoded cellular prion protein, PrPC. While mammalian PrPC is a highly conserved, monomeric cell surface glycoprotein, PrPsc forms protease-resistant oligomers with amyloid characteristics [1-3, 7]. PrPC and PrPsc monomers appear to be identical in their covalent structures [7], but an increased β-sheet content has been demonstrated for PrPsc when compared with PrPC [8, 9]. Different kinetic models, such as the 'nucleation-polymerization' model [10] and the 'template assistance' model [11], have been proposed for the mechanism of self-replication of the infectious oligomer (reviewed in [3]).

The murine prion protein, mPrP(23-231), consists of 208 amino acids (residues 23-231 in the numeration of PrP from Syrian hamster, with deletion of residue 55 [12]). It has a single disulfide bond (Cys179-Cys214), two N-glycosylation sites (Asn181 and Asn197) and a glycosyl-phosphatidyl-inositol (GPI) anchor at its carboxy-terminal Ser231 [7]. We previously demonstrated that the recombinant segment 121-231 of mPrP represents a distinct domain that folds autonomously and reversibly [13] and has a well-defined three-dimensional structure formed by three α-helices and a two-stranded antiparallel β-sheet [14].

The exact size of the domain mPrP(121-231) was deduced from the observation that expression of the PrP fragments 95-231 and 107-231 in the periplasm of Escherichia coli was accompanied by amino-terminal degradation, with cleavage at multiple sites in the segment 100-120 [13]. In this paper, we report the purification of the complete, recombinant murine PrPsc protein with intact disulfide bond, its spectroscopic characterization, and comparisons of the polypeptide segment 121-231 in full-length mPrPC with the isolated carboxy-terminal domain, mPrP(121-231).

2. Materials and methods

2.1. Materials

Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany) and SP-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Tryptone and yeast extract were from DIFCO (Detroit, USA) and isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from ACS (Reidelberg, Germany). 1,4-Dithio-DL-threitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and dithiobis(meso-benzozic acid (DTNB) were purchased from Sigma-Aldrich (Deisenhofen, Germany). All other chemicals were of analysis grade.

2.2. Expression and purification of recombinant mPrP(23-231)

The gene coding for mPrP(23-231) was amplified by the polymerase chain reaction from a plasmid harboring the mPrP(23-231) cDNA [15] using the oligonucleotide primers as listed in Table 1.

Table 1

| N-terminal primer | 5'-GACTGATGTCCATACATATGCTAAAGCGTCCAAAGCCTGGAGGGTGGA-3' |
| C-terminal primer | 5'-AGGAGGGGGGGGGGCGGCTGAAGCAGGAGGGGGGGGGGGGGGGG-3' |

The amplified gene was cloned into the plasmid pRBI-PDI-T7 [16] via the NdeI and BamHI restriction sites. In the resulting expression plasmid, termed pPrP(23-231), the PrP gene is under control of the T7 promoter/lac operator sequence. As recombinant mPrP(121-231) [13], the polypeptide expressed with pPrP(23-231) contains an additional Ser at the carboxy-terminus. According to the N-end rule in bacteria [17], we also introduced a Ser at the amino-terminus of...
mPrP(23-231) to minimize proteolytic degradation in the cytoplasm. Thus, recombinant mPrP(23-231) used in this study consists of 210 amino acids. The correct sequence of the amplified gene in pPrP(23-231) was verified by DNA sequencing.

For the production of unlabeled mPrP(23-231) and uniformly 15N-labeled mPrP(23-231), cells of E. coli BL21(DE3) [18] harboring pPrP(23-231) were grown at 37°C in 10 L LB medium, or in 1 L medium with unlabeled glucose (5 g/l) and (15)NH4)2SO4 (1 g/l), respectively. All media contained ampicillin (100 μg/ml). At an optical density (590 nm) of 0.1-0.3 (rich medium) or 0.9-1.1 (minimal medium), IPTG was added to a final concentration of 1 mM and the cultures were grown for another 16 h. The cells were harvested by centrifugation and suspended in 100 ml 150 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl pH 8.0 supplemented with 1 mM PMSF and two protease inhibitor cocktail tablets (COMPLETE® EDTA-free; Boehringer Mannheim, Germany). The bacteria were disrupted in a French Pressure cell (18 000 PSI) and the lysate was centrifuged (4°C, 39 000 x g, 1 h). The insoluble inclusion bodies were washed twice with the above buffer and solubilized in 100 ml 8 M urea, 10 mM Tris/HCl pH 8.0, 1 mM EDTA, 10 mM DTT. After centrifugation (59 000 x g, 22°C, 1 h) the pH of the supernatant was adjusted to 7.0 with HCl and applied to a SP-Sepharose column (20 ml) equilibrated with 8 M urea, 10 mM MOPS/NaOH pH 7.0. mPrP(23-231) was eluted with a linear NaCl gradient (400 ml, 0-600 mM). Fractions containing mPrP(23-231) were combined and the protein was reduced with 10 mM DTT at pH 8.0 (10 mM Tris/HCl) for 1 h at 37°C. After addition of 10% (v/v) acetic acid the solution was applied to a SP-Sepharose column (20 ml) equilibrated with 8 M urea, 100 mM NaCl, 10% (v/v) acetic acid, then washed with 8 M urea, 10 mM MOPS/NaOH pH 7.0, and eluted with a linear NaCl gradient (400 ml, 100-600 mM NaCl).

Oxidation of mPrP(23-231) was performed for 3-16 h at 22°C at a protein concentration of 0.1 mg/ml in 8 M urea containing 50 mM Tris/HCl pH 8.7 and 1 mM CuSO4. The reaction was analyzed by separation of acid-solubilized samples (pH ≤ 2) on an analytical reversed-phase HPLC column of Vydac C18 4.6 x 250 mm at 55°C with a linear gradient from 28% to 40% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA). After addition of one protease inhibitor tablet, 10 μM EDTA and 10 μM PMSF, the denaturant was removed by dialysis against distilled water. Unfolded proteins were precipitated by addition of 10 mM sodium phosphate pH 7.0, 10 μM EDTA and 10 μM PMSF, and the precipitate was removed by centrifugation (39 000 x g, 30 min, 4°C). The supernatant was applied to a SP-Sepharose column (15 ml) and mPrP(23-231) was eluted with a linear NaCl gradient (300 ml; 200-600 mM). Fractions containing homogeneous mPrP(23-231) were pooled and dialyzed against distilled water. The preparations were concentrated by ultrafiltration (39 000 x g, 22°C, 1 h) the pH of the supernatant was monitored by analytical HPLC and the denaturant was subsequently removed by dialysis. mPrP(23-231) with intact disulfide bond was then purified to homogeneity by cation exchange chromatography at pH 7.0 under non-denaturing conditions (Fig. 1). Overall, 5 mg of homogeneous mPrP(23-231) were obtained per liter of bacterial culture with rich medium, and 2.5 mg per liter for growth in minimal medium with 15N-ammonium sulfate as the sole nitrogen source. mPrP(23-231) was purified in H2O at concentrations of 1.5 mM between pH 4.0 and 7.0.

Recombinant mPrP(23-231) was found to be rather sensitive towards proteolytic digestion. Therefore, protease inhibitors were added during the purification of the protein. In the absence of protease inhibitors, we found proteolytic cleavage after residues 116, 118, and 120 (Fig. 1B). Long-term incubation of mPrP(23-231) at 20°C and pH 4.5 in the absence of protease inhibitors resulted in complete degradation of the amino-terminal polypeptide segment 23-120, while the carboxy-terminal domain was not degraded.

2.4. Circular dichroism and fluorescence spectroscopy

Spectroscopic measurements were performed at 22°C with filtered buffer solutions (0.2 μm pore size). Far-UV and near-UV circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter at protein concentrations of 0.2-0.5 mg/ml in 50 mM sodium phosphate pH 7.0. The spectra were recorded in a 0.2 mm cuvette in the far-UV region (180-250 nm) and in a 10 mm cuvette in the near-UV region (250-350 nm).

Fluorescence measurements were carried out with a Hitachi F-4500 fluorescence spectrophotometer in 0.4 x 1 cm cuvettes. An excitation wavelength of 280 nm was used in all experiments.
those of mPrP(121–231). Both mPrP(23–231) and mPrP(121–231) exhibit typical α-helical far-UV CD spectra, with minima at 222 and 208 nm (Fig. 2A). Although the shapes of the spectra are very similar, the mean residue ellipticities of full-length mPrP are significantly less negative than those of its carboxy-terminal domain, indicating that the percentage of residues located in regular secondary structures is higher in mPrP(121–231) than in the full-length protein. In accordance, the recently reported mean residue ellipticities of the segment 90–231 of hamster PrP are also less negative in the far-UV region when compared with mPrP(121–231) [24].

Corresponding observations were made for the near-UV CD spectra of mPrP(23–231) and mPrP(121–231) (Fig. 2B), where spectra of similar shape but lower mean residue ellipticities were obtained for mPrP(23–231) when compared to mPrP(121–231). Since the near-UV CD data relate to the tertiary structure of a protein [25], this would be compatible with the assumptions that the structure of the carboxy-terminal
Fig. 3. Fluorescence spectra of mPrP(23-231) (solid line) and mPrP(121-231) (dashed line) at 22°C in 50 mM sodium phosphate pH 7.0. Identical protein concentrations of 0.75 μM were used. The excitation wavelength was 280 nm.

domain observed in mPrP(121-231) is retained in the full-length protein, and that the amino-terminal segment 23-120 is not significantly involved in tertiary structure formation.

The emission maximum at 345 nm in the fluorescence spectra of mPrP(23-231) (Fig. 3) indicates that most of the 7 Trp residues in the segment 23-120 are solvent exposed and thus not in a hydrophobic environment. Reference values are 320-335 nm for tryptophans in the hydrophobic protein core, and 348 nm for free tryptophan [25].

3.3. One-dimensional $^1$H-NMR spectra

A $^1$H-NMR spectrum of mPrP(23-231) at pH 4.5 in a mixed solvent of 90% H$_2$O/10% D$_2$O (Fig. 4A) shows a chemical shift dispersion and resonance linewidths that are typical for a monomeric protein of size about 200 residues. The lines near 0 ppm are representative for ring current-shifted methyl resonances in well structured globular proteins [26]. The 30 glycyl residues in the segment 23-120 of mPrP(23-231) give rise to narrow, intense lines around 4 ppm. Near 10.2 ppm a group of lines represents the resonances of the indole NH-groups of the 8 Trp residues in mPrP(23-231). In accordance with the fluorescence properties of mPrP(23-231), the small chemical shift dispersion of these lines suggests that the indole rings are not in the interior of a densely packed structural element. The Fig. 4 also affords a comparison of the $^1$H-NMR spectrum of mPrP(23-231) (Fig. 4A) with that of the isolated carboxy-terminal domain mPrP(121-231) (Fig. 4B), for which some well separated resonances are labeled with the previously obtained assignments [14]. Similar patterns of resonance lines near 0 ppm and from 5.0 to 6.3 ppm are seen in mPrP(23-231) and mPrP(121-231), which would be compatible with a situation where the structure observed for the isolated domain mPrP(121-231) would be largely retained in mPrP(23-231).

3.4. Conclusions

Biophysical and structural studies on the cellular prion protein have been hampered in the past by the lack of an efficient expression and purification system that allows the production of milligram quantities of homogenous PrP with intact disulfide bond. We believe that the protocol presented in this study, i.e. production of mPrP in the cytoplasm of E. coli, oxidative refolding from solubilized inclusion bodies and purification by conventional chromatography, provides a suitable tool for obtaining large quantities of pure PrPC for biochemical experiments and isotope-labeled PrPC for NMR studies. Similar to mPrP(121-231) [13,14], mPrP(23-231) is soluble and does not aggregate irreversibly in aqueous solution. Its solubility between pH 4 and pH 7 is $\approx$1.5 mM and thus even higher than that mPrP(121-231), which is around 1 mM [13,14]. Thus, recombinant mPrP(23-231) is suitable for structural studies by NMR, as evidenced by the one-dimensional $^1$H-NMR spectrum in Fig. 4A. The following paper [27] presents a NMR study of the conformational state of the segment 23-120 in the full-length protein and a compar-
ison of the isolated carboxy-terminal domain mPrP(121–231)
with the corresponding polypeptide segment in mPrP(23–231).

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NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231)

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Abstract The recombinant murine prion protein, mPrP(23-231), was expressed in E. coli with uniform 15N-labeling. NMR experiments showed that the previously determined globular three-dimensional structure of the C-terminal domain of mPrP(121-231) is preserved in the intact protein, and that the N-terminal polypeptide segment 23-120 is flexibly disordered. This structural information is based on nearly complete sequence-specific assignments for the backbone amide nitrogens, amide protons and α-protons of the polypeptide segment of residues 121-231 in mPrP(23-231). Coincidence of sequential and medium-range nuclear Overhauser effects (NOE) showed that the helical secondary structures previously identified in mPrP(121-231) are also present in mPrP(23-231), and near-identity of corresponding amide nitrogens and amide proton chemical shifts indicates that the three-dimensional fold of mPrP(121-231) is also preserved in the intact protein. The linewidths in heteronuclear 1H-15N correlation spectra and 15N(1H)-NOEs showed that the well structured residues 126-230 have correlation times of several nanoseconds, as is typical for small globular proteins, whereas correlation times shorter than 1 nanosecond were observed for all residues of mPrP(23-231) outside of this domain.

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Key words: Prion protein; NMR; Protein structure and dynamics; Transmissible spongiform encephalopathies; Correlation time

1. Introduction

The 'protein-only' hypothesis [1,2] claims that transmissible spongiform encephalopathies (TSE) are distinct from infectious processes caused by bacteria, viruses or viroids in that nucleic acids are apparently not essential for the propagation of the infectious agent [3], which has been termed 'prion' [4]. TSEs have been linked with a conformational polymorphism of the 'prion protein' (PrP) [5], where a disease-related transformation of the ubiquitous cellular form of the protein, PrPC, into the infectious scrapie form, PrPSc, is believed to consist of a change from a predominantly α-helical protein to a β-sheet-containing fold [6,7]. Considering the central role thus attributed to PrP, investigations of the three-dimensional structure of this protein are of keen interest. We previously solved the NMR structure of a self-folding C-terminal domain of the cellular form of the murine prion protein, mPrP(121-231) [8-10]. Here, we report a preliminary structural characterization of the intact polypeptide chain of mature murine PrP, mPrP(23-231), in aqueous solution.

Besides the NMR structure determination of mPrP(121-231) [9], discussions on PrP conformations have so far been founded primarily on optical spectroscopy. PrPSc consists of a single polypeptide chain that contains two glycosylation sites, and is attached to the cell surface by a glycosyl-phosphatidylinositol anchor at its carboxy-terminus [11]. After separation from the cell membrane, it is a water-soluble, protease K-sensitive protein for which circular dichroism (CD) spectroscopy indicates a high content of helical secondary structure [12,13]. PrPSc has so far only been observed as an insoluble oligomer that displays resistance to protease K digestion and has characteristics of an amyloid [6,7]. Based on Fourier transform reflection infrared spectroscopy it was concluded that a significant percentage of the polypeptide chain in PrPSc forms β-sheet secondary structure [12,13]. Collection of more detailed structural data is all the more important as no one has succeeded so far to generate infectious PrPSc in vitro, either from previously denatured infectious material, or from recombinant or synthetic PrP or fragments thereof (see also ref. [14]). Three-dimensional structure determinations of both functional forms of PrP, and possibly of refolding intermediates arising in the course of the disease-causing conformational transition, promise to contribute in essential ways to a rational basis for continued investigations on the role of PrP and possibly additional factors in the pathology of TSEs, such as the Creutzfeldt-Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle.

2. Materials and methods

NMR experiments were carried out either with uniformly 15N-labeled murine prion protein, mPrP(23-231), that was prepared as described in the preceding paper [15], or with the 15N-labeled C-terminal domain of mPrP(121-231) [8,9]. The protein concentration was 0.8 mM in a solvent of 90% H2O/10% D2O containing 0.01 mM EDTA, 0.01 mM PMFSF, 0.1 μM Pepstatin and the protease inhibitor cocktail COMPLETE (Boehringer-Mannheim) at pH 4.5 and T=293 K. NMR spectra were recorded on a 750 MHz Varian UNITYPlus spectrometer and on a 500 MHz Bruker DRX spectrometer. Two-dimensional (2D) [1H,15N] COSY spectra [16] were acquired using spectral widths of τ0x=1800 Hz and τ0y=10000 Hz. For mPrP(121-231) the maximal evolution times where tmax=45 ms and tmax=204 ms, and the time domain data size was 160x4096 points. For mPrP(23-231) we used tmax=36 ms, tmax=204 ms and a time domain data size of 128x4096 points. 15N(1H)-NOEs of mPrP(23-231) were measured using the procedure described by Dayie and Wagner [17] with a relaxation delay of 3 s and a proton saturation length of 3 s achieved by applying a train of 120 degree pulses in 10 ms intervals. The dataset was recorded with t0x=2440 Hz, t0y=7000 Hz, tmax=33 ms, tmax=37 ms and a time domain data size of 160x512 points. Three-dimensional (3D) 1H,15N-NOESY spectra [18] were measured with t0x=1800 Hz, t0y=10000 Hz, t0z=10000 Hz, tmax=14 ms, tmax=23 ms, tmax=102 ms, a mixing time τm=40 ms and a time domain data size of 50x460x2048 points. Data process-
Fig. 1. Survey of the global structural characterization of mPrP(23–231) achieved in this paper. The fold that was previously observed in the isolated C-terminal domain, mPrP(121–231) [9], is preserved in the intact protein, and the backbone \(^{15}\text{N}-\text{H}\) moieties of the well-structured residues 126–230 manifest rotational correlation times of several ns, which is typical for a folded protein of this size. Dots represent the 98 residues of the N-terminal segment 23–120, which shows features of a flexible, 'random coil-like' polypeptide, with rotational correlation times for the \(^{15}\text{N}-\text{H}\) groups of \(\tau_c < 1\) ns.

**3. Results and discussion**

For ease of presentation Fig. 1 visualizes the key conclusions from the work reported in this paper: the previously determined three-dimensional fold of the C-terminal domain mPrP(121–231) is preserved in the intact protein. The backbone \(^{15}\text{N}-\text{H}\) moieties in this domain have effective rotational correlation times, \(\tau_c\), of several nanoseconds, which is typical for small globular proteins [21]. The N-terminal polypeptide segment 23–120 has a small dispersion of the proton chemical shifts and \(\tau_c\)-values < 1 ns for the \(^{15}\text{N}-\text{H}\) moieties, which is typical for a flexible 'random coil-like' polypeptide chain. In the following we describe the NMR data that lead to this global structural characterization of mPrP(23–231).

The NMR-spectral analysis in this paper is largely based on the previously obtained, nearly complete sequence-specific resonance assignments for the isolated C-terminal domain mPrP(121–231) [9] and on sequence-specific assignments for the backbone \(^{15}\text{N}, {^1}\text{H}^\alpha\) and \(^{1}\text{H}^\delta\) atoms of the residues 121 to 231 in intact mPrP(23–231). The assignments for mPrP(23–231) were based on sequential NOE connectivities [22–24], using 2D \(^{15}\text{N}, {^1}\text{H}\)-COSY and 3D \(^{15}\text{N}\)-resolved \(^{1}\text{H},{^1}\text{H}\)-NOESY. The assignments are complete with the following exceptions: The Xxx-Pro connectivities in positions 136/137 and 157/158, the segment 165–170 and Phe\(^{170}\), which could be only partially assigned also in mPrP(121–231) [9], and the segment 220–223 (Fig. 2).

Comparison of corresponding \(^{15}\text{N}\) and \(^{1}\text{H}^\alpha\) chemical shifts for the residues 121–231 in mPrP(121–231) and mPrP(23–231) suggests that the three-dimensional structure of this polypeptide segment is very similar in the two proteins. The deviations of the chemical shifts in mPrP(121–231) from the random coil shifts (Fig. 3a and b) are typical for a globular protein and indicate that even minor conformational rearrangements would be manifested by readily measurable shifts [24]. The shift differences between the two proteins (Fig. 3c and d), however, are very small. The ensemble of the chemical shift data in Fig. 3 thus provides strong evidence that the three-dimensional fold of the structurally well-defined polypeptide segment 126–230 in mPrP(121–231) [9] is preserved in the intact mPrP(23–231). In addition, in mPrP(23–231) three helices and a single helical turn near the C-terminus could be identified from the patterns of \(d_{\text{SN}}\), \(d_{\alpha\beta}(i,i+3)\) and \(d_{\alpha\beta}(i,i+4)\) NOE connectivities (Fig. 2) obtained from a 3D \(^{15}\text{N}\)-resolved \(^{1}\text{H},{^1}\text{H}\)-NOESY spectrum recorded with a mixing time of 40 ms [25]. These regular secondary structures are in exactly corresponding sequence locations to those of the helices that were previously identified in mPrP(121–231) [9]. A long-range NOE Tyr\(^{136}\)H\(^\alpha\)-H\(^\delta\)Met\(^{170}\) identified in mPrP(23–231) has a counterpart in the antiparallel β-sheet of mPrP(121–231) [9], and thus supports the presence of a corresponding β-sheet in the intact protein, which is independently implicated by the chemical shifts.
NMR data for mPrP(23–231)/ secondary structure of mPrP(121–231)

Fig 2. Amino acid sequence of the polypeptide segment 121–231 and survey of the sequential and medium-range NOE connectivities observed in mPrP(23–231). The sequential NOE connectivities $d_{NN}$, $d_{QN}$ and $d_{BN}$ are indicated with black bars, and the medium-range connectivities $d_{QN}(i,i+3)$ and $d_{QN}(i,i+4)$ are shown by lines starting and ending at the positions of the residues that are related by the NOE. The sequence locations of the regular secondary structure elements identified in the isolated C-terminal domain mPrP(121–231) are shown above the sequence, with $\alpha$ for helices and $\beta$ for $\beta$-strands.

The structural characterization of mPrP(23–231) as summarized in Fig. 1 is also supported by NMR data that relate to the segmental flexibility of the polypeptide chain, i.e., the linewidth of the $[^{15}\text{N}]^{1}\text{H}]$-COSY cross peaks, and the sign and magnitude of the $[^{15}\text{N}]^{1}\text{H}]$-NOEs [24]. In the $[^{15}\text{N}]^{1}\text{H}]$-COSY spectrum of mPrP(23–231) (Fig. 4a) two qualitatively different kinds of peaks can be distinguished, i.e., a first group of about 100 broad peaks with full linewidths at half height along $[^{15}\text{N}]^{1}\text{H}]$ of about 25 Hz, and a second group of sharp, more intense peaks with linewidths of about 12 Hz (Fig. 5a). Comparison with the $[^{15}\text{N}]^{1}\text{H}]$-COSY spectrum of mPrP(121–231) (Fig. 4b) showed that the positions of the broad peaks in the intact protein coincide very closely with the peaks in the free domain, where the linewidths are about 20 Hz. On the basis of the sequence-specific assignments (Fig. 2) all the broad peaks in mPrP(23–231) could be attributed to residues of the polypeptide segment 126–230, and 6 sharp peaks were attributed to residues 122–125, 231 and 232 (Ser232 is an additional residue in the construct used to express the protein). A count of the remaining sharp peaks revealed approximately 50 peaks in the spectral area outside of the two rectangles in Fig. 4a, and of the order of 25 Gly peaks inside the solid rectangle. Thus, there are about 75 sharp peaks that can be attributed as a group to the total number of 86 non-proline amide groups in the polypeptide segment 23–121, and there is not a single broad peak that could be attributed to the residues 23–121.

$[^{15}\text{N}]^{1}\text{H}]$-NOEs were recorded using a 2D$[^{15}\text{N}]^{1}\text{H}]$-COSY-relayed $[^{15}\text{N}]^{1}\text{H}]$-NOE experiment recorded at a $^{1}\text{H}$ frequency of 500 MHz [17]. Here, one expects peaks with positive sign and relative intensity +1 for $[^{15}\text{N}]^{1}\text{H}]$ groups with effective rotational correlation times $\tau_c \approx 1$ ns, peaks with negative sign and relative intensity $\approx -4$ for $\tau_c \approx 1$ ns, and peaks with very small positive or negative intensities for $\tau_c$-values near 1 ns. These three limiting situations are illustrated in Fig. 5b, where Met129, Ile138, Phe141 and Val180 show positive NOEs, Val122 is almost nulled, and there are three strong negative NOEs that were attributed to $[^{15}\text{N}]^{1}\text{H}]$ moieties in the segment 23–121. In Fig. 4c and d, the subspectra of negative and positive $[^{15}\text{N}]^{1}\text{H}]$-NOEs have been separated. Fig. 4d mimics the spectrum of broad $[^{15}\text{N}]^{1}\text{H}]$-COSY peaks that were as-
Fig. 3. Plots of chemical shift differences for the amide $^{15}\text{N}$ and $^1\text{H}$ resonances of the residues 121-231 in mPrP versus the amino acid sequence. (a) and (b): Difference between the experimental shifts for mPrP(121-231) and the random coil shifts for $^{15}\text{N}$ and $^1\text{H}$, respectively. (c) and (d): Difference between corresponding chemical shifts of $^{15}\text{N}$ and $^1\text{H}$, respectively in mPrP(121-231) and mPrP(23-231). The sequence locations of the regular secondary structures of mPrP(121-231) are indicated in (d), with $\alpha$ for helices and $\beta$ for $\beta$-strands.

4. Conclusions and outlook

The structural characterization summarized in Fig. 1 is to be attributed to the cellular form PrP$^C$, of the mouse prion protein, since it is based on experimental data collected in aqueous solution without addition of chemical denaturants or detergents. The present study answers a key question that has been raised (e.g. [6,26,27]) following the NMR structure determination of mPrP(121-231) [9]: The absence of the N-terminal segment of residues 23-120 in mPrP(121-231) does not significantly affect the C-terminal domain, which has the same three-dimensional fold in the full-length protein (Fig. 1). This is an important result because the polypeptide segment 90-145 in mammalian prion proteins has been reported to have polymorphic traits in structure predictions as well as in experimental studies such as, for example, structure characterization in different solvents [26].

The presence of an extensive polypeptide segment with the properties of a flexible extended coil in PrP$^C$ could enable structural transitions to PrP$^S$ aggregates that might display sizeable $\beta$-sheet content [6,7,28] without major conformational rearrangements in the C-terminal domain 121-231. In particular, the combination of the results presented in this paper with earlier findings that the polypeptide segment 90-120 is also protected against protease K digestion in PrP$^S$ [6,7] emphasizes that there is a major change in the structural arrangement of the residues 90-120 in PrP$^C$ and PrP$^S$.

The global view of the mPrP(23-231) structure in Fig. 1 leaves room for future refinements. For the C-terminal globular domain, extension of the sequence-specific backbone assignments to the amino acid side chains will provide a platform for studies of possible effects of the N-terminal chain extension on subtle features of the tertiary structure. For the N-terminal flexible coil of residues 23-120, sequence-specific assignments will enable a search for possible variations in the degree of flexibility along the sequence. For example, since only about 75 $^{15}\text{N}-^1\text{H}$ peaks have so far been attributed to this molecular region of 86 $^{15}\text{N}-^1\text{H}$ moieties, it will be of interest whether the remainder of the resonances might have escaped detection because of some slow, local exchange processes in this overall highly flexible polypeptide segment. This
might, among others, provide new insight into the structural and functional roles of the Pro-containing octapeptide repeats in positions 51–91 of mPrP(23–231).

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Fig. 5. (a) Cross sections along the ω₂(1H) frequency taken at the positions indicated with horizontal bars in the [15N,1H]-COSY spectrum of Fig. 4a. To allow for the fact that the ω₁(15N) positions of the individual peaks contained in a given cross section may be slightly different, cross sections taken exactly on-resonance for each individual peak in the cross section were added to obtain the correct representation of the relative peak intensities. (b) Cross sections through the [15N,1H]-COSY-relayed 15N{2H} NOE spectrum of Fig. 4, c+d. The resonances belonging to residues in the assigned segment 121-231 of mPrP(23-231) are identified by residue type and sequence position.

Fig. 6. Cross sections through the spectra (a) and (c+d) in Fig. 4, showing the resonances of the indole $^{15}\text{N}^4\text{H}$ moieties of the eight tryptophan residues in mPrP(23–231). The cross sections were obtained as described in the caption to Fig. 5. The peak of the single Trp residue from the assigned polypeptide segment 121–231 is identified in the top trace.
A scrapie-like unfolding intermediate of the prion protein domain PrP(121–231) induced by acidic pH

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ABSTRACT The infectious agent of transmissible spongiform encephalopathies is believed to consist of an oligomeric "scrapie" isoform of the prion protein, PrPSc, of the monomeric cellular prion protein, PrP0. The conversion of PrP0 to PrPSC is characterized by a decrease in α-helical structure, an increase in β-sheet content, and the formation of PrPSc amyloid. Whereas the N-terminal part of PrPSc comprising residues 23–120 is flexibly disorderd, its C-terminal part, PrP(121–231), forms a globular domain with three α-helices and a small β-sheet. Because the segment of residues 90–231 is protease-resistant in PrPSc, it is most likely structured in the PrP0 form. The conformational change of the segment containing residues 90–120 thus constitutes the minimal structural difference between PrPSc and a PrP0 monomer. To test whether PrP(121–231) is also capable to undergo conformational transitions, we analyzed its urea-dependent unfolding transitions at neutral and acidic pH. We identified an equilibrium unfolding intermediate of PrP(121–231) that is exclusively populated at acidic pH and shows spectral characteristics of a β-sheet protein. The intermediate is in rapid equilibrium with native PrP(121–231), significantly populated in the absence of urea at pH 4.0, and may have important implications for the presumed formation of PrPSc during endocytosis.

Prions constitute the infectious agents of fatal neurodegenerative illnesses such as the Creutzfeldt–Jakob disease, the Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia, and kuru in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep (1–3). According to the "protein-only" hypothesis (4–6), the prion is believed to consist mostly, if not entirely, of the oligomeric scrapie isoform prion protein PrPSc, of the host-encoded monomeric cellular prion protein PrP. PrPSc is most likely identical in its covalent structure with PrPSc (7). Both forms of the prion protein thus appear to differ only in their three-dimensional structure, as Fourier-transform infrared spectra and CD spectra indicate that the β-sheet content of PrPSc is significantly increased compared with PrP0 (8, 9).

Two different kinetic models for the self-replication of PrPSc have been proposed. In the first model, the rate-limiting step would be the irreversible autocatalytic conversion of monomeric PrPSc to a monomeric PrPSc subunit, followed by fast oligomerization of the subunits (10). This model has, however, been questioned recently by simulations of the kinetics of prion formation (11). In the second model, a fast equilibrium between monomeric PrPSc and a monomeric PrPSc precursor is proposed and the rate-limiting reaction would be the formation of a PrPSc oligomer, which acts as nucleus for the growth of larger oligomers of the infectious agent (12).

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Abbreviations: PrP, prion protein; PrPSc, cellular PrP; PrP0, PrPSc, insoluble oligomeric "scrapie" isoform of the PrP; PrP(121–231), C-terminal domain of PrPSc comprising residues 121–231.

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pendent of segment 90–120, fully reversible, and an intrinsic property of the C-terminal domain PrP(121-231). We present quantitative data on the population of the intermediate at acidic pH and discuss its possible implications for the present models of prion propagation and the presumed formation of PrPSc during endocytosis.

MATERIALS AND METHODS

Expression and Purification of PrP(121-231). Murine PrP(121-231) was expressed in the periplasm of Escherichia coli BL21(DE3) as described (14), except that the bacteria were grown at 37°C prior to induction and that the expression vector pPrP-CRR was used. This plasmid differs from the described plasmid pPrP-C (14) at the codons for Arg-228 and Arg-229 that were replaced by the most frequent Arg codons caused by the natural codons of Arg-228 and Arg-229 in the mouse PrP gene, which are the rarest Arg codons in E. coli genes. The modified gene encoding PrP(121-231) was amplified from pPrP-C by the PCR with the following oligonucleotide primers: N-terminal primer, 5'-GGCATGGCCGATCCGATGCGGATC-3'; C-terminal primer, 5'-AGAAGAGGGAGGG-GATCCAAGCTTACTAGCTGGAACGACGCCCGTGT-3'. The amplified gene was cut with NruI and BamHI and cloned into the plasmid pRBI-PDI-T7 (23) that had been digested with StuI and BamHI. pPrP-CRR provided about 2-fold higher yields of PrP(121-231) compared with pPrP-C. In addition, a modified form of PrP(121-231) with an apparent molecular mass that was about 1 kDa higher on SDS/polyacrylamide gels and that accumulated to about 10% was also observed. pPrP-CRR was used.

Urea-Induced Unfolding Transitions of PrP(121-231). We investigated the pH dependence of the urea-induced unfolding/refolding transition of mouse PrP(121-231) at 22°C and a constant ionic strength of 88 mM. The transitions were monitored by the far-UV CD signal of PrP(121-231) at 222 nm. Fig. 1A shows the urea-induced unfolding and refolding of PrP(121-231) between pH 4.0 and 7.0. All transitions were completely reversible, and no protein aggregation was observed under the conditions used. The transitions at pH 6.0 and 7.0 are apparent two-state transitions with similar cooperativities, transition midpoints, and free energies of folding/denaturation (excluding PrP(121-231) at pH 5.0, 6.0, and 7.0, but the plateau is only marginally developed (Fig. 1A). The pH dependence of the intermediate at pH 4.0 is reflected by its fraction in the absence of denaturant, which is 0.2% at pH 4.0 and 0.0002% at pH 4.5.

Acid-Induced Unfolding of PrP(121-231) at Constant Denaturant Concentration. The pH dependence of the formation of the intermediate was followed at a constant urea concentration of 3.5 M where all molecules are native at pH 7.0 and the intermediate is maximally populated at pH 4.0 (see Fig. 1A). Two pH-dependent processes were observed (Fig. 2). The first transition with an apparent pK_a of 4.5 corresponds to the transition from native PrP(121-231) to the unfolding intermediate. The second process with an apparent pK_a of 3.3 was assigned to the transition from the intermediate to an unfolded state at acidic pH. The apparent pK_a of 4.5 of the first transition suggests that acidic residues in PrP(121-231) are titrated upon formation of the intermediate. The transitions are significantly steeper than expected from two single acid/base equilibria, demonstrating that several acidic side chains are protonated in each of the transitions.

RESULTS
Fig. 1. (A) Reversible unfolding transitions of PrP(121-231) at 22°C in the presence of urea at pH 4.0 (■), 4.5 (▲), 5.0 (●), 6.0 (▲), and 7.0 (●). (A) Mean residue ellipticities at 222 nm. The solid lines result from fitting the data according to a two-state model for pH 5.0, pH 6.0, and pH 7.0 and according to a three-state model for pH 4.0 and pH 4.5. The corresponding refolding experiments are represented by open symbols. (B) Three-state analysis of the fractions of the native (○), intermediate (●), and unfolded state (▲) of PrP(121-231) at pH 4.0.

Fig. 2. Acid-induced unfolding of PrP(121-231) in the presence of 3.5 M urea monitored by the mean residue ellipticity at 222 nm. Three states were recorded at pH 4.0 (Fig. 3). The shape of the spectrum of native PrP(121-231) is identical with the spectrum at pH 7.0 with minima at 208 and 222 nm (mean residue ellipticity at 222 nm, −13,600 degrees-cm²/dmol) that are diagnostic for the high α-helix content in PrP(121-231). In contrast, the spectrum of the intermediate has a minimum at 215 nm with a mean residue ellipticity of −7,000 degrees-cm²/dmol that indicates a high content of β-sheet secondary structure and a strong decline of α-helix content in the intermediate (26).

Kinetics of the Formation of the Intermediate. The kinetics of formation of the equilibrium unfolding intermediate were analyzed at pH 4.0. The native protein and the unfolded protein (in 8 M urea) were diluted with buffer to a final urea concentration of 3.5 M where the intermediate is maximally populated. In both cases, the CD signal of the intermediate was formed within the dead time of manual mixing (about 15 s; data not shown), demonstrating that the equilibria between the native or unfolded state and the intermediate of PrP(121-231) are very rapid.

DISCUSSION

In this study we have demonstrated that the C-terminal domain of the prion protein (i.e., the only segment in PrPC with defined tertiary structure) can adopt an alternative conformation at acidic pH with spectral characteristics of a β-sheet protein. Therefore, structural differences between PrPC and PrPSc subunits may not be restricted to the prion protein segment 90–120, and the C-terminal domain PrP(121-231) may also undergo a conformational change during the conversion of PrPC into PrPSc. This is strongly supported by the fact that the epitope recognized by the only PrPSc-specific antibody that is presently available is composed of three segments from PrP(121-231) (27). An acid-induced unfolding intermediate with β-sheet properties has recently also been observed during guanidinium chloride-induced unfolding transitions of the recombinant fragment 90–231 from human PrP (21). We have

| Table 1. Thermodynamic parameters of urea-induced folding of PrP(121-231) between pH 4.0 and pH 7.0 at 22°C |
|----------------------------------|----------------|----------------|----------------|----------------|
| Parameter                        | pH 7.0         | pH 6.0         | pH 4.5         | pH 4.0         |
|                                  | ΔGnu, kJ mol⁻¹  | mNu, kJ mol⁻¹ M⁻¹ | Urea, M      | ΔGnu, kJ mol⁻¹  | mNu, kJ mol⁻¹ M⁻¹ | Urea, M      |
| ΔGnu, kJ mol⁻¹                   | −28.6 ± 0.95   | 4.73 ± 0.16    | 6.05          | 3.65           | 8.53 ± 0.41      | 3.65          |
| mNu, kJ mol⁻¹ M⁻¹                | 6.05           | 5.72           |              | 8.53 ± 0.41    | 7.12 ± 0.48      |              |
| Urea, M                         |              |                |              | 3.65           | 2.18            |              |
| ΔGhi, kJ mol⁻¹                   | −31.1 ± 1.3    | 7.12 ± 0.48    | 5.86          | −22.4 ± 5.4    | 5.54 ± 0.64      | 4.94          |
| mhi, kJ mol⁻¹ M⁻¹                | 8.53 ± 0.41    | 7.12 ± 0.48    |              | 5.86           | 4.94            |              |
| Urea, M                         | 3.65           | 2.18           |              | 5.86           | 4.94            |              |

In this study we have demonstrated that the C-terminal domain of the prion protein (i.e., the only segment in PrPC with defined tertiary structure) can adopt an alternative conformation at acidic pH with spectral characteristics of a β-sheet protein. Therefore, structural differences between PrPC and PrPSc subunits may not be restricted to the prion protein segment 90–120, and the C-terminal domain PrP(121-231) may also undergo a conformational change during the conversion of PrPC into PrPSc. This is strongly supported by the fact that the epitope recognized by the only PrPSc-specific antibody that is presently available is composed of three segments from PrP(121-231) (27). An acid-induced unfolding intermediate with β-sheet properties has recently also been observed during guanidinium chloride-induced unfolding transitions of the recombinant fragment 90–231 from human PrP (21). We have
unfolded state. This is consistent with the nucleation/high absorbance of the denaturant the presence of urea could not be recorded below 210 nm due to the was measured at pH 2.0 in 3.5 M urea (see Fig 2) Spectra recorded 231) and the intermediate were recorded at pH 4.0 and zero M and 3.5 M urea, respectively. The spectrum of acid denatured PrP(121-231) was measured at pH 2.0 in 3.5 M urea (see Fig 2). Spectra recorded in the presence of urea could not be recorded below 210 nm due to the high absorbance of the denaturant.

now shown that the formation of the acid-induced intermediate is an intrinsic property of the C-terminal domain PrP(121-231) and independent of segment 90–120.

Monomeric folding intermediates of amyloidogenic proteins have previously been proposed as precursors of amyloid for a variety of amyloidogenic diseases (28, 29). We have demonstrated that the equilibrium unfolding intermediate of PrP(121–231) is in a rapid equilibrium with the native and unfolded state. This is consistent with the nucleation/condensation model for the formation of the infectious PrPSc oligomer (12), which postulates a fast equilibrium between monomers of PrPSc and PrPc and a rate-limiting association of PrPSc monomers to an oligomer of critical size that forms a nucleus for further irreversible incorporation of PrPSc monomers into a growing PrPSc oligomer. Within the framework of this model, any change in solvent conditions that shifts the PrPc/PrPSc equilibrium toward PrPSc would accelerate the formation of oligomeric PrPSc. A change in solvent conditions, namely a shift from physiological to acidic pH, may indeed occur during the conversion of PrPc into PrPSc in vivo: The cell surface protein PrPc is normally exposed to physiological pH. PrPc appears to be clustered in cholesterol-rich invaginations of the plasma membrane termed caveolae (30), which may bud from the membrane and fuse with endosomes in a clathrin-independent endocytosis pathway. Intriguingly, insoluble PrPSc amyloid accumulates in the endosomal lumen of scrapie-infected cells (31, 32) where the pH varies between 4.0 and 6.0 (33), indicating that the propagation of the infectious scrapie agent may occur during endocytosis at acidic pH. This agrees with our experimental data that show that the unfolding intermediate of PrP(121–231) with β-sheet characteristics is only populated below pH 5.0 (Fig 2). A quantitative analysis demonstrates that the intermediate is also significantly present in the absence of urea at pH 4.0. The following scheme presents a deduced model for the propagation of PrPSc in endosomes, where the unfolding intermediate (I) should be populated in equilibrium in addition to the native (N) and unfolded (U) state and could be removed from the equilibrium by irreversible incorporation into the PrPSc oligomer.

physiological pH:

\[ N \rightleftharpoons U \]

endosomal pH:

\[ N \rightleftharpoons I \rightleftharpoons U \]

\[ (\text{PrPSc})_n \]

In this context, it is important to note that equilibrium unfolding intermediates are rather frequently observed during protein folding studies at acidic pH and have been extensively analyzed in the case of apomyoglobin (25) and staphylococcal nuclease (34). However, these intermediates generally contain native-like secondary structures, whereas the far-UV CD spectrum of the unfolding intermediate of PrP(121–231) lacks the native α-helical features. The apparent pKs of 4.5 measured for the transition from native PrP(121–231) to the intermediate (Fig 2) is reminiscent of the conformational change in influenza hemagglutinin that is also induced by mildly acidic conditions (pH 5.0) and required for fusion of the viral membrane with the endosomal membrane of infected animal cells (35). The conformational transition in hemagglutinin is, however, irreversible and kinetically controlled (35). For the domain PrP(121–231), the conformational change is reversible but may be followed by irreversible oligomerization of PrP. Importantly, acidic pH has also been shown to promote the formation of amyloid fibrils of another amyloidogenic protein, transthyretin, which represents the main β-sheet amyloid deposit of familial amyloidotic polyneuropathy (36, 37).

The model of an acid-induced unfolding intermediate of PrPSc that acts as possible monomeric precursor of the scrapie agent is also supported by the known amino acid exchanges in human PrP that have been associated with inherited human prion diseases (2): Like the protonation of acidic residues at low pH, the disease-related point mutations in human PrP that involve acidic residues (Asp178Asn and Glu200Lys) remove negative charges. Asp178 and Glu200 are located at the N terminus of the second and third helix of PrP(121–231), respectively (15). The mutations may thus eliminate favorable interactions of these residues with the helix dipoles and facilitate the formation of the intermediate. The fact that the intermediate can be almost quantitatively populated at equilibrium opens the possibility to determine its structure in solution by NMR spectroscopy. Acidic pH and endocytosis may also represent important experimental parameters for successful propagation of the infectious scrapie agent in vitro.

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Hypothesis

Prion protein structural features indicate possible relations to signal peptidases

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Abstract Transmissible spongiform encephalopathies (TSEs) in mammalian species are believed to be caused by an oligomeric isoform, PrPSc, of the cellular prion protein, PrPc. One of the key questions in TSE research is how the observed accumulation of PrPSc, or possibly the concomitant depletion of PrPc can cause fatal brain damage. Elucidation of the so far unknown function of PrPc is therefore of crucial importance. PrPc is a membrane-anchored cell surface protein that possesses a so far unique three-dimensional structure. While the N-terminal segment 23-120 of PrPc is flexibly disordered, its C-terminal residues 121-231 form a globular domain with three α-helices and a two-stranded β-sheet. Here we report the observation of structural similarities between the domain of PrP(121-231) and the soluble domains of membrane-anchored signal peptidases. At the level of the primary structure we find 23% identity and 41% similarity between residues 121-217 of the C-terminal domain of murine PrP and a catalytic domain of the rat signal peptidase. The invariant PrP residues Tyr-128 and His-177 align with the two presumed active-site residues of signal peptidases and are in close spatial proximity in the three-dimensional structure of PrP(121-231).

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Key words: Transmissible spongiform encephalopathy; Cellular prion protein; Signal peptidase; Three-dimensional structure; Sequence similarity

1. Introduction

Prions are the infectious agents of fatal transmissible spongiform encephalopathies (TSEs) such as scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans (for reviews see [1-4]). The ‘protein only’ hypothesis [5-7] proposes that the pathogenic component in prions is an oligomeric, protease-resistant form, PrPSc, of the monomeric cellular prion protein, PrPc. PrPSc is most likely identical with PrPc in its covalent structure [8] but possesses a different tertiary structure [9,10] and may propagate by imposing its fold on PrPc [11]. Research on the prion protein has long been dominated by studies of PrPSc because it can be purified in reasonable quantities from diseased brains, and its presence is related with the appearance of clinical symptoms of prion diseases. The discovery that PrPc is a benign, host-encoded protein is of more recent origin [12]. Besides numerous uncertainties about the nature of the infectious agent [13] and the mechanism of propagation of PrPSc [4], one of the key questions in TSE research is how the observed accumulation of PrPSc, or possibly concomitant depletion of PrPc during TSEs can cause neuronal cell death and fatal brain damage [1]. Elucidation of the natural function of PrPc is therefore a most important goal. Although the presence of PrPc is required for the development of TSEs [14,15], mice devoid of PrPc develop normally [14], and only small abnormalities such as reduced survival of Purkinje neurons [16], altered sleep patterns [17] and possibly impaired synaptic function [18,19] have been reported for some strains of knock-out mice. Despite the nearly normal phenotypes of PrP-deficient mice, it cannot be excluded that PrPc might have important functions in wild-type mice, since knock-out mice could possibly adapt during early development to the lack of PrP [1]. The high degree of sequence identity (generally above 90%) among the known mammalian prion protein sequences [20,21] could most readily be rationalized by the assumption of a life-supporting PrPc function.

PrPc from mammals is a cell surface glycoprotein of 209 amino acids (residues 23-231; amino acid numbering according to human PrP [20]), which is anchored to the cell membrane via a glycosyl phosphatidyl inositol (GPI) anchor at its C-terminus and expressed in most cell types [2,8,20]. Rapid progress has recently been made in the physico-chemical and structural characterization of recombinant PrPc expressed in Escherichia coli. Most importantly, the three-dimensional structure of recombinant PrPc in solution has been determined [22-25]. While its N-terminal segment 23-120 is unstructured, the C-terminal segment PrP(121-231) forms a self-folding domain with three α-helices and a two-stranded β-sheet [22,26]. Due to the limited availability and the poor solubility of PrPc it seems unlikely that correspondingly detailed structural and biochemical data will be obtained for this aggregated form of the prion protein in the near future. This situation further underlines the importance of continued investigations on PrPc, since these may also lead to novel insights into the mechanisms which lead to the transformation of PrPc into PrPSc.

Apart from the recent finding that the flexible N-terminal segment of PrPc appears to bind Cu2+ ions in vitro [27] and in vivo [28], the function of PrPc in the cell is still a mystery. This note describes new observations on the level of the primary and tertiary structure of PrPc that indicate a possible relation to membrane-anchored signal peptidases. The initial motivation for this study came from the observation that
preparations of recombinant mammalian prion proteins tend to be subject to partial proteolysis after prolonged incubation under the conditions used for nuclear magnetic resonance (NMR) spectroscopy [29]. As we could not exclude autocatalytic proteolysis of PrP\textsuperscript{Sc}, we analyzed the PrP sequence for possible similarities with proteases. We found striking similarities between the structured C-terminal domain of PrP and the catalytic domains of bacterial and eukaryotic signal peptidases. A relation between PrP\textsuperscript{Sc} and signal peptidases is further supported by inspection of the three-dimensional structure of PrP(121–231).

2. Results

2.1. Identification of sequence similarities between PrP and signal peptidases

In search for a structural basis for a possible proteolytic activity of PrP\textsuperscript{Sc}, a comparison of the mouse PrP sequence with sequences of a variety of proteases did not reveal significant sequence similarities with the major protease families. However, we observed a surprising sequence homology of the C-terminal domain of murine PrP with the catalytic domains of monomeric bacterial signal peptidases, and with the catalytic subunits of eukaryotic signal peptidases. For these membrane-anchored enzymes a three-dimensional structure has so far not been determined. The three previously identified conserved regions in the sequences of bacterial and eukaryotic signal peptidases [30,31] are strikingly homologous to corresponding segments in murine PrP(121–231) (Fig. 1). Within these three consensus regions, PrP is more similar to the catalytic subunits of signal peptidases from mammalians and yeast than to bacterial and mitochondrial signal peptidases (Fig. 1). Specifically, the mouse PrP segment 121–217 is 22.7% identical and 40.9% similar with residues 49–137 of a catalytic subunit of rat signal peptidase (cf. legend of Fig. 1). Seventeen out of the 20 identical residues in mouse PrP and this segment of the rat signal peptidase are invariant in the mammalian PrP sequences [20,21].

The most highly conserved segment among the signal peptidase subunits from rat and yeast, and murine PrP corresponds to the turn between helix 2 and helix 3 in the structure of murine PrP(121–231), i.e. PrP residues 193–197 (cf. Fig. 1). Interestingly, the signal peptides from E. coli and from yeast mitochondria have large insertions of 109 and 28 residues, respectively, before this conserved segment (Fig. 1). Two other strongly conserved regions between PrP(121–220) and the rat signal peptidase which correspond to the PrP segments 121–129 and 159–165, coincide with the antiparallel strands of the \( \beta \)-sheet in the structure of PrP(121–231). A fourth region of high homology between the rat signal peptidase and murine PrP, corresponding to PrP residues 175–186, coincides with the N-terminal half of helix 2 in PrP(121–231). Thus the most highly conserved segments of signal peptidases and PrP are found in the regular secondary structures of PrP(121–231) and the region preceding helix 3. Conversely, non-conserved segments typically correspond to loop regions in the structure of PrP(121–231). For example, the tripeptide insertion of residues 169–171 which is characteristic for PrP when compared to the signal peptidases, is located in the loop between the second \( \beta \)-strand and helix 2, which is the least well defined part in the NMR structure of PrP(121–231) [22]. Variability in this loop is also indicated by the fact that this loop is even longer by 8 amino acids in the chicken prion protein. The only strongly different region between murine PrP and the rat signal peptidase that lies within a regular secondary structure of PrP(121–231) corresponds to the residues 151–157 in the C-terminal half of helix 1. This heptapeptide is absent in the catalytic subunits of the rat and yeast signal peptidases (Fig. 1). Since helix 1 is rather isolated in the structure of PrP(121–231), unusually hydrophilic and does not significantly contribute to the hydrophobic core [22], it seems conceivable that this element of regular secondary structure might be either significantly shorter or possibly absent in an otherwise PrP-like three-dimensional structure of catalytic eukaryotic signal peptidase subunits. The invariant disulfide bridge of mammalian prion proteins between Cys-179 and Cys-214, which forms an important part of the hydrophobic core of PrP(121–231) and connects the helices 2 and 3 [22], is absent in signal peptidases.

2.2. Location of the PrP residues that align with the active-site residues of signal peptidases in the three-dimensional structure of PrP(121–231)

Previous work on the catalytic mechanism of the signal peptidase from E. coli indicates that the enzyme represents a new type of serine protease with a catalytic diad consisting of a nucleophilic serine (Ser-90), which is located in the consensus region 1, and a lysine residue (Lys-146) contained in the consensus region 2 [32–34] (Fig. 1). In the eukaryotic signal peptidases this lysine is replaced by histidine [30,31] (Fig. 1). This histidine is also present in PrP (His-177) and is invariant in the mammalian PrP sequences [20,21]. The PrP residue corresponding to the presumed nucleophilic serine of signal peptidases is an invariant tyrosine (Tyr-128) in all mammalian PrP proteins [20,21]. Strikingly, Tyr-128 and His-177 are in close proximity in the NMR structure of PrP(121–231) (Fig. 2). Although we did not observe a direct contact between Tyr-128 and His-177 in the refined, energy-minimized solution

![Fig. 1. Sequence comparison of the segment 121–220 of the mouse prion protein (residues 121–220) with the three conserved regions in monomeric bacterial signal peptidases and the catalytic subunits of eukaryotic signal peptidase.](image-url)
structure of PrP(121-231), rotation of Tyr-128 about $\chi^1$ and His-177 about $\chi^2$ can readily place the hydroxyl group of Tyr-128 in hydrogen bond distance to the N$^\omega$ nitrogen of His-177 bond (Fig. 2). The side chains of Tyr-128 and His-177 are thus principally capable to form a charged hydrogen bond. Further inspection of the structure of PrP(121-231) and modelling studies revealed that the side chains of Asn-174 and Asn-173, which are located on the opposite side of the ring of His-177, could form an additional hydrogen bond with the N$^\omega$ nitrogen of His-177, resulting in a triad with Tyr-128, His-177, and Asn-174 (or Asn-173) (Fig. 2). This modelled local structure is reminiscent of the active sites of cysteine proteases [35]. Asn-174 and Asn-173 in PrP belong to the flexible loop between the second $\beta$-strand and helix 2, which contains the above mentioned tripeptide insertion found in all mammalian prion proteins. Asn-173 is invariant in all mammalian prion protein sequences. Asn-174 is either strictly conserved or replaced either by Ser or Thr [20,21].

3. Discussion and outlook

The presently described similarity between mammalian prion proteins and signal peptidases may provide a platform for the design of future experiments to elucidate the cellular function of PrPC. The 23% sequence identity between the segment 121-217 of murine PrP and residues 49-137 of the rat signal peptidase alone is only slightly below the limit of 25% sequence identity that is generally assumed to be required for a reliable prediction of structural relationships between different proteins. There are however additional factors that support a relationship between PrPC and these proteases, especially the catalytic subunits of microsomal signal peptidases from eukaryotes. First, the most highly conserved regions of microsomal signal peptidases are also preserved in the prion protein sequence and so are their order and relative distances. The conserved regions also correspond to regular secondary structures in the tertiary structure of PrPC. Second, the PrP residues Tyr-128 and His-177, which align with the presumed active-site residues of signal peptidases [32-34] are in close proximity in the three-dimensional structure of PrP(121-231). Third, signal peptidases and PrPC are both membrane-anchored proteins with similar membrane topology in that they are located at the extracytoplasmic face of the membrane (Fig. 3). The main topological difference is the membrane anchor itself, which is an N-terminal transmembrane domain in the case of signal peptidases and a C-terminal GPI anchor in the case of PrPC. A C-terminal membrane anchor is however also observed for the signal peptidase of the inner mitochondrial membrane [31] (Fig. 3C). The N-terminal part of the soluble catalytic domain of signal peptidases is best known in the enzyme from E. coli where the periplasmic domain starts at residue 76, i.e. 14 residues before the essential Ser-90 [36,37]. This is similar to the C-terminal domain of murine PrPC [23,26,29], which begins seven residues before Tyr-128.

The serine/lysine diad proposed to be the active site of the E. coli signal peptidase is a common motif for the catalytic centers of hydrolases and has also been found in class A $\beta$-lactamases [38,39] and LexA-type proteases [40]. In PrPC the invariant Tyr-128 aligns with the nucleophilic serine of the signal peptidases. Tyrosines are not common nucleophiles in proteases, but are known as essential nucleophiles in type I and type II topoisomerases, where they form a transient phos-
Fig. 2. Location of the PrP residues which align with the active-site residues of signal peptidases in the refined NMR structure of PrP(121-231). A ribbon drawing of the energy-minimized mean solution structure of murine PrP(121-231) [22] is shown with the α-helices in red, the β-strands in light blue and the loop regions in dark blue. The side chains of Tyr-128, His-177 and Asn-174 in the energy-minimized mean structure are shown as yellow stick drawings. A hydrogen bond network reminiscent of the catalytic triad of thiol proteases was generated by rotation of these side chains about the χ^1 angles and χ^2 angles of His-177 and Asn-174. The resulting positions of the side chains are shown as ball and stick models with functional colors, and the hydrogen bonds are shown in green. The figure was generated with the program MOLMOL [48].

In a first attempt to demonstrate an enzymatic activity in the C-terminal domain of murine PrP^C, we tested a series of chromogenic protease substrates for trypsin-, chymotrypsin- and elastase-like protease specificities, but could not detect proteolytic activity (S. Hornemann and R. Glockshuber, unpublished data). This leaves the possibilities that PrP^C could have a very narrow specificity for an unknown substrate or is hydrolytically active only in conjunction with other proteins. The latter seems plausible based on observations made with the signal peptidases of Fig. 1: The microsomal signal peptidases from eukaryotes are hetero-oligomers of five different membrane-anchored subunits. Two of these subunits are believed to be the catalytically active proteins. The mitochondrial signal peptidases are heterodimers of two almost identical, catalytically active subunits [31]. PrP^C might similarly associate with a so far unknown protein at the surface of the cellular membrane and might be active only as a heterodimer. An obvious candidate for this presumed partner protein would be the so-called protein X, which has been postulated to be a species-specific protein involved in the conversion of PrP^C to PrP^{sc} in vivo [45]. Another intriguing aspect of the observed similarities between PrP^C and signal peptidases is the sequence relationship between signal peptidases and the proteases of the LexA family, which also possess a catalytic serine/lysine diad [40,46].

photorysme bond between enzyme and DNA phosphodiester backbone [41-43]. Furthermore, the implicated formation of a triad between Tyr-128, His-177 and Asn-173/Asn-174 in PrP (Fig. 2) is reminiscent of the active sites of cysteine proteases, such as papain with a triad of Cys, His and Asn [35,44].
The crystal structure of the UmuD' protein, a member of this protease family, has been solved [40]. UmuD' is a homodimer of 137 residues generated by autocatalytic cleavage of UmuD through removal of its N-terminal 24-residue segment (cf. [40]). The X-ray structure of UmuD' has shown that the subunits of UmuD' have an unusual β-sheet fold and that the dimers form long filaments in the crystal, which are held together by extended N-terminal tails of the subunits [40].

UmuD' in the crystal thus shares physical properties with the PrP^Sc amyloid [2,9,10,12]. The autocatalytic self-cleavage of UmuD is also reminiscent of our previous finding that the murine PrP^Sc segments 95-231 and 107-231 were N-terminally cleaved during expression in the periplasm of E. coli [26], so that we cannot a priori exclude the presence of in vivo self-cleaved PrP. Although there is no significant sequence similarity between UmuD' and the prion protein, a search for possible relationships between the PrP^Sc oligomer and the filaments of UmuD' dimers observed in the X-ray structure of UmuD' appears to be of potential interest.

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**References**

Erratum

FEBS 20420

Erratum to: Prion protein structural features indicate possible relations to signal peptidases (FEBS 20139)


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The legend of Figure 2 contained some mistakes. The figure and its legend are republished below.

Fig. 2. Location of the PrP residues which align with the active-site residues of signal peptidases in the refined NMR structure of mouse PrP(121-231). A ribbon drawing of the energy-minimized mean solution structure of murine PrP(121-231) [22] is shown with the α-helices in red, the β-strands in light blue and the loop regions in dark blue. The side chains of Tyr-128, His-177 and Asn-174 in the energy-minimized mean structure are shown as yellow stick drawings. A hydrogen bond network reminiscent of the catalytic triad in thiol proteases was generated by rotation of these side chains about the $\chi^1$ and $\chi^2$ angles. The resulting positions of the side chains are shown as ball and stick models with functional colors, and the hydrogen bonds are shown in green. The figure was generated with the program MOLMOL [48].

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Prion protein NMR structure and familial human spongiform encephalopathies

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ABSTRACT The refined NMR structure of the mouse prion protein domain mPrP(121–231) and the recently reported NMR structure of the complete 208-residue polypeptide chain of mPrP are used to investigate the structural basis of inherited human transmissible spongiform encephalopathies. In the cellular form of mPrP no spatial clustering of mutation sites is observed that would indicate the existence of disease-specific subdomains. A hydrogen bond between residues 128 and 178 provides a structural basis for the observed highly specific influence of a polymorphism in position 129 in human PrP on the disease phenotype that segregates with the mutation Asp-178→Asn. Overall, the NMR structure implies that only part of the disease-related amino acid replacements lead to reduced stability of the cellular form of PrP, indicating that subtle structural differences in the mutant proteins may affect intermolecular signaling in a variety of different ways.

A novel class of infectious pathogens, the prions, have been proposed to be the cause of transmissible spongiform encephalopathies (TSE) (1, 2). Prions are distinct from bacteria, viruses, or viroids in that nucleic acids are apparently not essential for the propagation of the infectious agent (3). TSEs include kuru, Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann-Straussler-Scheinker syndrome in humans, scrapie in sheep, and bovine spongiform encephalopathy. They have been reported as sporadic and familial human diseases (1, 2), and all amino acids that are in direct contact with these residues in the refined three-dimensional structure of mPrP are also identical in the two species. On this basis we use the NMR structure of mPrP to investigate likely structural and functional consequences of the disease-related amino acid substitutions in human PrP (APrP) and to critically evaluate a previously advanced general concept that inherited TSEs might be related to destabilization of the three-dimensional structure of PrP (15–17).

MATERIALS AND METHODS

Isotope-labeled mPrP(121–231) was prepared as described previously (14, 19). NMR measurements were performed on Bruker AMX500 and AMX600 and Varian Unity+750 spectrometers. For data processing and spectral analysis we used the programs PROSA (20) and XEASY (21), respectively. Stereo-specific assignments for the methyl groups of Val and Leu were obtained with 10% biosynthetically directed 13C-labeling of the protein (22).

The input of nuclear Overhauser effect (NOE) distance constraints for the structure calculation was obtained from the following 750-MHz data sets: three-dimensional 13C-resolved [1H,15N]-NOE spectra (23) with τm = 50 ms, using 13C/15N-labeled protein; three-dimensional 13N-resolved [1H,15N]-NOE spectra (24) with τm = 50 ms, using 15N-labeled protein; and

inositol anchor at its carboxyl terminus (12). After separation from the cell membrane, mammalian PrP is a water-soluble, protease K-sensitive protein. The NMR structures of intact recombinant mouse PrPc, mPrP(23–231) (13), and its C-terminal domain, mPrP(121–231) (14), have been determined, and this paper presents a refinement of the mPrP(121–231) structure.

Correlations between the molecular structure of prion proteins and their role in the pathology of TSEs previously have been discussed on the basis of structure predictions (15–17). The NMR structure of mPrPc (Figs. 1 and 2, refs. 13 and 14) now provides a basis for such work. The mouse and human prion proteins have identical global folds for the domain of residues 121–231 (R. Zahn, R.R., G.W., and K.W., unpublished work), as expected from the 93% sequence identity (18). All eight sequence positions in this domain for which amino acid substitutions have been related to human genetic TSEs (1) contain identical amino acids in the wild-type mouse and human proteins (18), and all amino acids that are in direct contact with these residues in the refined three-dimensional structure of mPrPc are also identical in the two species. On this basis we use the NMR structure of mPrPc to investigate likely structural and functional consequences of the disease-related amino acid substitutions in human PrP (APrP) and to critically evaluate a previously advanced general concept that inherited TSEs might be related to destabilization of the three-dimensional structure of PrP (15–17).

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Abbreviations: CJD, Creutzfeldt-Jakob disease; PrP, prion protein; PrPc, cellular form of PrP; PrPsc, scrapie form of PrP; APrP, human PrP; mPrP, mouse PrP; mPrP(121–231), fragment of mPrPc; mPrP(22–231), complete polypeptide of mature mPrP; NOE, nuclear Overhauser effect; TSE, transmissible spongiform encephalopathy.

Data deposition: The atom coordinates of the refined mean structure of mPrP(121–231) have been deposited in the Protein Data Bank, Biology Department, Brookhaven National Laboratory, Upton, NY 11973 (PDB ID code 1AG2).

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two-dimensional [1H,1H]-NOE spectra (25) in H2O and 2H2O solution with $\tau_m = 50$ ms, using unlabeled protein. The NOE intensities were calibrated with the tools of the program DYANA (26). Other tools of DYANA were used to eliminate NOEs that represent no true conformational constraints and, where required, pseudo-atoms were introduced with appropriate corrections (27). Supplementary constraints were: From $C^\alpha$ chemical shifts deviating from the random coil values, $-120^\circ < \phi < -120^\circ$ and $-100^\circ < \psi < 0^\circ$ for $\Delta\delta(C^\alpha) > 1.5$ ppm, $-200^\circ < \phi < -80^\circ$ and $40^\circ < \psi < 220^\circ$ for $\Delta\delta(C^\alpha) < -1.5$ ppm (28). With these constraints and intraresidual and sequential NOEs, HABAS (29) yielded 229 constraints on dihedral angles $\phi$, $\psi$, and $\chi^1$. The usual limits were used for the disulfide bond Cys-179—Cys-214 (30).

When using ASNO (31) a final input for the structure calculation of 1,592 NOE upper-limit distance constraints, 229 dihedral angle constraints and the six disulfide bond constraints (30) were obtained. The calculation was started with 50 randomized conformers, and the 20 best DYANA conformers were energy-minimized in a water bath using the AMBER force field (32) in the program OPAL (33). The mean structure of mPrP(121-231) used for the illustrations was obtained by averaging the coordinates of the 20 refined conformers after superposition of the backbone atoms of residues 124-166 and 172-226 and subsequent energy minimization.

RESULTS AND DISCUSSION
The NMR structure of the soluble form of the mouse PrP mPrP(23-231) consists of a globular C-terminal domain of residues 126-226 (Fig. 1a), a flexible unstructured 103-residue N-terminal tail, and a flexibly disordered C-terminal segment...
227–231 (13, 34). Suitably isotope-labeled mPrP(121–231) preparations were used to collect NMR data for a structure refinement of the globular domain. Although the initial structure determination (14) described the global fold and the spatial distribution of the amino acid side chains, the refined structure further affords a detailed description of the side-chain packing (Figs. 1b and 2).

The Refined NMR Structure of mPrP(121–231). Table 1 gives a survey of the solution conditions used, the input of conformational constraints, and the quality of the structure determination. Key data are that the structurally defined polypeptide segments include residues 124–166 and 172–226, as compared with 125–166 and 177–219 in the previous work (14), and that the rms deviation values for the backbone and all heavy atoms of these residues are 0.8 Å and 1.2 Å, respectively, as compared with 1.4 Å and 2.0 Å in ref. 14. The thickness of the yellow cylindrical rod in Fig. 1a represents the variable precision of the backbone structure determination along the sequence from residues 124–226.

The global polypeptide fold of mPrP(121–231) contains three $\alpha$-helices with residues 144–154, 175–193, and 200–219, two $\beta$-strands with residues 128–131 and 161–164, and a short segment of helix-like structure with residues 222–226 (Fig. 1a). Comparison with the initial NMR structure (14) (Fig. 1c) shows that helices 2 and 3 have been extended by four residues at the N-terminal end and by two residues at the C-terminal end, respectively (Fig. 1c) (35), which is a consequence of the improved definition of these segments (36), and the residues 220–226 are somewhat better defined (see below). During the refinement the axis of helix 3 has been slightly shifted relative to the other regular secondary structures.

Although the local precision of the structure determination generally was improved during the refinement, some short peptide segments remain poorly defined. In addition to the flexibly disordered chain-terminal segments 121–125 and 227–231 (14), these segments include residues 167–171 in the loop connecting the second $\beta$-strand with helix 2 and the last two turns of helix 2 with the sequence –Thr188–Val-Thr-Thr-Thr-Thr189. With regard to the pathology-related conformational polymorphism in position 129 of hPrP (37), the $\beta$-sheet in PrPC attracts particular interest. Although residues 129, 131, 161, 163, and 164 show typical $\beta$-sheet deviations of the $^{13}$Cα chemical shifts from the random coil values (38), the $\text{H}^\alpha$ chemical shifts and the $\nu_{\text{HNO}}$ coupling constants in the first
Table 1. Characterization of the energy-minimized NMR structure of \( \text{mPrP}(121-231) \)

<table>
<thead>
<tr>
<th>Quantity</th>
<th>20 conformers</th>
<th>Mean structure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number ±0.1 Å</td>
<td>2.8 ± 1.6</td>
<td>0</td>
</tr>
<tr>
<td>Maximum (Å)</td>
<td>0.10 ± 0.0</td>
<td>0.08</td>
</tr>
<tr>
<td>Residual dihedral angle constraint violations Number ±1.5 deg.</td>
<td>1.7 ± 1.0</td>
<td>0</td>
</tr>
<tr>
<td>Maximum, deg.</td>
<td>1.9 ± 0.3</td>
<td>1.3</td>
</tr>
<tr>
<td>AMBER energies, kcal/mol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>-5,061 ± 67</td>
<td>-4,777</td>
</tr>
<tr>
<td>Van der Waals</td>
<td>-330 ± 19</td>
<td>-348</td>
</tr>
<tr>
<td>Electrostatic</td>
<td>-5,679 ± 73</td>
<td>-5,303</td>
</tr>
<tr>
<td>rms deviation from ideal geometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths, Å</td>
<td>0.0078 ± 0.002</td>
<td>0.0074</td>
</tr>
<tr>
<td>Bond angles, deg.</td>
<td>2.3 ± 0.05</td>
<td>1.62</td>
</tr>
<tr>
<td>Peptide bonds, deg.</td>
<td>9.7 ± 2.3</td>
<td>10.7</td>
</tr>
<tr>
<td>rms deviation to the averaged coordinates, Å</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

\( \text{mPrP}(121-231) \) was studied in aqueous solution containing 0.8 mM protein, \( \text{pH} = 4.5 \), \( T = 20°C \). The structure determination was based on sequence-specific assignments for 98% of all \( ^1 \text{H}, ^{13} \text{C}, \) and \( ^{15} \text{N} \) nuclei. The missing \( ^1 \text{H} \) assignments include all resonances of Asp-167, the backbone resonances of Glu-168, \( ^{13} \text{C} \) of Tyr-169, Ser-170, and Asn-171, the nonaromatic resonances of Phe-178, and the side-chain resonances of Glu-220. The input for the final structure calculation consisted of 1,592 NOE upper distance limits (388 intraresidual, 428 sequential, 413 medium range, 363 long range) and 229 dihedral angle constraints. The average residual target function value for the 20 best DYANA conformers was 1.02 ± 0.48 Å.

*The energy-minimized mean structure differs from the average of the atom coordinates of the 20 conformers by a rms deviation of 0.8 Å calculated for the backbone atoms of residues 124-166 and 172-226.

†Best-defined side chains are those with a displacement of the heavy atoms smaller than 1.0 Å and include residues 130, 132-134, 137, 139, 141, 146, 149, 153, 158-161, 178-184, 188-192, 199, 201-203, 205-207, 209, 210, and 213-216.

The major result of the present structure refinement for \( \text{mPrP}(121-231) \) is the precise structural definition of a large fraction of the amino acid side chains. Twenty residues form a tightly packed hydrophobic core. In Fig 1b the outer confines of the core are indicated by a translucent sheet. Thirty residues in the hydrophobic core come from the antiparallel helices 2 and 3, which have mutual interactions through the residues Phe-175, Val-176, Val-180, Ile-184, Val-203, Met-206, Val-210, and Met-215, and the disulfide bridge Cys-179–Cys-214, whereas Met-205, Val-209, and Met-213 interact with the four core residues Met-134, Pro-137, Ile-139, and Phe-141 in the loop between the first \( \beta \)-strand and the first helix (Fig 1b). Pro-158 and Val-161 are adjacent and within the \( \beta \)-sheet, respectively, and Phe-198 is located in the loop between helices 2 and 3.

The hydrophobic core is surrounded by an outer shell of protein structure that contains 22 hydrogen bonds with amino acid side chains (Fig 1b and Table 2), which include medium-range interactions in all three helices and a variety of longer-range interactions. The N terminus of the first helix contains the hydrogen bond Asn-143H=O-Glu-146 (b in Fig 1b), which is directly supported by NOEs between \( ^\text{NH} \) of Asn-143 and \( ^\text{CHO} \) of Glu-146. A hydrogen bond between the backbone oxygen of Asn-171 and the side chain of Asn-174 (i in Fig 1b) initiates the second helix. The side chain of Thr-183, which also acts as an acceptor for a long-range hydrogen bond with the amide proton of Tyr-162, forms a hydrogen bond to the carbonyl oxygen of Cys-179, as is supported by the fact that the hydroxyl proton of Thr-183 exchanges sufficiently slowly to be observed by \( ^1 \text{H} \) NMR. The N terminus of the third helix is stabilized by a capping box (41) of Thr-199H–O–Asp-202 and Asp-202H–O–Thr-199, and the side chain–side chain hydrogen bond Thr-199H–O–Asp-202 (r, s, and t in Fig 1b).

Among the longer-range hydrogen bonds connecting different groups within the outer shell there are the two hydrogen bonds Tyr-128H–O–Asp-178 and Tyr-162H–O–Thr-183 (a and j in Fig 2b), and the salt bridge Arg-164–Asp-178 (k in Fig 1b), which hold the \( \beta \)-sheet against helix 2. The salt bridge Arg-
with wild-type mPrP(121-231) The phenotype of the prion disease that segregates with the mutation in position 178 has been shown to be determined by the nature of the amino acid residue in position 129. The observed specific interactions between positions 129 and 178 (Fig. 2a) then would also provide a rationale for the observation that the Val/Met-129 polymorphism does not affect the phenotypes of the inherited TSEs that segregate with the other known mutations (Fig. 1d).

The mutation Thr-183-Ala (Fig 2a) eliminates two hydrogen bonds that establish a link between helix 2 and the β-sheet, i.e., Thr-183-O-Cys-179 and Tyr-162-H-O-Ala-133 (j and m in Fig. 1b), indicating reduced stability for this protein variant.

For the amino acid replacement Gln-217-Arg (Fig 2b) the NMR structure indicates reduced stability of PrP0: in the wild-type protein, Gln-217 is surrounded by hydrophobic groups (Fig. 2b), and its side chain forms a long-range hydrogen bond to the carbonyl oxygen of Ala-133, thus stabilizing a distinct position of the loop between the first β-strand and helix 1 relative to the hydrophobic core. The replacement Gln-217-Arg introduces a positive charge into this otherwise uncharged region (the nearest charged group is about 10 Å away), and it appears most unlikely, for steric reasons, that a satisfactory hydrogen bond geometry with the carbonyl oxygen of Ala-133 could be achieved with the Arg side group.

The replacement of Phe-198 in the hydrophobic core by Ser is a pronouncedly nonconservative mutation. In the absence of follow-up structural changes this mutation would lead to an empty cavity that could accommodate 2-3 water molecules (Fig 2c). In the wild-type protein the aromatic ring is surrounded by numerous hydrogen-bonded polar side chains (g, r, s, and t in Fig. 1b) and its replacement by serine is likely to result in a modified pattern of polar interactions. These interactions could trigger a collapse of the cavity, which, in turn, would influence the surface structure and thus could also alter the ligand binding properties of PrP0. In view of the anticipated complex structural rearrangement, it is difficult to estimate the consequences of the charge change. In the NMR structure of the mutant proteins First, three point mutations were found in this mutant, which are located at the loop connecting the two helices. Two hydrogen bonds in wild-type mPrP(121-231) (Fig. 2a) link the strictly conserved residues Asp-178 and Arg-156 (i in Fig. 1f) to the side chain of Asp-178 with two side chains in the β-sheet, one of which is sequentially adjacent to position 129. Thus, the Asp-178-Arg exchange may affect the hydrogen bonding network involving Arg-164, Tyr-128, and Asp-178 somewhat differently depending on the nature of the amino acid residue in position 129. The observed specific interactions between positions 129 and 178 (Fig. 2a) then would also provide a rationale for the observation that the Val/Met-129 polymorphism does not affect the phenotypes of the inherited TSEs that segregate with the other known mutations (Fig. 1d).

Finally, there are four amino acid replacements for which the NMR structure predicts no or at most minor variations in stability. The mutations Gln-200-Lys and Arg-208-His (Fig. 1g) are located on the protein surface, so that even the change in overall charge of the protein should not have a major impact on the global structure. For the replacement of Val by Ile in either positions 180 or 210 (Fig. 2d) there is enough space to accommodate the somewhat larger Ile side chain.

CONCLUSIONS

The refined NMR structure of mPrP(121-231) and the recent determination of the solution structure of the intact mPrP, mPrP(23-231) (13) show convincingly, in contrast to earlier suggestions (15, 16), that familial human TSEs generally cannot be rationalized by the assumption of reduced stability of the PrP0 form of the mutant proteins. First, three point mutations that segregate with inherited TSEs are in the flexibly extended polypeptide segment N terminal to residue 121 (13), where it is unlikely that individual amino acid substitutions would measurably affect the global stability of PrP0. Second, for the eight protein species with disease-related amino acid exchanges in the globular C-terminal domain the refined NMR structure of wild-type mPrP(121-231) predicts largely different effects on the protein stability, from very small variations relative to the parent protein to major desta-
bibilization caused by loss of internal hydrogen bonds and salt bridges. These structure-based predictions on the relative thermodynamic stabilities of the individual variant proteins coincide qualitatively with experimental measurements using the corresponding recombinant protein variants of mPrP(121-231) (R. Glockshuber, personal communication). The refined NMR structure of mPrP(121-231) further shows that the mutation sites related to the human TSE phenotypes Gerstmann-Sträussler-Scheinker syndrome (GSS) and CJD do not form separate clusters in the three-dimensional structure of PrP^C (Fig. 1d), excluding the presence of GSS- and CJD-specific subdomains, and it presents a rationale for the specific influence of the residue type in the polymorphism site 129 on the TSE phenotypes that segregate with the mutation Asp-178→Asn (44).

We acknowledge the Centro Svizzero di Calcolo Scientifico for use of the NEC SX-4 computer, the Eidgenössische Technische Hochschule for the use of the Cray J-90 cluster, and Mrs. M. Geier and Mrs. M. S. Straussler-Scheiker syndrome (GSS) and CJD do not coincide qualitatively with experimental measurements using the corresponding recombinant protein variants of mPrP(121-231) (R. Glockshuber, personal communication). The refined NMR structure of mPrP(121-231) further shows that the mutation sites related to the human TSE phenotypes Gerstmann-Sträussler-Scheinker syndrome (GSS) and CJD do not form separate clusters in the three-dimensional structure of PrP^C (Fig. 1d), excluding the presence of GSS- and CJD-specific subdomains, and it presents a rationale for the specific influence of the residue type in the polymorphism site 129 on the TSE phenotypes that segregate with the mutation Asp-178→Asn (44).

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