I. Equilibrium unfolding studies of cytochrome C. II. The role of helix 1 aspartates in the stability and conversion of prion protein

Jonathan O'Connor Speare
The University of Montana

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I. EQUILIBRIUM UNFOLDING STUDIES OF CYTOCHROME C

II. THE ROLE OF HELIX 1 ASPARTATES IN THE STABILITY AND CONVERSION OF PRION PROTEIN

by

Jonathan O. Speare

B.A. The Colorado College 1994

Presented in partial fulfillment of the requirements
for the degree Doctor of Philosophy
The University of Montana

2003

Approved by:

[Signatures]
Chairperson

Dean, Graduate School

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Speare, Jonathan O. Ph.D., August 2003
Chemistry

I. Equilibrium Unfolding Studies of Cytochrome c
II. The Role of Helix 1 Aspartates in the Stability and Conversion of Prion Protein

Chair: Kent Sugden, Ph.D.

ABSTRACT

In order to improve methods to study protein structure and to obtain a detailed structural understanding of cytochrome c equilibrium unfolding, spectra have been obtained for horse heart cytochrome c at equilibrium in solutions of 0 to 7 M guanidine hydrochloride (GdnHCl) using absorption, fluorescence, circular dichroism (CD), and attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopies. Unfolding studies using ATR-FTIR spectroscopy were performed using deuterium substituted GdnHCl which enhanced the ability to measure the true protein IR spectrum in the amide I region where secondary structure can be deduced. Results of ATR-FTIR studies showed reductions in α-helix and increases in β-sheet at high denaturant concentrations, contrary to expectations of finding primarily random coil structure. Collectively, results of the various spectroscopic unfolding studies show that there are three regions of major conformational change. At low denaturant concentrations there is a slight decrease in the Fe-Met80 coordination, a loosening of α helices, and an increase in β-sheet structure. At mid-range GdnHCl concentrations, the changes include a significant decrease in α-helix, a complete loss of Fe-Met80 coordination, a dramatic increase in the Trp59-Heme distance, and increases in β-sheet, random structure, and turns. At high denaturant concentrations there is a slight continuation of the increase in the Trp59-Heme distance, a continued increase in β-sheet, and a decrease in random structure. These results suggest the unfolding of cytochrome c is not a two-state transition, but rather occurs through multiple structures with significant β-sheet structure in the denatured state.

In a separate study, the role of helix 1 aspartates in the stability and conversion of prion protein was investigated. Prion protein is involved in the pathogenesis of transmissible spongiform encephalopathy diseases, which include chronic wasting disease in deer and elk and Creutzfeldt-Jakob disease in humans. A key event in the pathogenesis of transmissible spongiform encephalopathies is the conversion of the normal α-helical prion protein, PrP-sen, to the abnormal, high β-sheet protease resistant form, PrP-res. It was proposed that the conversion mechanism involves critical interactions at helix 1 (residues 144-153) and that the helix is stabilized by intra-helix salt bridges between two aspartate-arginine ion pairs at positions 144 and 148 and 147 and 151, respectively. Three mutants designed to destabilize the helix 1 salt bridges by replacing the aspartates with either asparagines or alanines were compared to wild type PrP using CD spectroscopy and cell-free conversion reactions to assess differences in secondary structure stability and conversion efficiency. Thermal and chemical denaturation experiments indicated that the overall structures of the asparagine mutants are not substantially destabilized but they appear to unfold differently. Cell-free conversion reactions performed using conditions unfavorable to salt bridge formation showed no significant differences between conversion efficiencies of mutant and wild type proteins. Using conditions more favorable to salt bridge formation, the mutant proteins converted with up to four-fold higher efficiencies than the wild type protein. Thus, while spectroscopic data indicate that the salt bridges do not substantially stabilize PrP-sen, the cell-free conversion data suggest that D144, D147 and their respective salt bridges stabilize the molecule against the conversion of PrP-sen to PrP-res.
To my father,

Joel Carl Speare

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ACKNOWLEDGEMENTS

The work described in this dissertation would not have been possible without the assistance of many individuals. I begin by expressing my deepest thanks to Dr. Byron Caughey for taking me under his wing and providing mentorship and encouragement of the highest quality. I would also like to express my sincerest gratitude to Greg Raymond for his generous instruction and encouragement. Dr. Gerry Baron has my thanks for donating numerous hours of instruction in molecular biology and biochemistry. I would like to thank Drs. Mike DeGrandpre and Kent Sugden for being on my committee and for their additional guidance, without which I would have had a much more difficult time in accomplishing my goals. Thanks also go out to Drs. Ed Rosenberg and Dick Field for being on my committee and encouraging me to pursue my Ph.D.

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I would like to thank my family for their love and support. I would especially like to thank my mother for her endless love and confidence. I would not have been able to complete my Ph.D. without her. She is a remarkable person and mother. Finally, I would like to thank Kristie Scheel for her love, companionship, and encouragement, and for believing in me.
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<tr>
<td>A</td>
<td>alanine</td>
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<tr>
<td>Å</td>
<td>angstroms</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethyl-carbazole</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>amu</td>
<td>atomic mass units</td>
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<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>attenuated total reflectance Fourier-transform infrared</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>c</td>
<td>concentration in moles per liter</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>cm⁻¹</td>
<td>reciprocal centimeter (wavenumber)</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>Cyt c</td>
<td>cytochrome c</td>
</tr>
<tr>
<td>D</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DCyt c</td>
<td>deuterated cytochrome c</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------------------------</td>
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<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption ionization-time of flight</td>
</tr>
<tr>
<td>mCi</td>
<td>millicuries</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
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<tr>
<td>mg</td>
<td>milligrams</td>
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<tr>
<td>min</td>
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<tr>
<td>mL</td>
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<td>mm</td>
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<tr>
<td>mM</td>
<td>millimoles per liter</td>
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<td>mol</td>
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<tr>
<td>N</td>
<td>asparagine</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram(s)</td>
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<tr>
<td>nm</td>
<td>nanometer(s)</td>
</tr>
<tr>
<td>nM</td>
<td>nanomoles per liter</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>nsec</td>
<td>nanosecond(s)</td>
</tr>
<tr>
<td>NZY</td>
<td>NZ-amine yeast</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAS</td>
<td>protein A sepharose</td>
</tr>
<tr>
<td>PBBS</td>
<td>phosphate-buffered balanced salts</td>
</tr>
<tr>
<td>pBS</td>
<td>pBluescript vector</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<table>
<thead>
<tr>
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<td>PK</td>
<td>proteinase K</td>
</tr>
<tr>
<td>pmol</td>
<td>picomoles</td>
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<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>PrP-res</td>
<td>protease resistant, disease associated prion protein</td>
</tr>
<tr>
<td>PrP-sen</td>
<td>protease sensitive, cellular prion protein</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>R</td>
<td>arginine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SABU</td>
<td>sample buffer</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>T</td>
<td>temperature in Kelvin</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetic acid –EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-boric acid –EDTA buffer</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with tween 20R</td>
</tr>
<tr>
<td>TCB</td>
<td>Tris-chloride buffer</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>$T_m$</td>
<td>transition midpoint</td>
</tr>
<tr>
<td>TN-sark</td>
<td>Tris sodium chloride buffer with sarkosyl</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>TSE</td>
<td>transmissible spongiform encephalopathy</td>
</tr>
</tbody>
</table>
UV  ultraviolet
V  volt(s)
v/v  volume to volume
w/v  weight to volume
xg  times the force of gravity
βME  β-mercaptoethanol
ΔG  Gibbs free energy of unfolding
ΔG(H2O)  Gibbs free energy of conformation in the absence of denaturant
ΔN  difference in index of refraction from water
ε280  extinction coefficient at 280 nm
μg  micrograms
μL  microliter(s)
μM  micromoles per liter
θ  molar ellipticity
θobs  observed ellipticity
[θ]  ellipticity of sample
[θ]D  ellipticity of denatured protein
[θ]N  ellipticity of native protein
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This dissertation is composed of two different chapters covering research performed in the laboratory of Dr. Tom Rush III at the University of Montana from 1998 to 2000, and Dr. Byron Caughey at Rocky Mountain Labs from 2000 to 2003. The first chapter describes work performed with Dr. Rush on the equilibrium unfolding of cytochrome c. The goal of these studies was to use spectroscopic methods to elucidate structural details of equilibrium-unfolded cytochrome c and to investigate whether the protein unfolds via a two-state transition. Portions of the results from this chapter were published in the journal *Biopolymers* (Speare and Rush 2003). The second chapter describes work on the role of helix 1 aspartates in the stability and conversion of prion protein. These studies were started with Dr. Rush at The University of Montana in 2000 and, following Dr. Rush's departure, were completed with Dr. Caughey at Rocky Mountain Laboratories branch of the National Institutes of Health. The goal of the helix 1 studies was to determine if helix 1 salt bridges stabilize the structure of PrP-sen and play a role in the conversion of PrP-sen to the transmissible spongiform encephalopathy disease associated PrP-res. Portions of the results of this chapter were published in the *Journal of Biological Chemistry* (Speare et al. 2003). While these studies cover different areas of protein chemistry, they are complementary in nature as their fundamental goals are to understand and determine changes in protein structure using spectroscopic methods.
I. Equilibrium Unfolding Studies of Cytochrome C
A. INTRODUCTION

The determination of protein structure is critical for developing a detailed understanding of protein function, protein misfolding related to disease, and the fundamental thermodynamic factors that drive protein folding (Caughey et al. 1991b; Creighton 1990; Creighton et al. 1996; Dill and Shortle 1991; Dong et al. 1990; Kim and Baldwin 1982; Kim and Baldwin 1990; Muga et al. 1991a). Protein structure and folding have been studied since Anfinsen showed that denatured ribonuclease could be folded back to its native conformation in vitro (Anfinsen et al. 1961). Initially folding was thought to proceed by random sampling of all conformational possibilities to attain the three-dimensional structure with the lowest free energy. In 1969 however, Levinthal pointed out the large amount of time it would take to sample all of the possible conformations, even for small proteins (Levinthal 1969). This fact, combined with the knowledge that certain proteins refold in vitro within a few seconds, initiated efforts for further understanding changes in protein structure during folding (Creighton et al. 1996).

In order to be biologically active all proteins must adopt specific three-dimensional structures. The ability of proteins to fold into specific structures must be dictated by their primary linear amino acid sequence because genes only contain information that results in the creation of the primary linear sequence of amino acids that make up proteins and most purified proteins refold to their original native structure after being completely unfolded in vitro. How proteins fold into specific structures has come to be known as ‘the protein folding problem’. The problem can be broken down to three
different but related questions (Creighton 1990): 1) by what kinetic process or pathway
do proteins adopt their native and biologically active folded conformation? 2) What is the
physical basis of the stability of folded conformations? 3) Why does the amino acid
sequence determine one particular folding process and resultant three-dimensional
structure instead of some other? To address the questions of protein folding, one must be
able to directly determine protein structure. A number of spectroscopic techniques are
available for investigating protein structure including absorbance, fluorescence, X-ray
diffraction, Nuclear Magnetic Resonance (NMR), circular dichroism (CD), Raman, and
Fourier-transform infrared (FTIR) spectroscopies. Of these, X-ray diffraction and NMR
methods provide the most highly resolved details of structure. However, it is not always
feasible to use these techniques to study the more dynamic aspects of protein structure
(e.g., folding/misfolding and allosteric transitions), and their complexes with large
biological molecules or structures (e.g., protein aggregates and membranes). Protein
structure is influenced by solvent composition (i.e., the dielectric constant), temperature,
and pH. Thus, spectroscopic methods that are sensitive to protein structure combined
with chemical denaturants or temperature can be used to denature proteins and investigate
changes that occur during folding and unfolding (Arrondo et al. 1999; Eloé et al. 1992;
Filosa et al. 1999; Hamada et al. 1996; Johnson 1990; Johnson 1999; Sreenthan and

In the case of chemical denaturation, absorbance, fluorescence, CD, and FTIR
spectroscopies have been used extensively to monitor protein structural changes.
However, the amount of secondary structure information obtainable in CD and FTIR
spectroscopies is limited by complete absorbance of the signal by the denaturant in the spectral region of interest (Chen et al. 1998; Chen et al. 1999; Johnson 1990; Surewicz et al. 1993). Protein unfolding experiments using CD spectroscopy and the denaturant guanidine hydrochloride (GdnHCl) are limited to wavelengths above 210 nm because of significant absorption by the denaturant. This limits the amount of structural information that can be gained using CD because wavelengths down to 178 nm must be used to make accurate estimates of protein secondary structure (Johnson 1990). Unfolding experiments monitored by FTIR spectroscopy are also limited because water and the denaturant GdnHCl absorb strongly (peak centers of 1645 and 1673 cm\(^{-1}\), respectively) across the entire amide I region (1600-1700 cm\(^{-1}\)) which is the region relevant to secondary structure. While D\(_2\)O can be used to make non-denaturing aqueous studies more feasible (Byler and Susi 1986; Castresana et al. 1988; Surewicz and Mantsch 1988), there are currently few accurate methods for studying proteins in solutions containing high denaturant concentrations. Since folding intermediates have been implicated in protein folding diseases such as transmissible spongiform encephalopathies, Parkinson's, Huntington's, and Alzheimer's disease (Maiti and Surewicz 2001; Swietnicki et al. 2000; Taubes 1996), improving methods to study unfolded protein structure could help elucidate the mechanisms associated with these diseases.

Cytochrome c (Cyt c) is a model protein for investigating protein structure and folding. The biological function of Cyt c involves shuttling electrons as part of energy transduction in mitochondria and it can exist in two oxidation states with the heme iron either in the Fe(III) (as in ferricytochrome c) or Fe(II) (as in ferrocytochrome c) oxidation
states. This investigation uses Cyt c isolated from horse heart, which is made up of 104 amino acid residues and a covalently attached heme giving a molecular mass of 12,384 Da (from this point forward all mention of Cyt c refers to ferricytochrome c). Figure 1 shows a schematic diagram of Cyt c from X-ray diffraction coordinates (Bushnell et al. 1990) with structural elements of interest highlighted. Overall, Cyt c is composed of three major and two minor helical elements interconnected by strands of polypeptide chain folded into a roughly spherical shape within which the heme pocket resides. The specific secondary structural elements within the protein include five α-helices (residues 6 to 14, 49 to 54, 60 to 69, 70 to 75, and 87 to 102), a short two-stranded anti-parallel β-sheet (residues 37 to 40 and 57 to 59), and six β-turns (residues 14 to 17, 21 to 24, 32 to 35, 35 to 38, 67 to 70, and 75 to 78). The heme prosthetic group is covalently attached to two cysteine residues at positions 14 and 17 by thioether bonds. The heme iron is coordinated by two ligands, a histidine at position 18 and a methionine at position 80.

The structure and folding of Cyt c have been well characterized because of its small size which makes structural interpretation easier, it is easy to purify, has a reversible folding transition, has excellent spectroscopic properties, and is well behaved in aqueous solution. Its structure and folding have been well characterized using various spectroscopic techniques including fluorescence (Tsong 1973; Tsong 1974; Tsong 1975; Tsong 1976), visible absorption (Myer 1984; Sreenthan and Taylor 1971), CD (Hamada et al. 1994), NMR (Feng et al. 1990; Qi et al. 1996; Roder et al. 1988), X-ray diffraction (Bushnell et al. 1990), and FTIR (Bowler et al. 1994; Byler and Susi 1986; de Jongh et
Figure 1. Diagram of horse heart cytochrome c (MW 12,384 daltons, 104 residues) based on X-ray diffraction data from (Bushnell et al. 1990). Structural components involved in the spectroscopic probes used in this report are highlighted. Red ribbons represent helices. Turquoise bands represent β-sheets.

In previous unfolding studies using fluorescence and CD spectroscopies, Cyt c has been shown to unfold in a two-step process from a native folded structure to a non-native, unfolded structure. It was shown previously by Tsong that the tryptophan 59 (Trp59) fluorescence of Cyt c is sensitive to overall protein conformation. A major structural transition can be monitored by fluorescence because in its native conformation the indole ring of the single Trp residue in Cyt c donates a hydrogen bond to the heme prosthetic group, resulting in the complete quenching of the Trp fluorescence by Förster energy transfer to the heme. Upon denaturation, the Trp59 indole ring hydrogen bond to the heme is lost as the protein unfolds, resulting in a decrease in quenching by the heme and, therefore, a stronger fluorescence signal (Rush and Spiro 1998; Tsong 1976). Tsong reported that a major Cyt c structural transition occurs from 2.5 to 3.5 M GdnHCl at pH 7.0 with a transition midpoint (T_m) at 2.7 M GdnHCl. Tsong also reported that certain residual structures of the protein are retained, as judged by the change in Trp59 fluorescence, after the major conformational transition is over (Tsong 1974; Tsong 1975; Tsong 1976).

Circular dichroism has also been used to monitor the changes in secondary structure of equilibrium unfolded Cyt c. Being a highly α-helical protein in its native state (Figure 1), Cyt c correspondingly has an α-helical CD spectrum at low denaturant concentrations (< 2 M GdnHCl). Hamada and coworkers reported that as the GdnHCl concentration is increased, Cyt c follows a cooperative, two-state equilibrium unfolding
transition with no α-helix component present above 3.5 M GdnHCl (Hamada et al. 1996). The midpoint of the unfolding transition was identical to the fluorescence monitored transition midpoint at 2.7 M GdnHCl.

While the fluorescence and CD unfolding experiments suggest a two-state unfolding process other equilibrium folding studies of Cyt c have shown that the folding is not entirely a two-state process. Rush and Spiro have shown using resonance Raman spectroscopy that at pH 7.0 the transition midpoints for the loss of tyrosine hydrogen bonds present in the native structure occur at slightly lower denaturant concentrations (~2.5 M) than the transition midpoints for tryptophan fluorescence and CD unfolding experiments (2.7 M) (Rush and Spiro 1998). Using small angle X-ray scattering to monitor radii of gyration of the GdnHCl equilibrium unfolded species of Cyt c, Segel and coworkers reported that Cyt c has an unfolded intermediate and is likely to have a number of denatured state populations (Segel et al. 1998).

Results from other experiments monitoring equilibrium unfolding of Cyt c also suggest Cyt c unfolding is not a two-state process. The absorption band of Cyt c at 695 nm was shown to be due to the binding of the sulphur contained in the methionine 80 (Met80) side chain to the heme iron (Dickerson et al. 1967; Schechter and Saludjian 1967) and has been used to monitor the conformational state of Cyt c (Schjeter and George 1964; Sreenthan and Taylor 1971). The Fe(III)→Met80 coordination was shown to be essentially gone at GdnHCl concentrations above 3.0M at pH 7.0, suggesting a slightly lower transition midpoint for the conformational change than reported for the fluorescence and CD experiments described above (Ferri et al. 1996; Sreenthan and
Taylor 1971). In addition, results from experiments where equilibrium unfolded Cyt c samples were monitored by cyclic voltammetry suggested the presence of intermediate Cyt c structures at GdnHCl concentrations between 1.0 and 4.0 M (Ferri et al. 1996). Time-resolved studies of Cyt c folding using fluorescence, CD and FTIR spectroscopies also indicate that multiple structures occur along the folding pathway (England et al. 1998; Roder et al. 1988; Shastry et al. 1998; Telford et al. 1998; Yeh et al. 1998).

In the case of structural investigations of equilibrium unfolded Cyt c using FTIR spectroscopy, Bowler and coworkers reported an improved method to study the states of GdnHCl denatured proteins (Bowler et al. 1994). They showed that the spectral contributions from up to 3.0 M GdnHCl could be reliably subtracted from the experimental spectrum of Cyt c allowing for a qualitative comparison of the denatured state protein spectra. They reported that structural changes monitored by second derivative Fourier-transform infrared spectroscopy analysis start at 1.5 M GdnHCl. Saturation of the Hg/Te/Cd detector at the cell path length they used (6 μm) limited the concentration of GdnHCl to 3.0 M. Thus to date, the highest GdnHCl concentration reported for studying equilibrium unfolded Cyt c using FTIR is 3.0 M GdnHCl (Bowler et al. 1994). Considering that fluorescence (Tsong 1974), CD (Hamada et al. 1996), absorbance (Ferri et al. 1996; Sreenthan and Taylor 1971), X-ray scattering (Segel et al. 1998), and cyclic voltammetry (Ferri et al. 1996) Cyt c unfolding experiments have shown that transitions occur anywhere from 2.0 to 6.0 M GdnHCl. The FTIR limit at 3.0 M GdnHCl does not allow for the complete observation of the unfolding transition, nor does it allow for the observation of proteins in more fully denaturing concentrations.
of GdnHCl (~6.0 M GdnHCl). An improved method for studying denatured proteins in higher concentrations of GdnHCl using FTIR spectroscopy is therefore desirable.

There have recently been two reports describing methods that improve FTIR monitoring of protein denaturation at high denaturant concentrations. Oberg and Fink demonstrated using attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy that spectra could be obtained at 6 M GdnHCl concentrations that were well within the linear range of the instrument (Oberg and Fink 1998). In addition, Fabian and Mantsch showed that infrared detector saturation in the amide I region resulting from high urea concentrations could be reduced using carbon-13 labeled urea which causes the carbonyl band of urea to shift out of the amide I region (Fabian and Mantsch 1995). Results from experiments that used the high sensitivity of ATR-FTIR spectroscopy and isotope labeling of the denaturant in order to develop an improved method for studying the denatured structures of Cyt c are described (Speare and Rush 2003). We have obtained amide I infrared spectra of horse heart Cyt c in the presence of deuterium substituted GdnHCl (DGdnHCl) using ATR-FTIR spectroscopy. We show that the substitution of all exchangeable protons in GdnHCl with deuterium shifts the C=N vibrational peak of GdnHCl, thereby minimizing the distortion of the amide I region by the denaturant. Changes in secondary structure of equilibrium unfolded Cyt c, monitored from 0 to 6.6 M DGdnHCl and 0 to 6.7 M GdnHCl, were investigated.

Together, the investigations of equilibrium unfolding of Cyt c described above strongly indicate that Cyt c unfolding is not a two-state transition. None of the previous investigations of the structural changes that Cyt c undergoes during equilibrium
unfolding have been performed using the various probes together in a systematic and even-handed way. It is the purpose of this investigation to better characterize the various conformational states of horse heart Cyt c at equilibrium denatured in GdnHCl under equivalent experimental conditions (e.g., protein type, pH, buffer). In order to accomplish this task, coinciding equilibrium unfolding experiments were performed using fluorescence, CD, ATR-FTIR, and visible absorption spectrosopies. Results indicate that equilibrium unfolding of Cyt c is not a two-state transition.

B. Experimental Methods

1. Materials—Cyt c from horse heart was obtained from Sigma (C-7752, >99% purity, molecular mass 12,384 Daltons [Da]). Deuterium oxide (D2O) was obtained from Cambridge Isotope Laboratories (DLM-4, 99.9% purity). All water used for sample preparation was purified to 18.0 megohm-cm resistivity. All buffer solutions were filtered using Millex-GV 0.22 μm Durapore™ (PVDF) filters.

2. Preparation of Cytochrome c Samples for Fluorescence, Absorbance, and Circular Dichroism Spectroscopy—Horse heart Cytochrome c (molecular mass 12,384 Da), from Sigma, was used without further purification. A 1.0 mM stock solution of Cyt c was prepared in 0.01 M sodium phosphate buffer at pH 7.0. The concentration was confirmed using the absorbance at 526.5 nm which is an isosbestic point (extinction coefficient = 11,000 /M cm) where ferricytochrome c and ferrocytochrome c have identical extinction
coefficients. An absorbance measurement was performed to determine the oxidation state by measuring from 350 to 650 nm to confirm complete oxidation to ferricytochrome c. A broad peak from 500 to 600 nm is characteristic of ferricytochrome c, whereas two sharper peaks at 520 and 550 nm are characteristic of ferrocytochrome c.

Stock solutions (0.0 to 7.0 M by increments of 0.5 M) of guanidine hydrochloride in 0.01 M sodium phosphate buffer at pH 7.0 were prepared in the following manner: The appropriate amount of guanidine hydrochloride was weighed out and placed in a 100 mL volumetric flask along with 6.1 mmoles of Na₂HPO₄ and 3.9 mmoles of NaH₂PO₄. The mixture was then diluted to 100 mL with H₂O. The pH of each solution was adjusted to 7.0 if necessary using NaOH or HCl. The GdnHCl concentrations of each solution were checked using an Abbe type refractometer. Concentrations in molarity were calculated using the following equation:

\[
[GdnHCl] = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3
\]

Equation 1

where \(\Delta N\) is the difference between the refractive index of the GdnHCl solution and water (H₂O)/deuterium oxide (D₂O) at the sodium D line (Nozaki 1972).

Using the stock guanidine hydrochloride solutions prepared as described above, 3.0 mL of each the 15 solutions was placed into individual vials. 45 \(\mu\)L of the 1.0 mM Cyt c solution prepared as described above was placed into each vial and mixed carefully. The resulting 15 \(\mu\)M Cyt c denaturant solutions were allowed to stand for 1 hour maximum before recording the fluorescence, absorbance, and CD spectra.
3. **Tryptophan Fluorescence Spectroscopy**—The fluorescence spectra for the Cyt c solutions were obtained at an excitation wavelength of 280 nm using a Shimadzu RF-1501 Spectrofluorophotometer with PC1501 software. The emission was recorded from 250 to 500 nm for all samples. The folding titration curves were prepared using Microsoft Excel. The fraction unfolded was determined using the signal change at 350 nm and was calculated using the following equation:

\[
\frac{(\text{Sample Intensity at } 350 \text{ nm}) - (0.0M \text{ GdnHCl Sample Intensity at } 350 \text{ nm})}{(7.0M \text{ GdnHCl Sample Intensity at } 350 \text{ nm}) - (0.0M \text{ GdnHCl Sample Intensity at } 350 \text{ nm})} \times 100
\]

**Equation 2**

The values obtained, using the equation above, were plotted against the corresponding concentrations guanidine hydrochloride to obtain the folding titration curve.

4. **Absorbance Spectroscopy**—The absorbance spectra for each of the 15 μM cytochrome c solutions used in the fluorescence experiments were recorded from 350 to 750 nm using a Perkin-Elmer Lambda 11 UV/Vis Spectrometer with UV Winlab software. Peak-fitting at 695 nm was performed using the curvefit macro on Galactic Grams32 software. The resulting peak intensities were recorded and plotted versus the concentration of guanidine hydrochloride on Excel.

5. **Circular Dichroism Spectroscopy**—Circular Dichroism spectroscopy was performed using an On-Line Instrument Systems Inc. (OLIS) CD module with Cary-16 Spectrophotometer Conversion Unit with accompanying OLIS Software. Instrument
calibration was performed using camphorsulfonic acid. Samples were scanned in a 1.0 mm cylindrical cell from 260 to 200 nm with a 0.5 nm scanning interval. Protein unfolding was monitored using the ellipticity at 222 nm after subtraction of spectral contributions due to buffer and denaturant (Hamada et al. 1996).

6. Unfolding Curve Analysis—Unfolding curves were analyzed using methods described by other investigators (Hamada et al. 1996; Pace 1986; Pace and Scholtz 1997) The folding was assumed to be a two-state transition from the native state (N) to the denatured state (D) where the free energy change of unfolding (ΔG) is defined as:

\[
\Delta G = -RT \ln K_U
\]

Equation 3

Where R is the molar gas constant, T is the temperature in Kelvin, and K_U is the equilibrium constant for the unfolding transition obtained using the equation:

\[
K_U = \frac{([\theta] - [\theta]_N)}{([\theta]_D - [\theta])}
\]

Equation 4

Where [\theta], [\theta]_N and [\theta]_D are the spectroscopic signals for the sample, and the native and denatured states respectively. The unfolding transition curves were analyzed by plotting ΔG for samples in the transition region versus the denaturant (GdnHCl) concentration. Assuming a linear dependence, the relationship of ΔG to GdnHCl concentration is described by the equation:
\[
\Delta G = \Delta G(H_2O) - m [\text{GdnHCl}]
\]

Equation 5

Where \( \Delta G(H_2O) \) is the free energy of the protein in the absence of GdnHCl, \([\text{GdnHCl}]\) is the concentration of GdnHCl in moles per liter, and \( m \) is the slope, which is indicative of the cooperativity of the transition (Pace 1986; Pace and Scholtz 1997).

7. Preparation of Samples for ATR-FTIR Spectroscopy—Denaturant solutions up to 7.0 M GdnHCl/DGdnHCl were prepared from stock solutions of 7.0 M GdnHCl/DGdnHCl in 0.1 M pH/pD 7.0 phosphate buffer. The pD of samples prepared in D\(_2\)O were determined using a pH meter with microelectrode as described by Glasoe and Long (Glasoe and Long 1960) using the following equation:

\[
pD = pH + 0.40
\]

Equation 6

All samples prepared in D\(_2\)O were adjusted to pD 7.0 using NaOD or DCl. GdnHCl and DGdnHCl concentrations were determined in moles per liter using refractometry as described above.

Deuterated guanidine hydrochloride was obtained by dissolving guanidine hydrochloride in D\(_2\)O at a 33% weight to volume ratio. After a 24 hour incubation the solution was placed on a Rotovap at 55 °C under vacuum until dry followed by another 24 hour incubation in D\(_2\)O to insure complete deuteration. The sample was then placed under high vacuum to remove residual water vapor. The completion of the deuteration
reaction was confirmed using FTIR where DGdnHCl has a C=N stretching mode at 1598 cm\(^{-1}\) compared to 1673 cm\(^{-1}\) for GdnHCl.

Denaturant solutions of Cyt c were prepared containing 5% protein by weight (50 mg/mL). To prepare denaturant solutions containing similar H\(_2\)O content due to waters of hydration from the protein, the denaturant solution was treated with 1.0 \(\mu\)L of 0.1 M phosphate buffer per milligram protein used. The resulting solutions were allowed to stand a minimum of 48 hours prior to spectroscopic analysis to allow complete H\(\rightarrow\)D exchange. H\(_2\)O/GdnHCl protein solutions were prepared by dissolving Cyt c in buffered GdnHCl stock solutions. These samples were allowed to incubate at least one hour prior to analysis.

8. ATR-FTIR Spectroscopy—ATR-FTIR scans were performed using a Perkin-Elmer System 2000 FT-IR with an Infrared Associates model MCT-NB detector, a Graseby Infrared DP-8000-1 preamplifier and a Spectra Tech diamond cell ATR attachment. The ATR attachment was continuously purged using a Whatman FTIR purge gas system. One thousand scans were collected for each sample from 2500 to 1000 cm\(^{-1}\) at 2 cm\(^{-1}\) resolution and a 1 cm\(^{-1}\) scanning interval with Kaiser-Bessel apodization. The diamond cell background was subtracted from the spectra of all samples.

9. ATR-FTIR Data Analysis—All spectral manipulations were performed using Galactic Grams/32 software. The main buffer/denaturant and transient H\(_2\)O vapor background was subtracted from the sample spectrum by the method by Dong et al. (Dong et al. 1990).
The buffer/denaturant spectra were first subtracted after multiplication by a subtraction factor, so that there were no negative lobes adjacent to the amide I spectrum above 1700 cm\(^{-1}\). The transient water vapor background was then subtracted from the resulting spectrum, after multiplication by an adjustment factor, so that the region from 1800 to 1700 cm\(^{-1}\) was free of spectral contributions from water vapor. The resulting protein spectrum was then baseline corrected. Second derivative spectra were obtained from the protein spectra using a third-degree polynomial function with a 9-point Savitsky-Golay smoothing function. Deconvolution of protein amide I spectra was performed using the Deconvolution application in Grams/32 based on the method developed by Griffiths and Pariente (Griffiths and Pariente 1986). Gamma factors were determined from the width of the widest resolvable peak in the corresponding second derivative spectrum (Arrondo et al. 1993). During deconvolution the gamma factor was adjusted so that there were no negative side lobes on the amide I peak. The final deconvolved spectra were smoothed using a Bessel apodization function and baseline corrected. Deconvolved spectra were curve-fit using the Grams/32 Curve-fit application. Only peaks identified by second derivative analysis were used for initial curve-fitting efforts. A linear baseline was used for the curve-fit calculations. Peak frequencies were initially fixed at the frequencies determined in the second derivative spectrum and limited to ± 1.0 cm\(^{-1}\). The widths for major peaks were initially set at 2.5 to 3.0 times the width of the second derivative peak (Arrondo et al. 1999). The remaining peak widths were initially set at 5 cm\(^{-1}\). Peak width iteration was limited to a range from 4 cm\(^{-1}\) to 30 cm\(^{-1}\) to aid in obtaining a reasonable fit. Peak intensities were initially set at 90% peak intensity at the assigned peak frequency for
the major peaks and peaks at the wings. The remaining peaks were set to 50% intensity (Abbott et al. 1991). Intensity limits were set from 0 to the intensity of the spectrum at the respective frequency. Peaks were assumed to be of mixed Gaussian-Lorentzian shape and were initially fixed at 10% Lorentzian and limited between 0 and 100%. The subsequent iteration process was carried out by fixing the frequencies and allowing the peak widths, intensities and shapes to vary. Then, only peak frequencies were allowed to vary while the other parameters were held fixed. An acceptable fit was determined both by the program's statistical analysis, and by visual inspection. Secondary structure estimates were made by calculating the secondary structure band percentage of the total amide I region (1600-1700 cm\(^{-1}\)) using the following frequency ranges: In H\(_2\)O - 1620-1645 cm\(^{-1}\), \(\beta\)-sheet; 1645-1652 cm\(^{-1}\), random; 1652-1662 cm\(^{-1}\), \(\alpha\)-helix; 1662-1690 cm\(^{-1}\), turns; In D\(_2\)O - 1613-1637 cm\(^{-1}\), \(\beta\)-sheet; 1637-1646 cm\(^{-1}\) random; 1646-1662 cm\(^{-1}\), \(\alpha\)-helix; 1662-1682 cm\(^{-1}\), turns. These assignments for curve-fitting estimates of secondary structure were made based on those used by other investigators (Byler and Susi 1986; de Jongh et al. 1995; Dong et al. 1990; Ernst-Fonberg et al. 1993; Filosa et al. 1999; Goormaghtigh et al. 1990).

**C. RESULTS AND DISCUSSION**

1. *Cytochrome c Equilibrium Monitored by Fluorescence Spectroscopy*— Fluorescence spectroscopy has been used to monitor the folded state of cytochrome c. When excited with 280 nm light, Trp59 fluoresces at 350 nm. In the folded state, the Trp59

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fluorescence is completely absorbed or quenched by the proximal heme which absorbs heavily from 300 to 400 nm. In the unfolded state the Trp59-Heme distance is increased, reducing the quenching and resulting in a strong fluorescence signal (Tsong 1973; Tsong 1974; Tsong 1975). Figure 2 shows the fluorescence spectra of cytochrome c in 0.0 to 7.0 M GdnHCl buffered in 10 mM sodium phosphate at pH 7.0. The spectra show the characteristic increase in fluorescence intensity resulting from the Trp59 and Heme groups becoming further separated as the protein unfolds. The unfolding curve (Figure 3) showed a slight signal change occurring prior to 2.5 M GdnHCl, which could be due to a number of structural changes, most likely tertiary structure changes and loosening of the native structure to that resembling the molten globule type structure. The gross conformation change or unfolding occurs from 2.5 to 3.5 M GdnHCl, as indicated by the large increase in fluorescence intensity. This intensity change is indicative of gross conformation change as the protein unfolds to a less ordered, non-native structure. Above 3.5 M the intensity continues to increase, although not as dramatically as the unfolding region from 2.5 to 3.5 M, indicating that the Trp59 and the heme continue to become farther apart. Tsong attributed this discrepancy to the possibility that unfolded Cyt c does not exist in a truly random coil state (Tsong 1975).

Unfolding curve analysis using Equations 3, 4, and 5 gave thermodynamic values of -24.9 kJ/mole for the Gibbs free energy of conformation in the absence of denaturant, a cooperativity of -7.97 kJ/mol/M, and a transition midpoint of 2.7 M (Table 1). It is important to note that the transition point is pH sensitive, so every precaution was made to control the pH at 7.0 for all experiments (Tsong 1975).
Figure 2. Fluorescence emission spectra of Cyt c (15 μM, 0.01 M sodium phosphate, pH 7.0) denatured with GdnHCl (0.0 to 7.0 M) resulting from 280 nm excitation. GdnHCl concentrations of selected spectra are indicated by the arrows. The fluorescence intensity of Cyt c increases as the protein unfolds with increasing GdnHCl concentration.
Figure 3. Fluorescence monitored unfolding curve for Cyt c buffered in 0.01 M sodium phosphate at pH 7.0 denatured in GdnHCl. The line represents the best-fit line determined by non-linear least squares regression. Thermodynamic parameters determined from this unfolding curve are shown in Table 1.
Table 1. Summary of Cyt c thermodynamic data from absorption, CD, and fluorescence unfolding studies.

<table>
<thead>
<tr>
<th>Thermodynamic Parameter</th>
<th>695 nm Absorption</th>
<th>Trp Fluorescence</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_m$ (M GdnHCl)</td>
<td>2.4</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>$\Delta G(H_2O)$ (kJ/mole)</td>
<td>-33.7</td>
<td>-24.9</td>
<td>-34.7</td>
</tr>
<tr>
<td>$m$ (cooperativity) (kJ/mole M)</td>
<td>-14.3</td>
<td>-8.0</td>
<td>-13.0</td>
</tr>
</tbody>
</table>

2. Cytochrome c Unfolding Monitored by Circular Dichroism Spectroscopy—The chemical unfolding of Cyt c using GdnHCl was monitored by CD spectroscopy. CD spectroscopy is useful for determining the major secondary structural components in a protein (Johnson 1990). Far-UV CD (185-250 nm) observes amide chromophore $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ electronic transitions. In a protein, interactions between amide portions of peptide bonds associated with $^2\alpha$ and $^3\alpha$ structure correlate to differences in the absorption of circularly polarized light. Spectral components associated with secondary structure have been assigned to 195 nm and 217 nm for random coil, 196 nm and 215 nm for $\beta$-sheet and 191, 208 and 222 nm for $\alpha$-helix (Johnson 1999). In order to get accurate estimates of secondary structure using standard CD basis sets it is necessary to obtain sample spectra down to 178 nm (Johnson 1990; Johnson 1999). The technique is limited in that a precise determination of the relative amounts of secondary structure is difficult in the presence of denaturants (e.g. guanidine hydrochloride) which absorb strongly in the UV region making spectral determinations below 210 nm impossible. However, for
cytochrome c the α-helix content has been monitored using the ellipticity at 222 nm (Goto et al. 1993). Plotting the ellipticity at 222 nm versus the concentration of denaturant provides an unfolding titration curve in the same manner as the unfolding curve was determined using the fluorescence signal at 350 nm.

Figure 4 shows the CD spectra of the native protein and selected spectra from the analysis of GdnHCl induced unfolding. The native Cyt c CD spectrum is typical for an α-helical protein with ellipticity minima at 222 and 208 nm and is consistent with what has previously been observed (Goto et al. 1993; Hamada et al. 1996). As denaturant is added, the spectrum gradually increases in ellipticity indicating a loss of α-helical structure. A plot of the ellipticity at 222 nm versus the concentration of denaturant provides an unfolding titration curve (Figure 5) (Bowler et al. 1993; Goto et al. 1993; Hamada et al. 1996). The transition midpoint of 2.7 M GdnHCl is consistent with previously reported results (Goto et al. 1993). The Gibbs free energy in the absence of denaturant and cooperativity were calculated as -34.7 kJ/mole and -13.0 kJ/mole M respectively (Table 1).

3. Cytochrome c Unfolding Monitored by Absorption Spectroscopy at 695 nm—Visible absorption spectroscopy can be used to monitor the Met80–Heme coordination. The peak at 695 nm, present in absorption spectra of native Cyt c resulting from the coordination of Met80 with the heme iron (Kaminsky et al. 1973; Sreenth and Taylor 1971), was monitored at various denaturant concentrations to determine the point at which the coordination was lost. Figure 6 shows that the peak was present in native Cyt c and at low
Figure 4. CD spectra of Cyt c (15 μM, 0.01 M sodium phosphate, pH 7.0) unfolded in GdnHCl used for determining the unfolding curve (Figure 6). GdnHCl concentrations are indicated adjacent to the corresponding spectrum. The spectrum of the native protein (0.0 M GdnHCl) is predicatively characteristic of an α-helical protein with negative ellipticities at 208 and 222 nm.
Figure 5. CD monitored unfolding curve of Cyt c in 0.01 M sodium phosphate at pH 7.0 denatured with GdnHCl. The line represents the best-fit line determined by non-linear least squares regression. Thermodynamic parameters determined from this unfolding curve are shown in Table 1.
GdnHCl concentrations. At GdnHCl concentrations above 3.0 M the peak was no longer present. An unfolding curve was prepared by plotting peak area versus denaturant concentration to analyze the transition. Figure 7 shows that the change in peak area at 695 nm is essentially complete at 3.0 M GdnHCl. Analysis of the unfolding curve yielded a Gibbs free energy of conformation in the absence of denaturant of -33.7 kJ/mole, a cooperativity of -14.3 kJ/mole M, and a transition midpoint of 2.4 M (Table 1). While the Gibbs free energies and cooperativities are comparable, the transition midpoint value suggests that the loss of the iron-Met80 coordination occurs earlier in the unfolding of the protein relative to the changes in secondary structure monitored by CD and the Trp59-Heme distance changes monitored by fluorescence.

4. Cytochrome c unfolding monitored by ATR-FTIR Spectroscopy—Attenuated total reflectance (ATR) FTIR has been used extensively to determine relative amounts of secondary structure in proteins (Arrondo et al. 1993; Byler and Susi 1986; Surewicz et al. 1993). The carbonyl stretch vibrations of protein backbone carbonyl groups give rise to absorption in the amide I region from 1600-1700 cm⁻¹. The energy of this vibration is highly sensitive to hydrogen bonding associated with secondary structure. It is possible to estimate amounts of secondary structure using band-narrowing methods such as second derivatives and Fourier deconvolution to resolve individual vibrational contributions. The individual bands can then be quantified using curve-fitting analysis. In addition, unlike CD spectroscopy, proteins denatured by chemical denaturants (e.g., guanidine hydrochloride) can be monitored. Spectral contributions by the denaturant can be
subtracted so as not to distort the protein spectral contributions (Dong et al. 1990). In this study D$_2$O is used to allow for the deuteration of the denaturant guanidine hydrochloride. Guanidine hydrochloride has a C=N stretching mode at 1675 cm$^{-1}$ that overlaps the protein amide I region interfering with spectral subtraction. Deuterated guanidine hydrochloride has a C=N stretching vibration shifted to the lower wavenumber portion of the amide I region at 1600 cm$^{-1}$. This allows the view of the main portion of the amide I region containing the bulk of the secondary structure of Cyt c with less overlap of the denaturant band. Equilibrium unfolded solutions of Cyt c were analyzed using ATR-FTIR with deuterated GdnHCl to gain a higher resolution viewpoint of conformational differences between equilibrium-unfolded solutions of Cyt c in D$_2$GdnHCl. A comparison was made to spectra of denatured Cyt c in H$_2$O to show that spectra of Cyt c in D$_2$GdnHCl have less amide I region distortion after denaturant spectral subtraction than spectra of Cyt c in GdnHCl.

The advantages of using deuterated GdnHCl are shown clearly in Figures 8a and 8b, which show representative spectra of native Cyt c and deuterated Cyt c (DCyt c) overlapped with the corresponding denaturant spectrum (GdnHCl or D$_2$GdnHCl). Natural abundance GdnHCl has a normal mode vibration at 1671 cm$^{-1}$ primarily due to the stretch of the C=N bond. The same normal mode in D$_2$GdnHCl is shifted to lower energy at 1598 cm$^{-1}$ indicative of deuterium substitution and consistent with $ab$ initio calculations (Chakraborty and Manogaran 1994). The complete deuteration of GdnHCl was obtained by incubating GdnHCl in D$_2$O at a 33% weight to volume ratio for 24 hours with one solvent exchange. Figure 9 shows that after these two incubation periods, the GdnHCl is
Figure 6. Absorption spectra for Cyt c (15 μM, 0.01 M sodium phosphate, pH 7.0) unfolded in GdnHCl (0.0 to 7.0 M). GdnHCl concentrations of select spectra are indicated by the arrows. The absorbance at 695 nm decreases as the protein unfolds in higher denaturant concentrations.
Figure 7. Absorption at 695 nm monitored unfolding curve for Cyt c in 10 mM sodium phosphate at pH 7.0 denatured in GdnHCl. The line represents the best-fit line determined by non-linear least squares regression. Thermodynamic parameters determined from this unfolding curve are shown in Table 1.
completely deuterated judging by the fact that the peak does not red-shift further after an additional solvent exchange. The red-shift in absorption in the DGdnHCl denaturant spectrum significantly lessens the amount of overlap with the protein vibrations as compared to the non-deuterated sample thereby improving the sensitivity of methods to obtain the spectrum due to protein alone.

We explored the effects of over- and under-subtraction of the GdnHCl (Figure 10a) and DGdnHCl (Figure 10b) solution spectra in the amide I region following the procedure of Bowler et al. (Bowler et al. 1994). To obtain a protein amide I spectrum, the background buffer and denaturant (if applicable) spectrum must be subtracted from the corresponding protein spectrum. Before subtracting, the denaturant/buffer spectrum is multiplied by a subtraction factor to ensure that the resulting amide I spectrum is not over- or under-subtracted. Whether subtracting GdnHCl or DGdnHCl, care must be taken to avoid using an incorrect subtraction factor. Incorrect subtraction would result in a distorted amide I spectrum and may therefore lead to erroneous secondary structure interpretations. In Figure 10a, for the GdnHCl subtraction process, it can be seen that over- and under-subtraction lead to differences in intensity and slight differences in peak position throughout the entire amide I region. Over- and under-subtraction of DGdnHCl, on the other hand, only shows slight intensity differences in the amide I region below 1640 cm⁻¹ (Figure 10b). Little to no effect is seen above 1640 cm⁻¹. The adverse effects of poor subtraction are increased the closer you get to 1598 cm⁻¹, the vibrational frequency of the deuterated guanidinium C=N normal mode. The most pronounced effect is at 1617 cm⁻¹, where the second derivative peak becomes more negative and shifts to lower
frequency on over-subtraction. The changes however, are consistent with those reported by Bowler et al. who concluded that they are not significant enough to cause artifacts leading to poor spectral interpretation on a qualitative level (Bowler et al. 1994). While the effects of over- and under-subtraction were demonstrated on the spectra of the highest denaturant concentrations it serves as proof of higher spectral integrity for samples in D$_2$O with any concentration of DGdnHCl compared to samples in H$_2$O and GdnHCl.

To test the utility of this new method, the secondary structure of equilibrium mixtures of DCyt c and DGdnHCl were investigated by ATR-FTIR spectroscopy with second derivative analysis and amide I curve-fitting. Figure 11a shows the experimentally obtained amide I spectrum of native DCyt c with the atmospheric water vapor and solvent spectra subtracted (0.1 M phosphate buffer, pD 7.0). The inset shows the corresponding second derivative spectrum identifying individual bands in the amide I region used for curve-fitting. The second derivative spectrum of native DCyt c shows four major peaks, at 1674, 1653, 1636 and 1615 cm$^{-1}$, which have been previously assigned to turns, α-helix, β-sheet, and β-sheet respectively (Figure 11a inset and 12b) (Byler and Susi 1986; Dong et al. 1990; Dong et al. 1995; Filosa et al. 1999; Muga et al. 1991b). Figure 11b shows the curve-fitting results for native DCyt c. Secondary structure estimates were determined to be 45% α-helix, 23% β-sheet, 13% turns and 19% random coil. These results are comparable to values reported by other investigators (Table 2) using IR spectroscopy (de Jongh et al. 1995; Dong et al. 1990; Filosa et al. 1999) and X-ray crystallography (Bushnell et al. 1990).
Figure 8. ATR-FTIR spectra emphasizing the overlap between denaturant and protein bands. (a) 0.5 M guanidine hydrochloride and native Cyt c in H₂O with buffer and water vapor subtracted. (b) 0.5 M deuterated guanidine hydrochloride and native Cyt c in D₂O with buffer and water vapor subtracted.
Figure 9. Deuteration of GdnHCl monitored by ATR-FTIR spectroscopy. Non-deuterated GdnHCl (black spectrum) becomes partially deuterated after 3 minutes and 24 hours (red spectra) in D$_2$O as indicated by the red shift from $\sim$1675 cm$^{-1}$ to $\sim$1600 cm$^{-1}$. After exchanging the buffer with fresh D$_2$O and incubating for 5 minutes and 24 hours (blue spectra) deuteration is essentially complete as an additional buffer exchange with fresh D$_2$O does not change the peak center (green spectrum).
In order to determine the secondary structure of DCyt c as it unfolds, samples were prepared in increasing concentrations of DGdnHCl and allowed to equilibrate prior to analysis by ATR-FTIR spectroscopy. As the denaturant concentration increases the amide I spectra change from having one main peak at 1650 cm$^{-1}$ under native conditions to having two main peaks at 1618 and 1651 cm$^{-1}$ under denaturing conditions (Figure 12a). The amide I spectral changes are indicative of a cooperative two state process of unfolding. To better resolve the structural changes second derivative spectra were obtained and overlaid for comparison (Figure 12b). The most abundant component of the secondary structure of native Cyt c is α-helix with lesser amounts of turns, random structure and β-sheet. Qualitatively, the changes in secondary structure of equilibrium solutions of DCyt c with increasing concentrations of DGdnHCl, as monitored by second derivative spectra, reveal conformational changes where the content of α-helix decreases as β-sheet increases. The α-helix peak at 1653 cm$^{-1}$ appeared from 0.0 to 2.9 M DGdnHCl. At 1.5 M DGdnHCl the peak distinctly shifted to 1650 cm$^{-1}$ which is the wavenumber assigned to α-helical bands of proteins in D$_2$O (Byler and Susi 1986). In a previous study a similar shift was seen in the amide I spectrum of thermally denatured DCyt c (Muga et al. 1991b). Muga et al. attributed the shift to the exchange of interior amide protons of the helices as the overall structure of the protein changed from a compact native state to a more globular structure. Our findings are consistent with those of Muga et al. in that these protons are not initially exchanged when the protein is in the folded state, resulting in an amide I band which is closer to the one expected for the
protein in H$_2$O (1656 cm$^{-1}$). The gradual decrease in size of the 1650 cm$^{-1}$ peak over the
1.9 to 2.9 M DGdnHCl range indicates a loss of helical structure. The disappearance of
the 1650 cm$^{-1}$ peak at 3.4 M DGdnHCl is consistent with $\alpha$-helix changes observed using
CD spectroscopy (Hamada et al. 1996). The 1674 cm$^{-1}$ peak associated with turns is not
apparent above 3.4 M DGdnHCl. The broad peak at 1636 cm$^{-1}$ associated with $\beta$-sheet is
not present above 1.0 M DGdnHCl. The 1615 cm$^{-1}$ peak associated with $\beta$-sheet shifts to
higher wavenumber and becomes the dominant peak above 2.6 M DGdnHCl indicating
the protein has taken on a predominantly $\beta$-sheet structure.

To quantify the secondary structure of the denatured protein, curve-fitting was
performed on the amide I spectra of DCyt $c$ up to 6.6 M DGdnHCl (Figure 13, Appendix
I). Curve fitting was used to resolve amide I spectra into the multiple component bands
which together make up the entire amide I spectra and correlate to the different types of
secondary structure. The component bands were used to quantify the amounts of
secondary structure present in the DCyt $c$ samples. Table 3 shows the results of all curve-
fitting quantification. One can see that as the denaturant concentration increases the
amount of $\alpha$-helix decreases while the amount of $\beta$-sheet increases. The amount of turns
and random structure essentially remain the same. Figure 13 shows the curve-fit
performed on the deconvolved 6.6 M DCyt $c$ spectrum (additional curvefits are shown in
Appendix I). The spectrum has two main peaks, the dominant one at 1613 cm$^{-1}$ indicating
the protein has taken on more $\beta$-sheet secondary structure than the native protein. The
amount of $\beta$-sheet in the 6.6 M DGdnHCl sample was estimated to be 51% compared to
23% for the native protein (Tables 2 and 3). This result is surprising because one would
Figure 10. ATR-FTIR spectra of (a) Cyt c in 6.7 M GdnHCl, 0.1 M phosphate buffer at pH 7.0 and (b) DCyt c in 6.6 M DGDnHCl, 0.1 M phosphate buffer at pH 7.0 showing the effects of over- (lower spectrum) and under-subtraction (upper spectrum) of the respective denaturant spectrum. The insets show the second derivatives of the over- (upper spectrum) and under-subtracted (lower spectrum) amide I spectra.
Figure 11. (a) ATR-FTIR spectrum of native DCyt c, 0.1 M phosphate buffer, pH 7.0. An inset of the second derivative in the amide I region (1700-1600 cm⁻¹) is shown to demonstrate identification of component bands associated with secondary structure. (b) Curve-fit results for the deconvolved amide I band of native DCyt c in 0.1 M phosphate buffer, pH 7.0. Areas of individual component bands are representative of the secondary structure of the protein. The spectrum labeled with "+" is the deconvolved amide I spectrum.
Figure 12. (a) Stacked overlay of the amide I spectra of DCyt c (0.1 M phosphate buffer, pD 7.0) in the presence of increasing amounts of DGdnHCl. The molarity of DGdnHCl for each spectrum is indicated on the right. (b) Second derivative amide I spectra of DCyt c in the presence of DGdnHCl. The molarity of DGdnHCl for a given spectrum is as indicated by the arrows.
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<th>% Turns</th>
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<td>X-ray</td>
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<td>45</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>(Bushnell et al. 1990)</td>
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Table 2. Comparison of Secondary Structure Estimates of native and denatured DCyt c and Cyt c to X-ray diffraction estimate of native structure.
expect the protein to predominantly consist of more random structure at high denaturant concentrations rather than maintain ordered secondary structure. Previous IR studies of thermally denatured (Dong et al. 1995; Muga et al. 1991b; Surewicz et al. 1990) and aggregated (Caughey et al. 1991b) proteins have attributed peaks below 1620 cm\(^{-1}\) to intermolecular \(\beta\)-sheet formation and aggregation. While aggregation is a possibility given the high protein concentration used in our investigation (50 mg/mL), visual examination of samples and gel filtration chromatography showed no evidence of aggregation (data not shown). The \(\beta\)-sheet content did not appear to be concentration dependent given that the normalized amide I spectra of 50, 25 and 10 mg/mL Cyt \(c\) in 6.7 M GdnHCl were identical (Figure 14). The high \(\beta\)-sheet content is also confirmed by the high amount of \(\beta\)-sheet (93%) determined for Cyt \(c\) denatured in 6.7 M GdnHCl (Table 2, Appendix I).

For a qualitative comparison of the changes seen with the samples in D\(_2\)O and D\(\text{GdnHCl}\), equilibrium solutions of Cyt \(c\) in H\(_2\)O and GdnHCl were analyzed by ATR-FTIR in the presence of 0.0 to 6.7 M GdnHCl. While curve-fitting was not performed on all samples in the presence of denaturant because the amide I region is less precisely determined due to problems with denaturant subtraction, the amide I and second derivative spectra are still useful for identifying secondary structure because the wavenumbers for peak centers are approximately correct (Figure 10a). Starting with the Cyt \(c\)/GdnHCl amide I and second derivative spectra in Figure 15a, one observes 4 major peaks for native Cyt \(c\) (0.0 M GdnHCl) at 1681, 1655, 1633 and 1615 cm\(^{-1}\). These peaks have been previously assigned to turns, \(\alpha\)-helix, \(\beta\)-sheet at 1681, 1655, and 1633 cm\(^{-1}\).
Figure 13. Curve-fit bands of the deconvolved spectra of DCyt c in 6.6 M DGuHCl. The spectrum labeled with "+" is the deconvolved amide I spectrum.
Figure 14. Dependence of Cyt c amide I spectrum on concentration of Cyt c. Normalized (a) amide I spectra and (b) second derivative spectra of 5% (black), 2.5% (blue), and 1.0% (red) Cyt c (weight:volume) in 6.7 M GdnHCl suggest that amide I spectrum is not influenced by concentration of Cyt c.
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<td>6.6</td>
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<td>51</td>
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<td>15</td>
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</table>

Table 3. Curve-fitting results for amide I IR spectra of DCyt c denatured in DGdnHCl.
respectively (Bowler et al. 1994; Byler and Susi 1986; Dong et al. 1990; Ernst-Fonberg et al. 1993; Filosa et al. 1999; Goormaghtigh et al. 1990; Lee et al. 1990; Oberg and Fink 1998). The 1615 cm\(^{-1}\) peak has been assigned to side chain interactions not associated with secondary structure (Susi and Byler 1983). The secondary structure of native Cyt \(c\) was estimated by curve-fitting (Figure 15b) to be 42% \(\alpha\)-helix, 27% \(\beta\)-sheet, 6% turns and 5% random coil (Table 2). These results are also comparable to FTIR results reported by other investigators (Dong et al. 1990; Ernst-Fonberg et al. 1993; Filosa et al. 1999) and to X-ray diffraction estimates (Bushnell et al. 1990). Similar to the DCyt \(c\) data, the stacked overlay of amide I spectra of Cyt \(c\) in increasing concentrations of GdnHCl indicates a highly cooperative two-state equilibrium unfolding process (Figure 16a). With increasing denaturant concentrations the one main peak center shifts from 1652 cm\(^{-1}\) to 1635 cm\(^{-1}\) indicating a shift from \(\alpha\)-helical to \(\beta\)-sheet structure. Unlike the amide I spectra for DCyt \(c\) however, the Cyt \(c\) spectra have only one main peak at high denaturant concentrations. Following the second derivative spectra in Figure 7b the major peak at 1655 cm\(^{-1}\) associated with \(\alpha\)-helix is present from 0.0 to 2.6 M GdnHCl, and gradually shifts to 1653 cm\(^{-1}\) by 2.6 M GdnHCl; at 3.2 M GdnHCl the peak is no longer distinguishable indicating the loss of \(\alpha\)-helix structure similar to the changes seen with DCyt \(c\). This change in secondary structure is consistent with previous CD data (Hamada et al. 1996) and the overall unfolding of the protein as monitored by tryptophan fluorescence quenching (transition midpoint of 2.7 M GdnHCl) (Tsong 1974). The increase in the size of the doublet peak at 1638 and 1644 cm\(^{-1}\) with up to 6.7 M GdnHCl indicates an increase in \(\beta\)-sheet similar to the increase seen in the DGdnHCl samples up.
to 6.6 M (51% β-sheet) with an estimated 93% of the structure consisting of β-sheet (Table 2, Appendix 1). The reason for the discrepancy between the amounts of β-sheet in the GdnHCl sample versus the DGdnHCl sample is not clear. It is possible that the high absorbance of GdnHCl in the amide I region leads to distortion of the protein spectrum after subtraction of the denaturant spectrum.

The observed increase in β-sheet for both Cyt c in 6.7 M GdnHCl and DCyt c in 6.6 M DGdnHCl is, at first, surprising as proteins in the presence of GdnHCl have been previously shown to have the properties expected of random coil polypeptides (Creighton 1990; Ikai et al. 1973). However, more recently it has been shown that denatured proteins consist of a collection of partially folded states (Dill and Shortle 1991; Dobson et al. 1984; Griko et al. 1988; Howarth and Lian 1984; Privalov et al. 1989; Smith et al. 1996). In addition it has been pointed out that in a truly random coil polypeptide the energetics of the interactions between different parts of the polypeptide chain and the solvent must be exactly balanced and that this is highly unlikely given the broad range of chemical properties for the 20 amino acids (Creighton 1990). In the case of Cyt c, Kataoka et al. demonstrated using small-angle X-ray scattering that Cyt c in the presence of 4 M GdnHCl at pH 2.0 had a radius of gyration of 32.4 Å, which is lower than that estimated for a truly random coil suggesting that the denatured state contains residual structure (Kataoka et al. 1993). Our results suggest that denatured Cyt c contains residual native β-sheet and/or newly formed β-sheet.
Figure 15. (a) ATR-FTIR spectrum of native Cyt c, 0.1 M phosphate buffer, pH 7.0. An inset of the second derivative in the Amide I region (1700-1600 cm⁻¹) is shown to demonstrate identification of component bands associated with secondary structure. (b) Curve-fit results for the deconvolved amide I band of native Cyt c in 0.1 M phosphate buffer, pH 7.0. Areas of individual component bands are representative of the secondary structure of the protein. The spectrum labeled with “+” is the deconvolved amide I spectrum.
Figure 16. (a) Stacked overlay of the amide I spectra of Cyt c (0.1 M phosphate buffer, pH 7.0) in the presence of increasing amounts of GdnHCl. The molarity of GdnHCl for each spectrum is indicated on the right. (b) Second derivative amide I spectra of Cyt c in the presence of GdnHCl. The molarity of GdnHCl for a given spectrum is as indicated by the arrows.
5. Discussion—The folding studies presented in this report suggest that there are multiple events occurring within the structure of Cyt c as it unfolds. The data do not support the presence of a simple, two-state equilibrium between the native and unfolded states. The structural changes monitored by each spectroscopic method used in this study are summarized in Figure 17. The difference between the transition midpoints for the absorbance data and the CD and fluorescence data suggest that the Fe(III)-Met80 coordination is lost prior to the complete unfolding of the secondary structure and the movement of Trp59 beyond the distance required for complete quenching by the Heme. In addition, the FTIR studies suggest that the \( \alpha \)-helices loosen at denaturant concentrations below 1.5 M GdnHCl and that \( \beta \)-sheet content increases all the way up to the highest denaturant concentrations tested (6.7 M GdnHCl). The structural changes are summarized in Figure 17 as follows: Between 0.0 and 1.5 M GdnHCl there is a loosening of the Fe(III)-Met80 coordination and the \( \alpha \) helices, along with an increase in \( \beta \)-sheet structure; between 1.5 and 3.5 M GdnHCl there is a major loss in \( \alpha \)-helix, a complete loss of the Fe(III)-Met80 coordination, and significant increases in the Trp59-Heme distance, \( \beta \)-sheet, random structure, and turns; between 3.5 and 7.0 M GdnHCl there is a slight continuation of the increase in the Trp59-Heme distance, a continued increase in \( \beta \)-sheet structure, and a decrease in random structure.

The results suggest that there are multiple unfolded states for the protein at equilibrium conditions. The multiple bonding interactions associated with the native structure of the protein vary in strengths as do all forms of bonds. It seems intuitively correct to assume that these interactions occur over a narrow range of denaturant
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</table>

Figure 17. Summary of structural changes observed in Cyt c by spectroscopic methods used in this investigation. ↑ indicates an increase, ↓ indicates a decrease.
concentrations since non-covalent interactions are low energy interactions. Slight changes in the solvent environment apparently induce continual slight changes in protein structure until a critical point is reached. Or is it a critical point? The fluorescence unfolding curve (Figure 3) is deceptive in the sense that the fluorescence change upon unfolding appears as a dramatic jump. The efficiency of the energy transfer for fluorescence quenching, and absorbance-probed interactions, such as the Fe(III)-Met80 coordination are reduced by the acceptor-donor/transition dipole interaction distances raised to the 6th power. Slight changes in these distances lead to exponentially dramatic changes in probe response. This is not to say that dramatic structural changes do not occur but caution should be used when interpreting results of conformational probes of protein structures at equilibrium. It has been proposed that the rapid folding of Cyt c goes through a number of intermediate states before attaining native conformation (Ferri et al. 1996; Rush and Spiro 1998; Segel et al. 1998). The results of equilibrium studies support this proposal in the sense that there are differences in the denaturant concentrations at which native structural features change to non-native structure as monitored by fluorescence, CD, absorbance, and FTIR measurements. Slight changes in tertiary and secondary structure such as repositioning of loops and turns, can happen at any point prior, during, and after major structural changes occur, such as hydrophobic expansion upon unfolding or collapse upon folding.
D. Conclusions

In this study, it has been shown that amide I spectra at high denaturant concentrations (up to 6.6 M DGdnHCl) obtained using ATR-FTIR spectroscopy and DGdnHCl are less distorted than spectra obtained using GdnHCl. Using this method, significant amide I spectral changes were observed during chemical unfolding of horse heart Cyt c. These spectral changes have not previously been reported and are not entirely observable by other secondary structure techniques. Solutions of equilibrium unfolded Cyt c in D$_2$O and DGdnHCl were obtained from 0.0 to 6.6 M DGdnHCl. Qualitative analysis of the unfolding of the protein monitored by second derivative analysis revealed the protein unfolds from a mainly $\alpha$-helical to a primarily $\beta$-sheet protein. Similar changes were observed in the second derivative spectra of Cyt c in H$_2$O from 0.0 to 6.7 M GdnHCl. The secondary structure of the denatured protein in 6.6 M DGdnHCl and 6.7 M GdnHCl were estimated by curve-fit analysis to be 51% and 93% $\beta$-sheet respectively. While the reason for the discrepancy between the DGdnHCl and GdnHCl denatured protein secondary structure estimates is not clear, both methods suggest the denatured protein contains a substantial amount of secondary structure. This new method allows insights into secondary structure changes induced by DGdnHCl and should be applicable to many water-soluble proteins.

Combining the ATR-FTIR Cyt c unfolding data with the fluorescence, CD, and absorption data has provided a clear view of the equilibrium unfolded structures populated by Cyt c. The unfolding can be divided into three distinct domains defined by

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denaturant concentration: low concentration unfolding events (≤ 1.5 M GdnHCl), mid-range denaturant concentration events (between 1.5 and 3.5 M GdnHCl), and high concentration unfolding events (between 3.5 and 7.0 M GdnHCl). At low denaturant concentrations there is a slight decrease in the Fe(III)-Met80 coordination, a loosening of α-helices, and an increase in β-sheet structure. Many of the structural changes occur within the mid-range GdnHCl concentrations. These changes include a significant decrease in α-helix, a complete loss of the Fe(III)-Met80 coordination, a dramatic increase in the Trp59-Heme distance, and increases in β-sheet, random structure, and turns. At high denaturant concentrations there is a slight continuation of the increase in the Trp59-Heme distance, a continued increase in β-sheet, and a decrease in random structure. The results described in this report demonstrate that equilibrium unfolding of Cyt c is not a simple two-state process, but rather, is comprised of multiple complex structural transitions. Thus in order to improve understanding of the forces that drive the folding of Cyt c and the folding of proteins in general, more sophisticated assumptions of protein folding pathways and improved methods of determining protein structure (structural intermediates in particular) must be developed.
APPENDIX I

CURVE-FIT ATR-FTIR SPECTRA OF CYTOCHROME C
2.6 M DGDnHCl

Absorbance
5.2 M D	extsubscript{2}HCl

Absorbance
6.6 M DGdnHCl
II. The Role of Helix 1 Aspartates in the Stability and Conversion of Prion Protein
A. INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) or prion diseases are neurological disorders characterized by the accumulation of abnormal protease-resistant forms of prion protein (PrP)-e.g., scrapie-associated PrP or protease resistant PrP (PrP-res)-in the central nervous system of diseased animals. TSE diseases include Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome and kuru in humans, scrapie in sheep, mice and hamsters, bovine spongiform encephalopathy in cattle, and chronic wasting disease in deer and elk. Although not yet fully understood, the accumulation of PrP-res appears to lead to cerebral amyloidosis, dementia, motor dysfunction, and eventually death. However, PrP-res has been shown to accumulate as a result of the conversion of the normal, protease sensitive form of PrP (PrP-sen) which occurs as a post-translational process without any apparent requirement for covalent modifications (Caughey and Raymond 1991; Hope et al. 1986; Stahl et al. 1993).

Outside of its role in TSE diseases the normal biological function of PrP has not been clearly determined. However, mice lacking PrP have been observed to have altered sleep patterns and circadian activity rhythms (Tobler et al. 1996; Tobler et al. 1997). Additional studies have suggested other roles for the protein including regulation of copper levels (Brown 1999; Brown et al. 1997; Jackson et al. 2001; Hornshaw et al. 1995; Thackray et al. 2002; Viles et al. 1999; Waggoner et al. 2000), super oxide dismutase activity (Brown 1999), and signal transduction (Mouillet-Richard et al. 2000).
While the normal biological function of PrP-sen remains in question, much has been learned about its biochemistry. The gene for PrP encodes for polypeptides of 254 amino acids (Basler et al. 1986; Locht et al. 1986). An N-terminal signal peptide of 22 amino acids is removed from these molecules during biosynthesis (Bolton et al. 1987; Hope et al. 1986; Turk et al. 1988), and an additional 23 amino acids are removed from the C-termini during glycosylphosphatidylinositol (GPI) addition at Ser 231 (Stahl et al. 1990), resulting in a mature PrP polypeptide of 210 residues. The secondary structure of PrP-sen has been analyzed by CD and FTIR spectroscopies, revealing the secondary structure consists predominantly of α-helix with little β-sheet (Pan et al. 1993). Additional structural studies using nuclear magnetic resonance (NMR) have provided more detailed views of PrP-sen structure. The structure of PrP-sen as revealed by NMR spectroscopy (Figure 18) consists of a core domain (residues 125-228), containing 3 α-helices (helix 1, residues 144-154; helix 2, 172-193; helix 3, 200-227), two short anti-parallel β-strands (residues 129-131 and 161-163), and a largely unstructured N-terminal domain (residues 23-119) (James et al. 1997; Liu et al. 1999). A disulfide bond between cysteine residues 179 and 214 in PrP forms a loop (Turk et al. 1988), which contains two consensus sites for asparagine-linked glycosylation at residues 181 and 197. Addition of glycans at these sites generates unglycosylated, partially glycosylated, and fully glycosylated PrP glycoforms. Following translation of PrP by the ribosome, high mannose glycans are added to the protein in the endoplasmic reticulum which are subsequently converted to complex or hybrid glycans in the Golgi apparatus (Caughey et al. 1989). Approximately 60 individual oligosaccharide chain variants have been
Figure 18. Salt bridges proposed to stabilize helix 1 of PrP-sen. NMR structure of hamster PrP-sen (residues 125-228) reported by James et al. (left) (James et al. 1997). Enlarged areas show helix 1 with stabilizing salt bridges between aspartic acid 147 and arginine 151 (center) and side chain atoms involved in the ionic interactions (right).
identified on PrP, demonstrating that the partially- and fully-glycosylated proteins are composed of numerous minor glycoforms (Rudd et al. 1999; Stimson et al. 1999). Following processing in the Golgi apparatus, PrP-sen is translocated to the cell surface and anchored to the plasma membrane by the transmembrane insertion of the GPI anchor (Caughey et al. 1989; Caughey et al. 1990; Stahl et al. 1987; Stahl et al. 1990). Once on the cell surface, PrP-sen has a half-life of three to six hours (Borchelt et al. 1990; Caughey et al. 1989; Caughey and Raymond 1991) and most PrP appears to be degraded in non-acidic compartments bound by cholesterol-rich membranes (Taraboulos et al. 1995). Investigations on the endocytosis of PrP-sen indicate that it cycles between the cell surface and an endocytic compartment with a transit time of approximately 60 min (Shyng et al. 1993).

The prion hypothesis proposes that an abnormal form of PrP, such as PrP-res, is in fact the TSE infectious agent (Prusiner 1998). The importance of PrP in TSEs was clearly shown in infectivity experiments performed with PrP knockout mice. Mice completely lacking PrP were completely protected against TSE disease, and their ability to propagate infectivity was eliminated (Bueler et al. 1993). In diseased animals PrP-res forms insoluble aggregates and is partially resistant to proteinase K (PK), which removes approximately 67 amino acid residues (6-7 kDa) from the N-terminus (Hope et al. 1986; Meyer et al. 1986; Oesch et al. 1985; Rubenstein et al. 1986). These differences in biophysical properties most likely reflect different conformations of the two protein isoforms. While NMR and optical spectroscopic data indicate that PrP-sen isolated from normal brain is highly $\alpha$-helical (James et al. 1997; Liu et al. 1999; Pan et al. 1993),
FTIR studies indicate PrP-res contains a large proportion of β-sheet secondary structure (Caughey et al. 1991c; Safar et al. 1993). The conversion of PrP-sen into PrP-res is likely to be the key event in the pathogenesis of TSE diseases. Although TSE diseases may be sporadic, genetic or infectious in origin, it is commonly argued that in each circumstance the disease propagates itself through a self-catalytic mechanism involving existing multimers of PrP-res. This idea is supported by experiments such as studies using the cell-free conversion reaction which show that PrP-res can induce the conversion of PrP-sen into PrP-res-like aggregates in vitro (Caughey et al. 2001; Kocisko et al. 1994).

While much work has been done using such model systems, the precise molecular mechanism of the conversion between the normal and pathogenic forms of prion proteins remains poorly understood.

Currently, two theoretical models of PrP-res formation have been described: the heterodimer model (Prusiner 1998) and the nucleated polymerization model (Come et al. 1993; Jarrett and Lansbury, Jr. 1993; Lansbury, Jr. and Caughey 1995). The heterodimer model proposes that a stable PrP-res monomer binds PrP-sen, forming a heterodimer. Once PrP-sen is bound, the PrP-res portion of the heterodimer catalyzes a conformational change in PrP-sen leading to the formation of a homodimer of PrP-res. The PrP-res homodimer then dissociates yielding two PrP-res monomers. This model requires that PrP-res is more stable thermodynamically than PrP-sen, that conversion of PrP-sen to PrP-res is rare unless catalyzed by a preexisting PrP-res template, and that the PrP-res homodimer tends to dissociate into monomers. According to the model, conversion also requires the assistance of a hypothetical, species-specific cofactor termed “protein X”
(Bolton and Bendheim 1988; Griffith 1967; Prusiner 1998). In the nucleated polymerization model, PrP-sen binds to a preexisting polymer of PrP-res and subsequently converts. According to this model, seeded polymerization and conversion of multiple PrP-sen monomers is required in order to stabilize PrP-res enough to allow its accumulation to levels that have biological impacts. The spontaneous formation of PrP-res multimers that can seed the polymerization is rare because of the weakness of monovalent interactions between PrP-sen molecules and/or the rarity of the conformers that polymerize. However, once formed, polymeric seeds are stabilized by multivalent interactions that give PrP-res its characteristic resistance to disaggregation and proteolytic degradation (Griffith 1967; Gajdusek 1988; Jarrett and Lansbury, Jr. 1993; Lansbury, Jr. and Caughey 1995).

According to the proposed theoretical models described above, PrP-res and PrP-sen are expected to interact directly in the course of TSE pathogenesis. Empirical evidence suggesting that PrP-sen and PrP-res specifically interact has been provided in studies using animals (Prusiner et al. 1990), and scrapie-infected tissue culture cells (Priola et al. 1994; Priola and Chesebro 1995). More directly, specific binding of PrP-res to PrP-sen has been observed in lysates or culture supernatants from \[^{35}S\]-methionine-labeled cells expressing soluble PrP-sen lacking the GPI (GPI') anchor (Horiuchi and Caughey 1999). Under these conditions (pH 6.0, 1% sarkosyl), only GPI \[^{35}S\]PrP-sen bound, out of the many labeled proteins in the preparations. Work exploring the interaction and conversion of PrP-sen species containing the GPI anchor did not detect conversion of the membrane-bound PrP-sen by exogenous PrP-res (Baron et al. 2002). It
was, therefore, proposed by Baron and coworkers that membrane attachment of PrP-sen may limit accessibility to PrP-res or prevent the conformational conversion necessary for PrP-res formation. The inaccessibility may result from the PrP-res binding sites being located near the C-terminus of PrP-sen. These sites may be blocked by attachment to the membrane via the C-terminal GPI anchor (Horiuchi and Caughey 1999), or direct interactions between membrane surfaces and the PrP-sen polypeptide chain may block its access to PrP-res (Morillas et al. 1999; Sanghera and Pinheiro 2002), although data from recent cell-free conversion studies do not support this latter possibility (Baron and Caughey 2003).

As mentioned above, cell-free conversion reactions have provided only circumstantial proof of the protein-only hypothesis because the cell-free conversion generated PrP-res has yet to be shown to be infectious. This has resulted in questions regarding its use as a physiologically relevant model of conversion. There are a number of ways, however, in which cell-free conversion reflects the biology of TSE disease in vivo. Cell-free conversion reactions have demonstrated PrP sequence specificity between the PrP-sen and PrP-res that correlates with inter- and intra-species TSE transmissions in vivo, possibly reflecting a critical control point in interspecies TSE transmissions (Bossers et al. 1997; Bossers et al. 2000; Horiuchi et al. 2000; Kocisko et al. 1995; Raymond et al. 2000; Raymond et al. 1997). In addition, the strain specificity of the reaction products is consistent with in vivo PrP-res formation and provides a potential mechanism for TSE strain propagation by a protein-only mechanism (Bessen et al. 1995). When unfolded with GdnHCl, both the converting activity of PrP-res and its infectivity
titer in animals decrease, suggesting the two parameters are directly related (Caughey et al. 1997). Finally, since the *in vitro* conversion reaction has been adapted to physiologically compatible conditions, this suggests that it can also occur *in vivo* (Baron et al. 2002; Bessen et al. 1997; DebBurman et al. 1997; Horiuchi and Caughey 1999; Wong et al. 2001). Thus, the variety of observations on cell-free conversion studies warrants its use as a model for studying the conversion of PrP-sen to PrP-res.

A number of mechanistic details about the conversion of PrP-sen to PrP-res have been described in reports that used the cell-free conversion reaction. Generation of conversion product was shown to be dependent upon time and the concentrations of input PrP-res and PrP-sen in a complex and in a condition-dependent fashion (Caughey et al. 1995; Horiuchi and Caughey 1999). Converting activity was distinctly associated with PrP-res polymers but not with soluble monomeric forms of PrP from disaggregated PrP-res (Caughey et al. 1995; Caughey et al. 1997). There was no detectable dissociation of newly converted PrP-res which remained associated with the seed PrP-res aggregates and was not solubilized without GdnHCl treatment (Bessen et al. 1997; Callahan et al. 2001). Analyses of the kinetics of conversion provided evidence that the conversion process can be separated into two stages, an initial binding of PrP-sen to PrP-res, and then a slower conversion of the bound PrP-sen to PrP-res (Bessen et al. 1997; DebBurman et al. 1997; Horiuchi and Caughey 1999). Comparisons of conversion reactions containing GdnHCl (Kocisko et al. 1994) and reactions containing no GdnHCl and low amounts of detergent suggest that the binding and conversion of the latter reactions occur at earlier time intervals (Wong et al. 2001). Recently, Zou and Cashman found a stimulatory effect by
pretreatment of brain PrP-sen with acid pH and GdnHCl followed by exposure to a low
congeentration of sodium dodecyl sulfate (SDS) which could generate PrP-sen that is more
convertible to PrP-res (Zou and Cashman 2002). Collectively, the observations made on
the cell-free conversion reactions described above, and the fundamental characteristic of
PrP-res to form amyloid fibril polymers, are consistent with the conversion mechanism
following the nucleated polymerization mechanism.

More insights into the conversion mechanism have been gleaned from studies
using anti-PrP antibodies, which have been used to begin mapping the surfaces on PrP-
sen that are involved in its initial binding to PrP-res during cell-free conversion (Horiuchi
and Caughey 1999). So far, out of a number of antibodies directed against regions
throughout the PrP sequence, only antibodies directed against the C-terminus of mouse
PrP (α219-232 epitope) have inhibited binding and have resulted in conversion of PrP-
sen to PrP-res. However, removal of the α219-232 epitope from PrP-sen did not
eliminate binding suggesting that it is not the epitope itself that is required, but residues
close to it in space that are sterically hindered by antibody binding. Surfaces that are
close to the epitope through space include the extended chain of residues (~119-140)
close to helix 1, the loop of residues after helix 1 (~165 to 174) between the second β-
strand and the second α-helix, and helical residues (~206-223) in the third helix
(Horiuchi and Caughey 1999).

The same regions identified in antibody inhibition studies have been recognized
using peptide inhibition studies as regions of possible interaction between PrP-sen and
PrP-res. Peptides corresponding to residues 119-136 (Chabry et al. 1999; Chabry et al.
Peptides corresponding to residues 109-141 and 90-145 can also form protease-resistant complexes with PrP-sen (Kaneko et al. 1997a; Kaneko et al. 1995). Amino acid substitutions in these regions have been shown to affect PrP-res formation in scrapie-infected cells (Kaneko et al. 1997b). The ability of such mutants to inhibit PrP-res formation may be due to an ability to bind wild type PrP-res and, without converting themselves, to interfere with the binding and/or conversion of wild type PrP-sen as has been shown for heterologous PrP-sen molecules (Horiuchi et al. 2000; Priola et al. 1994).

Several considerations (Morrissey and Shakhnovich 1999; Homemann and Glockshuber 1998; Kaneko et al. 1995; Swietnicki et al. 2000b) suggest that helix 1 (residues 144-153) might be a site of interaction of PrP-sen and PrP-res, and that it plays a critical role in the initial stages of PrP-sen unfolding and conversion to PrP-res. Morrissey and Shakhnovich (Morrissey and Shakhnovich 1999) noted that helix 1 is unusually hydrophilic and lacking in non-covalent contacts with the remainder of the protein. They proposed that the stabilization of helix 1 is self-sustaining through the presence of intra-helix electrostatic interactions such as the two properly phased aspartate (Asp)-arginine (Arg) pairs at positions 144-148 and 147-151 respectively within the helix, which form strong salt-bridges and stabilize the helical turns (Figure 18) (Morrissey and Shakhnovich 1999). The directional ordering of the charges is expected to interact with the intrinsic dipole moment of the helix providing further stabilization. Molecular modeling studies suggested that the conserved amino acid sequence of hamster
PrP helix 1 (Table 4) might favorably adopt conformations necessary to form intermolecular β-sheet aggregates and that these aggregates might be stabilized by intermolecular salt bridges between the helix 1 Asp and Arg residues of adjacent molecules (Morrissey and Shakhnovich 1999). Consistent with this idea, Vorberg et al. reported that the deletion of helix 1 resulted in the complete inhibition of PrP-res formation in cell-free conversion reactions and scrapie infected cells (Vorberg et al. 2001). Other studies have shown that a de novo α-helix to β-sheet conformational change and aggregation of recombinant human PrP occurs in the presence of 1 M guanidine hydrochloride and at pH's below 5.0 (Hornemann and Glockshuber 1998; Kaneko et al. 1995; Swietnicki et al. 2000b). If the non-ionic denaturant urea was used, then the conversion to β-sheet also required NaCl. It is possible that the protonation of D144 and D147 at low pH interrupts the salt bridges in helix 1, and compromises its stability. The presence of the additional denaturant and salts may also help disrupt the overall structure and stability of the folded protein, and screen electrostatic charges that stabilize helix 1.

To address the question of the role of helix 1 Asp residues and their salt bridges to Arg residues in the stabilization of PrP-sen and its conversion to PrP-res, six mutants replacing D144 and D147 with either asparagines or alanines were prepared to eliminate the putative salt bridges. The results indicate that PrP-sen is not substantially stabilized overall by the Asp-Arg salt bridges and that intermolecular salt bridge formation involving the same residues is not essential for the formation of PrP-res. The unfolding pathway of PrP-sen and its conversion to PrP-res, however, are influenced by interactions involving the helix 1 aspartic acid residues that appear to be independent of PrP-sen.
<table>
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<th>Helix 1 Primary Sequence</th>
<th>Reference</th>
<th>Accession Number</th>
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<tr>
<td>Hamster</td>
<td>144-DWEDRYYREN</td>
<td>(Oesch et al. 1985)</td>
<td>UJHYIH</td>
</tr>
<tr>
<td>Mouse</td>
<td>143-DWEDRYYREN</td>
<td>(Westaway et al. 1987)</td>
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<tr>
<td>Human</td>
<td>144-DYEDRYYREN</td>
<td>(Liao et al. 1986)</td>
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<td>144-DYEDRYYREN</td>
<td>(Schatzl et al. 1995)</td>
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<td>(Goldmann et al. 1990)</td>
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<td>(O'Rourke et al. 1998)</td>
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<td>Dog</td>
<td>148-DYEDRYYREN</td>
<td>Unpublished</td>
<td>AAB99743</td>
</tr>
<tr>
<td>Cattle</td>
<td>155-DYEDRYYREN</td>
<td>(Hope et al. 1988)</td>
<td>P10279</td>
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Table 4. Primary sequence alignments of highly conserved helix 1 residues from a variety of species. The residue number for the N-terminal sides of the sequences are given at the beginning of each sequence. All sequences and Genbank accession numbers were obtained from the PubMed protein sequence databank (National Library of Medicine 2003).
binding to PrP-res. These results provide insight into the basic mechanism of PrP-res formation.

B. EXPERIMENTAL METHODS

1. Cloning of wild type and mutant hamster PrP for expression using E. coli—The plasmid encoding Syrian hamster PrP (HaPrP) (residues 23-231) was generously provided by Dr. Marshall Bloom. It was constructed in a pET24a(+) expression vector from Novagen inserted as an Ndel fragment using standard cloning techniques (Russell 2001). Mutations at positions 144, 147 and both 144 and 147 were introduced using the QuikChange Site-directed Mutagenesis Kit from Stratagene. The mutagenesis was performed by mixing the plasmid pET24a(+) DNA (~10 ng) containing the wild type hamster PrP insert in reaction buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris/HCl, 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin, pH 8.8) and excess free deoxynucleotide triphosphates (dNTP) with oligonucleotide primers (125 ng) that are complementary to a portion of the hamster PrP DNA sequence except where the desired mutation is encoded. Samples were then thermocycled using a Perkin-Elmer GeneAmp PCR System 9600 with PfuTurbo DNA polymerase (2.5 units) to extend and incorporate the mutant primers resulting in circular, double-stranded DNA using the following cycling parameters:
<table>
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<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>12 min*</td>
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</table>

* 2 min/kb of plasmid length; plasmid size was 5961 bp.

Oligonucleotides used in the mutagenesis were obtained from Genosys Biotechnologies and include the following: (D144N) 5'-GCATTCTGGAATCTGGGAGGACCGCTACTACCG-3' and 5'-CGGTAGTAGCGGTCCTCCCAGTTATTGCCAAAATGC-3'; (D147N) 5'-GCAATGACTGGGAGAACCGCTACTACCGTGAAAAC-3' and 5'-GTTTTCACGGTAGTACGGTTTCTCCCAGTCATTGC-3'; (D144N/D147N) 5'-GCATTCTGGGAATCTGGGAGGACCGGTACTACCG-3' and 5'-CGGTAGTAGCGGTCCTCCCAGTTATTGCCAAAATGC-3'. After thermocycling, the samples were treated with Dpn I endonuclease (2.5 units) for 1 hour at 37°C to remove the methylated template DNA leaving only the newly amplified, unmethylated mutant circular double-stranded DNA. The circular DNA was then transformed into XL-1 Blue supercompetent *Escherichia coli* (*E. coli*) for mutant DNA expression. Transformations were performed according to the supplier's directions. Briefly, supercompetent XL1-Blue cells were thawed on ice and then 50 μL aliquots were transferred to pre-chilled Falcon 2059 tubes. One-microliter aliquots of the Dpn I-treated DNA were transferred to the
cells which were subsequently incubated for 30 min on ice with occasional swirling. After the incubation, cells were heat shocked for 45 seconds at 42°C and then cooled on ice for 2 min using a Brinkmann RM6 thermostatted water bath. To grow cells containing the plasmid DNA, 0.5 mL SOC broth (20 g/L bacto tryptone, 5 g/L bacto yeast extract, 10 mM NaCl, 7 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) was added and cells were incubated at 37°C with shaking at 250 RPM for 1 hour. After the incubation, cells were plated on Luria-Bertani (LB) agar containing antibiotic (171 mM NaCl, 10 g/L tryptone, 5 g/L yeast extract, 20 g/L agar, 50 mM kanamycin, pH 7.0). Plates were incubated ~16 hours at 37°C to grow transformed colonies containing the mutant plasmid. Mutant plasmid DNA was purified from 1.5 mL overnight cultures of transformed colonies in LB broth using a Promega Wizard Miniprep Plasmid Purification Kit. The kit procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (Vogelstein and Gillespie 1979). The procedure is performed as follows: Cells from the overnight culture are pelleted by centrifugation for 2 min at 10,000 x g. After discarding the supernatant, the cells were resuspended in 300 μL resuspension buffer (50 mM Tris/HCl, 10 mM EDTA, 100 μg/mL RNase A, pH 7.5). After resuspension, 300 μL lysis buffer (0.2 M NaOH, 1% SDS) was added with gentle mixing to disrupt cell membranes. Following lysis, 300 μL neutralization solution (1.32 M potassium acetate, pH 4.8) was added with gentle mixing to return the solution closer to neutral pH causing the precipitation of cellular components. Following neutralization, the lysates were centrifuged at 10,000 x g for 5 min to remove the precipitated cellular components. The supernatant was then applied to
silica resin column. The silica resin preferentially binds the highly negatively charged plasmid DNA. The DNA-bound resin was then rinsed with wash buffer (80 mM potassium acetate, 8.3 mM Tris/HCl [pH 7.5], 40 μM EDTA, 55% ethanol). DNA was eluted by adding ~30 μL Tris-EDTA (TE) buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and spinning the column for 1 min at 10,000 x g. The plasmid DNA for all constructs was sequenced by MWG Biotech. For expression, plasmids containing the desired HaPrP construct were transformed into BL21(DE3) supercompetent cells from Stratagene for PrP expression. Recombinant proteins accumulated in cytoplasmic inclusion bodies and were purified as described below.

2. Prokaryotic Protein Expression and Purification—Cells of E. coli BL21(DE3) with the desired PrP construct were grown overnight in 5 mL of NZ-amine/yeast (NZY) medium (100 mM NaCl, 25 mM MgCl2, 10 g/L NZ-amine [Sigma], 5 g/L yeast extract, pH 7.2) containing 0.05 mg/mL kanamycin. The overnight culture was added to 500 mL NZY medium with 0.05 mg/mL kanamycin and grown at 37°C in a shaking incubator to an optical density at 600 nm of 0.5. Protein expression was then induced by adding isopropyl-β-D-thiogalactoside to 0.5 mM to the culture. The induced culture was grown for 2 hours at 37°C in a shaking incubator. Cells were then harvested by centrifugation.

Cell pellets were suspended in lysis buffer (50mM Tris/HCl pH 7.5, 2 mM EDTA, 0.1% Triton X-100) and sonicated to release the inclusion bodies containing the protein of interest. Inclusion bodies were collected by centrifugation and then solubilized for 2 hours in 8 M urea buffered at pH 8 with 0.1 M sodium phosphate and 0.01 M Tris.
The solubilized protein solution was then diluted 10-fold with 8.0 M urea in Tris-chloride buffer (TCB; 20mM Tris/HCl pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂). The solubilized protein solution was purified using His-Bind resin (Novagen). The resin was incubated with the solubilized protein in 8 M urea for 2 hours. The protein bound resin was loaded onto the column and washed with a 2 M step gradient down from 8 to 0 M urea in TCB. The protein was eluted using 60 mM imidazole (Fisher) in TCB followed by 200 mM imidazole in TCB. Fractions were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) to confirm the presence of PrP (molecular mass 23 kDa) and identify fractions containing impurities. SDS/PAGE was performed using precast 10% Nupage polyacrylamide gels (Novex). Samples containing volumes from individual fractions were boiled in sample buffer (SABU; 62.5mM Tris/HCl, 5% [v/v] glycerol, 3 mM EDTA, 5% [w/v] SDS, 0.02% [w/v] bromophenol blue, 4 M urea, 4% [v/v] β-mercaptoethanol [BME]) for 5 min prior to loading onto gels. Gels were run in Xcell II gel boxes (Novex) at 200 V for 1 hour. Gels were fixed for 20 min in fixative (50% methanol, 10% acetic acid), washed in water, and stained for 2 hours to overnight with GelCode Blue stain reagent (Pierce). Destaining was performed for 1 hour in water. Gels were documented using a Gel-doc imaging system (Bio-Rad). Fractions containing only PrP were pooled and dialyzed against 0.01 M sodium phosphate at pH 6.0 to remove the imidazole and then concentrated using a centrifugal filter device (Millipore, molecular weight cut-off 10 kDa) to approximately 0.5 mg/mL final protein concentration (ε₂₈₀ = 2.7 mL/mg cm).
3. **Protein purity and disulfide bond analysis**— Sample purity and the presence of the disulfide bond (between residues C179 and C214 of HaPrP) was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) with precast 14% Tris-glycine polyacrylamide gels (Novex) with SDS running buffer (0.1% [w/v] SDS, 25 mM Tris/HCl, 200 mM). For oxidized samples, 180 ng of protein was added to oxidizing sample buffer (62.5 mM Tris/HCl, 5% [v/v] glycerol, 3 mM EDTA, 5% [w/v] SDS, 0.02% [w/v] bromophenol blue, 4 M urea) and boiled for 5 min prior to loading onto gel. Reduced samples were prepared by added 180 ng protein to reducing sample buffer (62.5 mM Tris/HCl, 5% [v/v] glycerol, 3 mM EDTA, 5% [w/v] SDS, 0.02% [w/v] bromophenol blue, 4 M urea, 80 mM iodoacetamide, 4% [v/v] β-mercaptoethanol [βME]) and boiling for 5 min prior to loading onto gel. Electrophoresis was performed for 1.5 hours at 125 V using an Xcell II gel box (Novex) and a PowerPac 1000 power supply (Bio-Rad). Following electrophoresis, gels were fixed in fixative, stained in GelCode Blue (Pierce), and recorded as described above.

4. **Mass Spectrometry of Recombinant HaPrP**—Molecular masses for all purified recombinant *E. coli* protein constructs were confirmed following trypsin digestion using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Proteins were precipitated in methanol (3 volumes methanol: 1 volume buffer) to remove interfering storage buffer ions. Precipitated proteins were pelleted by centrifugation for 35 min at 20,800 x g (Eppendorf Centrifuge 5417R). Supernatants were removed by aspiration and then the precipitated proteins (0.2-0.3 μg) were suspended in
50 μl of 20 μg/mL sequence grade trypsin (Promega) in 25 mM NH₄HCO₃ at pH 8.0 and incubated overnight at 37°C. Salt contaminants were removed using ZipTipC₁₈ (Millipore) hydrophilic resin containing pipette tips. Prior to loading samples, tips were washed with 100% acetonitrile (MeCN; Sigma) and then equilibrated with 50% (v/v) MeCN, 0.1% (v/v) trifluoroacetic acid (TFA; Sigma) followed by just 0.1% TFA. Volumes (10 μL) of the trypsin-digested samples were acidified with 1 μL 10% TFA prior to loading onto the 0.1% TFA equilibrated ZipTips. Loaded samples were washed with 0.1% TFA and then eluted with 3 μL 50% MeCN, 0.1% TFA. Eluted samples were diluted volumetrically by a factor of ten in α-Cyano-4-hydroxycinnamic Acid (CHCA) matrix (10 mg CHCA/mL 50% MeCN, 0.1% TFA). Mass data was acquired using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems). Spectra were obtained using 100 nsec extraction mode delay, positive polarity, 20,000 V accelerating voltage, and 50 laser shots per spectrum. Spectra were calibrated using known trypsin and PrP masses (842.5100, 988.4601, and 1102.5322).

5. Generation of Mutant HaPrP GPT pSFF Expression Vectors— D-to-N mutant

HaPrP (residues 23-231) in the pET24a(+) vector were subcloned using Bsu36 I and Stu I restriction sites into a gene encoding full length HaPrP without the glycosylphosphatidylinositol (GPI) anchor inserted in a pBluescript vector generously provided by Dr. Bruce Chesebro. The pBS HaPrP construct lacked the GPI anchor because the gene lacked the C-terminal GPI attachment sequence (Kocisko et al. 1994). The cloning was performed using standard techniques (Russell 2001). For generating the
insert containing the D to N helix 1 mutations, DNA from the pET24a(+) vector containing the mutant HaPrP gene was transformed into SCS110 *E. coli* for expression of *dam* methylation deficient plasmid DNA. After transformation, overnight cultures of transformed SCS110 colonies containing the HaPrP plasmid DNA were grown and *dam* methylation deficient plasmid DNA was purified as described above. Purified *dam* methylation deficient plasmid DNA containing the desired helix 1 mutant HaPrP genes in the pET24a(+) vector and plasmid DNA containing the wild type HaPrP gene in a pBluescript vector were digested for 5 hours at 37°C with Bsu36 I (20 units; New England Biolabs) in 100 mM NaCl, 50 mM Tris/HCl, 10 mM MgCl₂, and 1 mM dithiothreitol (DTT) at pH 7.6. Samples were then digested for 5 hours at 37°C in the presence of Stu I (20 units; New England Biolabs) buffered with 100 mM NaCl, 50 mM Tris/HCl, 10 mM MgCl₂, 1 mM DTT, and 100 μg/mL bovine serum albumin at pH 7.6. Prior to electrophoresis, the Bsu36 I and Stu I digested wild type HaPrP pBluescript DNA was treated with shrimp alkaline phosphatase (USB) to dephosphorylate the 5' phosphate from the digested ends of the double-stranded DNA to minimize the chance of self-recombination of the linear DNA. The digested DNA was treated with shrimp alkaline phosphatase (10 units) buffered with 20 mM Tris/HCl and 10 mM MgCl₂ at pH 8.0 for 4 hours at 37°C. The enzyme was inactivated by heating at 65°C for 15 min.

Digestion products were analyzed using gel electrophoresis with 0.8% agarose gels buffered with Tris-boric acid-EDTA buffer (TBE, 90 mM Tris/HCl, 90 mM boric acid, 2 mM EDTA, pH 8) using a Wide Mini-sub Cell GT electrophoresis unit (Bio-Rad). A 1 kilobase DNA ladder (Gibco) was run with all electrophoresis samples to estimate the
molecular sizes of all DNA samples. DNA was visualized by staining with ethidium bromide for 15 min, illuminating with a transilluminator (Spectroline Model TR-302), and photographing (Polaroid DS 34 Direct Screen Instant Camera). DNA to be used for ligation reactions was separated electrophoretically in 0.8% Sea Plaque low melting point agarose (BioWhittaker Molecular Applications) in Tris-acetic acid-EDTA buffer (TAE, 40 mM Tris/HCl, 18 mM acetic acid, 2 mM EDTA, pH 8). The SeaPlaque agarose is a high quality agarose that is free of impurities that might interfere with ligation reactions. Gels were stained with ethidium bromide (15 min) and visualized with a transilluminator (Bio-Rad). The desired bands were then cut out from the gel and saved for extraction of the DNA from the agarose by electroelution. Electroelution was performed using a Model UEA Unidirectional Electroeluter (International Biotechnologies). Agarose gel containing the digested DNA fragments was electroeluted for 1 hour at 100 V in electroelution buffer (20 mM Tris/HCl, 0.2 mM EDTA, and 5 mM NaCl, pH 8.0) using 8.5 M ammonium acetate dyed with bromophenol blue as a salt cushion to collect the eluted DNA. The DNA collects in the salt cushion where it migrates more slowly due to the ionic interference from the high salt concentration.

After electroeluting for 1 hour, the salt cushions for each well containing sample were removed and saved for phenol/chloroform precipitation. Phenol/chloroform precipitation was used to remove any protein impurities that may have co-electroeluted with the digested DNA. An equal volume of phenol:chloroform:isoamyl alcohol (Gibco; 25:24:1 volume ratio) was added to the electroeluted DNA and vortexed to mix the aqueous and organic layers. The mixture was then centrifuged for 5 min at 14,000 x g
(Eppendorf Centrifuge 5417R) to separate the organic and aqueous phases. The organic layer, containing the bromophenol blue and protein impurities, was removed by pipette and the aqueous layer, containing the DNA, was ethanol precipitated to further purify the DNA in 95% ethanol at -20°C for 2 hours. The precipitated DNA was then pelleted by centrifugation at 14,000 x g for 35 min at 4°C. After discarding the supernatant, the precipitated DNA was purified further by washing with 70% ethanol. The washed and precipitated DNA was pelleted again by centrifuging 15 min at 14,000 x g. This last wash step helps to remove any residual salt impurities that may have co-precipitated with the DNA. After discarding the 70% ethanol wash supernatant, the DNA was resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and analyzed by agarose gel electrophoresis as described above to confirm the correctly sized digested insert and vector DNA were obtained.

The Bsu36I and Stu I digested insert DNA containing the desired HaPrP helix 1 mutation was ligated to the Bsu36I and Stu I digested dephosphorylated vector DNA containing the wild type HaPrP gene using T4 DNA ligase (New England Biolabs). Reactions were performed by mixing insert DNA with vector DNA at a 10:1 ratio with T4 DNA ligase (120 units) buffered with 1X T4 DNA ligase buffer (Gibco; 50 mM Tris/HCl, 10 mM MgCl2, 1 mM adenine triphosphate [ATP], 1 mM DTT, 5% [w/v] polyethylene glycol-8000, pH 7.6). The polyethylene glycol present in the ligase buffer acts as a molecular concentrator by increasing the number of collisions between insert and vector DNA through partitioning of the hydrophilic DNA molecules from the more hydrophobic ethylene glycol, thus making successful ligation interactions more probable.
Samples were incubated 10 hours at 15°C using a thermostatted water bath (Brinkman). Ligations were performed at low temperatures to minimize melting of the ends of the linear DNA molecules in order to increase the ligation efficiency.

Following ligation, samples were transformed into XL1 Blue supercompetent *E. coli* using the same protocol described above. The DNA from successfully transformed colonies was then purified as described above and screened using Bsu36 I and EcoR I restriction enzyme digestion. Conditions for the Bsu36 I digest were the same as described above. DNA was digested at 37°C for 2-5 hours using EcoR I (50 units; New England Biolabs) buffered with 50 mM NaCl, 100 mM Tris/HCl, 10 mM MgCl₂, and 0.025% Triton X-100 at pH 7.5. Digested DNA was separated using agarose gel electrophoresis as described above. DNA with the correct inserts were identified by the presence of a ~750 bp band, corresponding to the HaPrP gene insert, along with a ~3000 bp band corresponding to the pBluescript vector without the HaPrP insert. The Bsu36 I digest was used to confirm the presence of only one Bsu36 I cleavage site present on the HaPrP insert.

DNA that met the screening criteria were subsequently sequenced to confirm the exact DNA sequence. DNA was prepared for sequencing using a DNA Sequencing Kit with Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) with m13 primers (Amersham Biosciences) and primers directed to wild type hamster PrP (generously provided by Dr. Ina Vorberg). Primer sequences are as follows: m13 forward 5’-GTAAACGACGGCCAGT-3’; m13 reverse 5’-CAGGAAACAGCTATGAC; HaPrP 624 Forward 5’-CCCTGGAGGCAACCGTACCC-3’; HaPrP 604 reverse 5’-
TCGGTCTCCGTGAAGTTCTCCC. Sequencing reactions were performed by adding 1 μg DNA to 3 pmol primer in the presence of the Big Dye Terminator reaction mix. The reaction mix consisted of Tris/HCl (pH 9.0), magnesium chloride, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, and A, C, G and T-Big Dye Terminator nucleotides, which each contained unique fluorescent probes for nucleotide distinction. Reactions were thermocycled using the following parameters:

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Reaction products were purified using Centri-Sep columns (Princeton Separations) which remove salts and low molecular compounds, including unreacted deoxynucleoside triphosphates less than 16 bp from amplified sequence reaction mixtures. The purified reaction products were then transferred to a 96 well plate (Applied Biosystems) for sequence detection using an Applied Biosystems 3700 automated sequencer. The obtained sequences were aligned using Sequencher software.

For expression in mammalian cells, the sequenced helix 1 mutant pBluescript clones were subcloned into the pSFF retroviral expression vector generously provided by
Dr. Bruce Chesebro. PSFF vector and insert DNA containing the HaPrP gene with the helix 1 mutations were prepared by digesting the pBluescript clones with EcoR I, separating the digestion products, and then purifying and isolating them as described above. The vector DNA was also treated with shrimp alkaline phosphatase prior to the separation step as described above. Purified pSFF vector and insert DNA were ligated using T4 DNA ligase as described above using a 5:1 insert to vector molar ratio. Ligated DNA was transformed into JM109 supercompetent *E. coli* (Stratagene) which is a recombination deficient strain that minimize the chance of recombination events that large plasmids, such as pSFF, are more likely to undergo. Transformed plasmid pSFF DNA was screened for the correct orientation of the PrP insert using EcoR I and Bsu36 I restriction enzyme digestion as described above. The pSFF vector containing the mutant HaPrP gene insert has two Bsu36 I restriction sites: one with the pSFF vector and one in the HaPrP gene insert. As a result, vector DNA containing the HaPrP gene digested into two segments which varied in size based on the orientation of the HaPrP gene insert. Bsu36 I digests of plasmid DNA containing the correctly oriented insert produced 1968 and 7519 bp bands when analyzed by agarose gel electrophoresis, while the digests of plasmid DNA containing incorrectly oriented insert produced 2313 and 7174 bp bands. Samples were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized as described above.

The D-to-A HaPrP mutants were generated by site directed mutagenesis of the same wild type full length HaPrP pBluescript clone provided by Dr. Chesebro using a QuikChange Site-directed Mutagenesis Kit from Stratagene as described above.
Oligonucleotides were obtained from Genosys Biotechnologies and include the following: (D144A) 5'-GCATTTTGGCAATGCCTGGGAGGACCGCTACTACCG-3' and 5'-CGGTAGTAGCGGTCCTCCCAGGCATTGCCAAAATGC-3'; (D147A) 5'-GCAATGACTGGGAGGCCCGCTACTACCGTGAAAAC-3' and 5'-GTTTTACCGTAGTGCGGCCCTCCCAGTCATTGC-3'; (D144A/D147A) 5'-GCATTTTGGCAATGCCTGGGAGGCCCGCTACTACCG-3' and 5'-CGGTAGTAGCGGTCCTCCCAGGCATTGCCAAAATGC-3'. After mutagenesis and plasmid DNA purification using a Qiagen Qiaprep Spin Miniprep Kit (which follows the same basic protocol as the Promega Wizard Miniprep Kit described above), the pBS PrP clones were then sequenced with an Applied Biosystems 3700 automated sequencer and subcloned using standard techniques as described above into the pSFF retroviral expression vector using the EcoR I restriction site (Chesebro et al. 1993). All constructs were screened for orientation using Bsu36 I and EcoR I restriction enzymes as described above.

6. Generation of HaPrP expressing eukaryotic cells—Plasmid pSFF DNA containing the correctly oriented wild type and mutant HaPrP gene inserts were transfected into mixtures of PA317 and ψ2 mouse fibroblast cells (generously provided by Dr. Bruce Chesebro) using an Effectene Transfection Kit (Qiagen). In a single well of a 6 well plate (Corning), 1.25 x 10^5 ψ2 mouse fibroblast cells and 1.5 x 10^5 PA317 and mouse fibroblast cells (generously provided by Dr. Bruce Chesebro), suspended in RPMI medium (ICN Biomedicals) supplemented with 10% (v/v) fetal bovine serum (Hyclone),
2 mM L-glutamine (Gibco), 1 unit/mL penicillin (Gibco), and 1 μg/mL streptomycin (Gibco), were plated and incubated overnight at 37°C. The next day, transfections were performed on the plated cells. The transfections were performed by adding 1 μg pSFF plasmid DNA containing the desired HaPrP construct to the transfection reagents. The transfection kit reagents form micellar complexes with the added DNA, which are permeable to cell membranes. After the DNA complexes are taken up by cells, the DNA is incorporated into the chromosomal DNA of the host cell via retroviral mechanisms. The incorporated retroviral gene then proceeds to express, among other proteins, the retroviral virus capsid structural protein, Gag, and the inserted HaPrP mutant protein (Chesebro et al. 1993). Transfected cells were cultured and subsequently screened for protein expression by immunoperoxidase staining and used for metabolically labeled protein expression as described below based on methods described by others (Kocisko et al. 1994; Priola et al. 1994; Robertson et al. 1992).

Transfected cells were monitored for incorporation of the pSFF DNA by PrP and Gag immunoperoxidase staining. Immunoperoxidase staining was performed by suspending cells in 200 μL trypsin-EDTA (Gibco) and transferring them to a round bottom 96 well plate (Corning) with 50 μL fetal bovine serum (Hyclone). The plates containing the cells were centrifuged (Beckman GS-6R) 2 min at 1200 RPM to pellet the cells. Supernatants were discarded and 1 μL of cells were added to a 60 mm microscope slide (Fisher) and dried. Dried cells were fixed for 20 min in 3.7% formaldehyde in phosphate buffered saline (PBS; 20 mM sodium phosphate, 130 mM NaCl, pH 7.4). Fixed cells were blocked for 5 min using 0.1 M glycine buffered with PBS. Cells were
then permeablized for 5 min with 0.4% (v/v) Triton X-100 buffered with PBS. After permeablization, cells were washed once with PBS before adding the primary antibodies. For detection of HaPrP, supernatants of hybridoma cells expressing the HaPrP 3F4 antibody (generously provided by Dr. Bruce Chesebro) were incubated with the fixed, blocked, and permeablized cells for 30 min. For detection of the retroviral capsid protein, Gag, supernatants of hybridoma cells expressing the Gag antibody 18-8 (generously provided by Dr. Bruce Chesebro) were incubated with the fixed, blocked, and permeablized cells for 30 min. To remove the primary antibodies, cells were washed once for 5 min with PBS. The secondary antibody, goat anti-mouse IgG horseradish peroxidase (generously provided by Dr. Bruce Chesebro) was added to the 18-8 and 3F4 antibody bound cells and incubated for 30 min. Cells were washed for 5 min in PBS to remove the secondary antibody prior to substrate addition. Cells were incubated for 30 min in 3-amino-9-ethyl-carbazole (AEC) substrate (0.2 mg/mL AEC, 50 mM sodium acetate, 0.015% H$_2$O$_2$), which reacts with horseradish peroxidase forming a water insoluble red precipitate. Unreacted substrate was removed by washing three times for 5 min in PBS. Stained cells were air dried and mounted for microscopic analysis by adding a few drops of mounting solution (50% [v/v] glycerol, 2% [v/v] formaldehyde in PBS) to the stained cells and covering with a cover slip. Stained cells were viewed and photographed using an Olympus BH-2 light microscope with an Olympus OM System SC35 type 12 camera.
7. **Metabolic [³⁵S]-methionine Labeling of PrP-sen**—Metabolic labeling of PA317 and ψ2 mouse fibroblast cells with the wild type or mutant PrP expressing pSFF clones was performed using previously described methods (Caughey et al. 1999; Horiuchi and Caughey 1999; Kocisko et al. 1995). Cells were grown to 85-95% confluence in a 75 cm² flask (Corning) in RPMI medium (ICN Biomedicals) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 2 mM L-glutamine (Gibco), 1 unit/mL penicillin (Gibco), and 1 μg/mL streptomycin (Gibco). The growth medium was removed and cells were washed twice with 5 mL warm phosphate buffered balanced salts solution (PBBS; 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 1.3 mM CaCl₂, 4.3 mM KCl, 123 mM NaCl, 1 mM MgCl₂, 0.8 mM MgSO₄, 1% [w/v] phenol red, 5.6 mM glucose, pH 7.3). Cells were starved of L-methionine by adding warmed labeling medium (3.5 mL; methionine depleted RPMI medium [ICN Biomedicals] supplemented with 1.5 mg/L L-methionine, 15 mg/L L-glutamine, and 5% [v/v] dialyzed FBS [Hyclone]) to the PBBS-washed cells. Flasks were treated with air containing 5% CO₂ and incubated in a model 4200 CO₂ incubator (Napco), receiving 0.4 L/min CO₂ and 4 L/min air, for 30 min at 37°C. Following the starvation incubation, 3 mCi of [³⁵S]-methionine (NEN) was added to the labeling medium and cells were incubated for 2.5 hours at 37°C. Following the labeling incubation period, flasks were removed from the incubator and iced for 2 min to drain the labeling medium from the cell surface. The labeling medium was removed and cells were washed three times with 5 mL ice cold PBBS. Cells were lysed by adding 3 mL lysing buffer (0.5% [v/v] Triton X-100, 0.5% sodium deoxycholate, 5 mM Tris/HCl [pH 7.4 at 4°C], 150 mM NaCl, 5 mM EDTA) and incubating 2 min to allow cell lysis (Caughey
and Raymond 1991; Caughey et al. 1995). Cell lysates were then centrifuged for 5 min at 1000 x g using a J6B centrifuge (Beckman) to remove cellular debris. Supernatants were removed and methanol precipitated by adding 12 mL methanol and incubating at -20°C for two hours. Precipitated proteins were centrifuged for 20 min at 5,000 x g and the supernatant was discarded. Pelleted proteins were washed once with 3 mL TN-sark buffer (50 mM Tris/HCl [pH 7.0 at 4°C], 0.5 M NaCl, 1% [w/v] sarkosyl) and then resuspended in 1 mL detergent, lipid, protein complex (DLPC; 4.2 mg/mL L-α-phosphatidylcholine [Sigma], 50 mM Tris/HCl [pH 7.5 at 25°C], 2% sarkosyl, 120 mM NaCl) supplemented with protease inhibitors (0.1 mM pefabloc [Boerhinger-Mannheim], 0.15 nM aprotinin [Boerhinger-Mannheim], 1 nM leupeptin [Boerhinger-Mannheim], 1 nM pepstatin [Boerhinger-Mannheim], 0.5 mM EDTA) by sonication for 2 min at full power using a cephorn sonicator (Heat Systems Ultrasonics). After resuspending the proteins, 3.5 μL 3F4 monoclonal antibody (generously provided by Greg Raymond) directed towards HaPrP was added to the resuspended protein-DLPC solutions to immunoprecipitate the [35S]-labeled HaPrP proteins (Caughey et al. 1995). Solutions were incubated overnight at 4°C to allow antibody binding. Antibody-bound protein was removed from solution by adding 50 μL of a 50% (resin volume/total volume) CL-4B protein-A sepharose (PAS; Pharmacia) equilibrated in lysing buffer and incubating with rotation on a LabQuake rotisserie shaker (Barnstead-Thermolyne) for 1 to 2 hours at 4°C. Radiolabel led HaPrP bound PAS beads were centrifuged for 1 min at 20,000 x g and the supernatant was discarded. The pelleted PAS was washed once with 0.5 mL DLPC, twice with 1 mL TN-sark, and once with 1 mL distilled-deionized water. Following each wash, all liquid was
aspirated. [\textsuperscript{35}S]-labeled HaPrP protein was eluted by adding 45 µL 0.1M acetic acid (pH ~2.75) and incubating at 25°C for 35 min. Eluted [\textsuperscript{35}S]-labeled HaPrP was quantitated by scintillation counting 1 µL eluate in 5 mL Ready-Safe scintillation cocktail (Beckman) using a LS6500 Multi-purpose scintillation counter (Beckman). Radiolabel led eluates were stored at 4°C until use.

8. Circular Dichroism Spectroscopy and Denaturation Curves—Circular Dichroism spectroscopy was performed using an OLIS CD module with Cary-16 Spectrophotometer Conversion Unit with a NESLAB RTE-111 temperature control module. For thermal denaturation experiments the ellipticity from 225 to 207.5 nm of PrP samples at 0.3 mg/mL in 0.01 M sodium phosphate at pH 6.0 was measured at intervals between 25 and 72.5°C. Full spectra at 25, 62.5 and 72.5°C were scanned from 185 to 260 nm. Samples were scanned 3 times with a 0.5 nm scanning interval in a 1.0 mm cylindrical cell after equilibrating 15 min at the desired temperature. Scans were averaged using the OLIS spectral processing software supplied with the instrument. The fraction of unfolded protein for each construct was determined using the molar ellipticity at 222 nm and was plotted versus the temperature in degrees Celsius. The ellipticity at 72.5°C was used for the unfolded protein ellipticity. The ellipticity was converted to molar ellipticity, \( \theta \), using the following equation:

\[
\theta = \frac{100 \cdot \theta_{\text{obs}}}{c l}
\]

Equation 7
Where $\theta_{\text{obs}}$ is the observed ellipticity, $c$ is the protein concentration in moles per liter and $l$ is the cell path length in cm (Johnson 1990). All of the mutant protein signals were normalized to the wild type PrP unfolded signal. Averages and standard deviations of transition midpoints values and slopes were determined from three individual experiments for each protein.

For the GdnHCl denaturation, samples from 0 to 5.0 M GdnHCl were prepared by diluting a stock PrP solution (0.3 mg/mL) with varying amounts of a stock 8.0 M GdnHCl solution and buffer giving a final protein concentration of 0.1 mg/mL. All solutions were buffered at pH 6.0 in 0.01 M NaPO₄. Samples were allowed to equilibrate for at least one hour prior to analysis. Samples were scanned 3 times in a 1.0 mm cylindrical cell from 225 to 207.5 nm with a 0.5 nm scanning interval at 25°C. All spectral data were corrected for absorbance by the buffer and denaturant. The fraction of unfolded protein was determined for each construct using the ellipticity at 222 nm. Averages and ranges for the thermodynamic parameters were determined from two individual experiments for each protein. Thermal and GdnHCl denaturation curves were fit using the nonlinear regression analysis tool on GraphPad Prism. Thermal unfolding transition midpoints and GdnHCl denaturation thermodynamic parameters were determined assuming a two-state unfolding process as described by Pace and Scholtz (Pace 1986; Pace and Scholtz 1996). Plots of $\Delta G$ versus GdnHCl concentration were fit by linear regression according to Equation 6 as described above.

9. Preparation of Purified PrP-res—Purified PrP-res from 263K scrapie infected

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hamsters was generously provided by Greg Raymond. It was prepared using a revision of the Bolton et al. method (Bolton et al. 1987). Brains were harvested from hamsters inoculated with 263K scrapie showing middle to late-stage clinical symptoms. Animals were sacrificed by asphyxiation with CO₂ for 4 min. Brains were excised, rinsed in PBS to remove residual blood, dried by gently blotting onto filter paper (Whatman), flash frozen by placing into liquid nitrogen, and stored at -80°C. To prepare PrP-res, frozen brains were thawed, weighed, and rinsed 6 times with ~100 mL ice cold PBS. Brains were suspended to give a 10% (w/v) suspension in buffer 1 (10% [w/v] sarkosyl, 10 mM Tris/HCl [pH 8.3 at 4°C], 1 mM EDTA, 133 mM NaCl, 1 mM DTT, 0.5 mM pefabloc, 0.15 nM aprotinin, 1 nM leupeptin, 1 nM pepstatin). The suspension was homogenized using a dounce homogenizer (6 strokes with loose pestle, 10 strokes with tight pestle) and incubated at 25°C for 1 hour with occasional stirring. Homogenates were transferred to Optiseal tubes (Beckman) and ultracentrifuged at 22,000 x g for 30 min using a TL100.3 Ultracentrifuge (Beckman) with a type 50.2 rotor (Beckman). Supernatants were transferred to a new Optiseal tube and ultracentrifuged again at 150,000 x g for 3 hours at 4°C. Supernatants were discarded and the pellets were rinsed with 5 mL buffer 2 (10% [w/v] NaCl, 1% [w/v] zwittergent 3-14 [Calbiochem], 10 mM Tris/HCl [pH 8.3 at 4°C], 1 mM EDTA, 1 mM DTT) and then resuspended by douncing in buffer 2 (1-2 mL buffer 2 per g starting brain material) at 25°C. Resuspended pellets were transferred to Optiseal tubes and ultracentrifuged at 225,000 x g for 2 hours at 20°C. Supernatants were discarded and pellets were washed with 5 mL buffer 3 (10 mM Tris/HCl, 100 mM NaCl, 5 mM MgCl₂, pH 7.4). Washed pellets were removed and resuspended by douncing in
buffer 3 (0.25-0.5 mL per g starting brain material) with added protease inhibitors (0.5 mM pefabloc, 0.15 nM aprotinin, 1 nM leupeptin, 1 nM pepstatin, 1 mM EDTA). RNA was removed by treating the suspensions with RNase A (100 µg/mL; New England Biolabs) for 2 hours at 37°C. RNase treated suspensions were supplemented with CaCl₂ and treated with DNase I (20 µg/mL) for 2 hours at 37°C. After the digests, EDTA and NaCl were added to final concentrations of 20 mM and 10% [w/v] respectively. After mixing the digests thoroughly, zwittergent 3-14 (Calbiochem) was added to a final concentration of 1%. The total volume was adjusted with buffer 3 to a ratio of 0.73 mL solution to 1 g starting brain material. The suspension was transferred to an Optiseal tube and a pad, consisting of 1 M sucrose, 0.1 M NaCl, 10 mM Tris/HCl (pH 7.4 at 25°C), and 1% (w/v) zwittergent 3-14, was added underneath the solution. The suspension was ultracentrifuged at 225,000 x g for 2 hours at 20°C. The pellet was rinsed twice and then dounced into 1 mL of a solution containing 0.5% (w/v) zwittergent 3-14 in phosphate buffered saline (20 mM sodium phosphate, 130 mM NaCl). The dounced suspension was ultracentrifuged at 350,000 x g for 30 min at 20°C. After discarding the supernatant, the pellet was resuspended by sonication in a cuphom sonicator in 1 mL phosphate buffered saline with 0.5% (w/v) zwittergent 3-14 and stored at 4°C.

Preparations were analyzed for total protein content spectrophotometrically using the BCA assay (Pierce) with a DU-7 spectrophotometer (Beckman). Purity was analyzed by silver stain SDS/PAGE and PrP amount was determined by Western blotting. SDS/PAGE was performed as described above using 10% NuPAGE Bis-Tris gels (Novex). Silver staining was performed by fixing the gels for 30 min in fixative solution.
(50% [v/v] methanol, 10% [v/v] acetic acid, 37% [v/v] formaldehyde). Fixed gels were then washed twice for 10 min in 50% [v/v] ethanol. Gels were pretreated with sodium thiosulfate (0.2 mg/mL) and rinsed three times for 20 seconds with water. Gels were then impregnated in a solution of 0.2 mg/mL silver nitrate in 37% (v/v) formaldehyde for 20 min. Gels were then rinsed twice for 20 seconds in water and developed for ~5 min in developer (60 mg/mL Na$_2$CO$_3$, 37% formaldehyde, 4 µg/mL Na$_2$S$_2$O$_3$). Gels were washed twice for 1 min in water and developing was stopped by adding to fixative solution containing 50% methanol and 10% acetic acid. Western blotting was performed by transferring gels onto PVDF membranes (Millipore) using a semi-dry Fastblot B34 blotting apparatus (Biometra) at 151 mAmps for 20 min. Blotted membranes were blocked for 45 min with 5% (w/v) Blotto (Bio-Rad) in Tris-buffered saline-tween solution (TBST; 20 mM Tris/HCl, 150 mM NaCl, 0.05% [v/v] tween-20R [Sigma]). Blocked membranes were then incubated with a 1:10,000 dilution of the primary antibody (3F4) in 5% Blotto/TBST for 45 min. Membranes were then washed 3 times for 5 min with TBST and then a 1:10,000 dilution of the secondary antibody, goat anti-mouse IgG alkaline phosphatase, in 5% Blotto/TBST was added and incubated for 45 min. Membranes were then washed 5 times for 5 min prior to development. Development was performed using Attophos chemiluminescent alkaline phosphatase substrate (Promega). Membranes were exposed to 1.25 mL Attophos substrate for 5 min and then dried overnight. Developed membranes were scanned using a STORM system phosphoimager (Molecular Dynamics) at 450 nm. Scanned images were analyzed using ImageQuant software (Molecular Dynamics).
10. **Preparation of Normal Brain and Scrapie Brain Microsomes**—Crude normal and 263K infected hamster brain microsomes were generously provided by Dr. Gerald Baron and were prepared as described previously (Baron *et al.* 2002). For a preparation, one Syrian golden hamster brain, either a normal brain or a brain excised from a hamster showing clinical signs of scrapie, was minced using a razor blade and homogenized by douncing (12 slow strokes with loose pestle, 20 strokes with tight pestle) in enough phosphate buffered saline supplemented with 0.5 mM pefabloc, 0.15 nM aprotinin, 1 nM leupeptin, 1 nM pepstatin, 1 mM EDTA, and 1 mM DTT to make a 10% (w/v) brain homogenate. The homogenate was sonicated using a cuphorn sonicator for 3 min at full power. Homogenates were then centrifuged at 3000 x g (Beckman J6-B) for 10 min and the supernatant was removed and saved. The pellet was resuspended in the initial volume of PBS and the suspension was dounce homogenized again (20-30 strokes with the tight pestle). Suspensions were then pelleted again at 3000 x g and the supernatant was combined with the supernatant from the first spin. The pooled supernatants were transferred to an Optiseal tube and then ultracentrifuged in a TL100.3 ultracentrifuge (Beckman) with a Type 50.2 rotor at 100,000 x g for 1 hour at 4°C. The supernatant was discarded and the pellet was triturated using a 3 cc syringe with a ~700 µL of PBS. After transfer to a 15 mL tube (Corning), triturated pellets were suspended by sonicating for 7 min using a cuphorn sonicator at full power and by pipetting up and down using a P1000 pipettor (Rainin). Samples were stored at -20°C until use. Total protein and PrP
quantification was performed by BCA assay and by Western blot as described for the purified PrP-res preparations.

11. Cell-Free Conversion Reactions—Cell-free conversion reactions were performed following the basic procedures described previously with modifications (Baron et al. 2002; Caughey et al. 1995; Horiuchi and Caughey 1999; Kocisko et al. 1994). Two different types of cell-free conversion reaction conditions were used: 1) reactions using purified PrP-res and 2) reactions using PrP-res in brain microsomes. All reactions were performed at pH 6.0 and contained 100 ng of 263K PrP-res mixed with 10,000 to 20,000 cpm [35S]PrP-sen in the presence of the standard conversion reaction components: 50 mM sodium citrate, 12 mM NaCl, 2 mM sodium phosphate, and 18 mM acetic acid. Among the two conversion reaction types, there were a total of six different conversion reaction sub-types that varied by the containment of additional conversion reaction buffer components and presence of PrP-res pretreatments.

For reactions using purified PrP-res, five different conversion reaction subtypes were used: guanidine pretreated PrP-res, guanidine-free, denaturant-free, urea pretreated PrP-res, and denaturant/detergent free. Cell-free conversion reactions using guanidine pretreated purified PrP-res were performed by pretreating the PrP-res in 4.0 M GdnHCl for 1 hour at 37°C prior to adding the [35S]PrP-sen. Pretreated PrP-res was then added to [35S]PrP-sen and additional buffer components resulting in a solution containing, in addition to the standard reaction components listed above, 364 mM GdnHCl, 5 mM cetyl pyridinium chloride, 34 mM sarkosyl, and 1.25 mM zwittergent 3-14. For guanidine-free
conversion reactions, there was no pretreatment of PrP-res. Instead, purified PrP-res was mixed with \[^{35}\text{S}]\text{PrP-sen} and additional components resulting in a final conversion buffer containing the standard conversion reaction components, 21 mM sarkosyl, 1.25 mM zwittergent 3-14, 100 mM NaCl, and 5 mM MgCl\(_2\). Denaturant-free conversion reactions were performed by adding purified PrP-res without pretreatment to \[^{35}\text{S}]\text{PrP-sen} in the presence of the standard conversion reaction components, 1.25 mM zwittergent 3-14, 100 mM NaCl, and 5 mM MgCl\(_2\). For urea pretreatment conversion reactions, purified PrP-res was pretreated in 6.0 M urea for 1 hour at 37°C prior to preparing the conversion reaction mixture. Urea pretreated PrP-res was added to \[^{35}\text{S}]\text{PrP-sen} and additional buffer components resulting in a solution containing the standard conversion reaction components, 545 M urea, and 1.25 mM zwittergent 3-14. Denaturant/detergent-free conversion reactions were performed by, prior to setting up the conversion reaction, removing the zwittergent 3-14 detergent from the purified PrP-res storage buffer by pelleting the PrP-res for 30 min at 20,800 x g, removing the supernatant by aspiration, washing with PBS, and repeating two more times. The detergent free, purified PrP-res was then mixed with \[^{35}\text{S}]\text{PrP-sen} and additional components resulting in solutions containing the standard conversion reaction components, 100 mM NaCl and 5 mM MgCl\(_2\).

For conversion reactions using PrP-res in brain microsomes, two conversion reaction sub-types were performed: reactions with and without the detergent sarkosyl. For conversions using microsomal PrP-res without sarkosyl, final reaction mixtures contained the standard conversion reaction components in addition to 100 mM NaCl and 5 mM MgCl\(_2\).
MgCl\textsubscript{2}. For conversions using microsomal PrP-res with sarkosyl, final reaction mixtures contained 100 mM NaCl, 5 mM MgCl\textsubscript{2}, 20 mM sarkosyl, and the standard reaction components. All conversion reactions were incubated for 40-48 hours at 37°C. After incubation, 90% of the reaction mixture was treated with 20 \(\mu\)g/mL of proteinase K (PK; Calbiochem) at 37°C for 1 hour. The remaining 10% was analyzed without PK treatment. The PK digestion was stopped by adding Pefabloc (Boehringer Mannheim) to 2 mM, 20 \(\mu\)g thyroglobulin (Fluka) as a carrier protein, and four volumes of methanol. Precipitated proteins were collected by centrifugation, suspended by boiling for 5 min in sample buffer, and separated by SDS/PAGE using NuPAGE pre-cast, 10% Bis-Tris gels (Novex) as described above. Gels were fixed for 30 min in fixative (50% methanol, 10% acetic acid) and hydrated for 30 min in gel drying solution (4.0% glycerol, 10% ethanol) prior to drying for 1 hour at 60°C using a Speed Gel SG210D gel dryer (Savant). Radioactive proteins on dried gels were exposed to Storage Phosphor Screens (Molecular Dynamics), visualized, and quantified using a STORM Phosphorimager and ImageQuant software (Molecular Dynamics).

12. Analysis of PrP-sen binding to PrP-res—Two types of PrP-res binding experiments based on experiments performed by Horiuchi et al. (Horiuchi and Caughey 1999) were performed: binding to purified PrP-res in the presence of anionic detergents and binding to microsomal PrP-res without detergents and denaturants. For binding to purified PrP-res in the presence of anionic detergents, purified 10,000-20,000 cpm \[^{35}\text{S}\]-labeled PrP-sen and 100 ng purified 263K PrP-res were mixed yielding a total volume of 22 \(\mu\)l in
conversion reaction buffer containing detergent (0.05% [w/v] zwittergent 3-14, 1.25% [w/v] sarkosyl, 1.8 mM sodium phosphate, 14 mM acetic acid, 112 mM NaCl, 5 mM MgCl₂, 50 mM sodium citrate at pH 6.0). For detergent-free microsome binding reactions, 10,000-20,000 cpm purified [³⁵S]-labeled PrP-sen and microsomes containing 100 ng 263K PrP-res were mixed in 22 μl of the microsome conversion reaction buffer (1.8 mM sodium phosphate, 14 mM acetic acid, 112 mM NaCl, 5 mM MgCl₂, 50 mM sodium citrate at pH 6.0). All samples were mixed thoroughly and incubated for 3 days at 37°C. After incubation, reaction mixtures were centrifuged at 20,800 x g for 20 min. The supernatant was saved and the resulting pellet was washed once with 180 μl of the same conversion reaction buffer without the microsomes and centrifuged again. The final pellet was suspended in 1% SDS. The radioactivities in the supernatant and pellet were quantified by scintillation counting, and [³⁵S]PrP in both fractions was analyzed by SDS/PAGE and Phosphorimager analysis. In addition to the centrifuge fractions, the radioactivities of tube bound PrP and PrP lost during the wash step were also determined by scintillation counting. Tube bound [³⁵S]PrP was solublized by adding 1% SDS to the tubes that contained the reaction mixtures. The percentages of [³⁵S]PrP-sen were determined by dividing the component radioactivity by the sum of the radioactivities for the supernatant, pellet and tube bound [³⁵S]PrP-sen.
C. RESULTS AND DISCUSSION

1. Expression of Recombinant HaPrP Using E. coli—To investigate the roles of the helix 1 Asp residues and their proposed salt bridges on the stability of the secondary structure of PrP, three mutant proteins (D144N, D147N, and D144N/D147N) of full length hamster PrP (residues 23-231) were generated by site-directed mutagenesis of a clone provided by Dr. M. Bloom consisting of a full length wild type hamster PrP gene (residues 23-231) inserted into at the Nde I restriction site of the pET24a(+) expression vector. Sequencing of the mutagenized clones indicated that the all three mutations were incorporated successfully into the HaPrP gene (data not shown).

Wild type and mutant HaPrP proteins were expressed as cytoplasmic inclusion bodies in E. coli, and then purified and refolded for analysis by circular dichroism spectroscopy. The protein yields varied between the constructs. The highest yield was obtained for the wild type protein, which typically gave 5 mg protein per liter culture. The D144N and D147N mutants gave approximately 1.5 mg protein per liter culture, while the typical D144N/D147N mutant protein yield was the lowest at 1.0 mg protein per liter culture. The reasons for the variations remain to be determined. However, the variation does not appear to result from differences in protein expression after induction as SDS/PAGE analysis of induced and uninduced cultures indicated that the wild type and mutant E. coli expressed roughly the same amounts of protein (data not shown). The differences may have been because the proteins have different propensities to form inclusion bodies, may have different stabilities that affect protein refolding, or may have
different affinities for binding to the nickel affinity column during purification.

Regardless, the purification and refolding protocols yielded enough protein to perform the CD analysis.

The purified proteins were analyzed by SDS/PAGE to assess protein purity and the presence of the disulfide bond between Cys179 and Cys214 (Figure 19). The slower migration of the proteins on SDS/PAGE upon treatment with β-mercaptoethanol (lanes 1-4) than untreated samples (lanes 5-8) indicated that the disulfide bond was present in the untreated preparations of each construct (Rezaei et al. 2002). Each protein ran as a single band with an estimated purity of at least 90%.

The purified proteins were analyzed by MALDI-TOF mass spectrometry as an indirect confirmation of the presence of the correct mutations. The D144N and D147N HaPrP proteins are theoretically 0.98 amu lower in molecular mass than wild type HaPrP (theoretical molecular mass 22974.65 Da), while D144N/D147N HaPrP is theoretically 1.96 amu lower. In order to obtain the accuracy necessary for resolving 1 amu differences in molecular mass, since the error in measurement was 0.05% of the total molecular mass, it was necessary to proteolytically digest the wild type and mutant HaPrP proteins using Trypsin to generate smaller fragments of PrP. The mass spectra (Figure 20) show numerous peaks due to the proteolytic digestion of HaPrP into multiple fragments. Analysis of the molecular masses of the peaks identified the peak in the wild type spectrum (Figure 20a) at 1534.57 amu as resulting from a PrP fragment containing both D144 and D147 (theoretical molecular mass for the fragment is 1534.6207). The spectra
Figure 19. Disulfide bonds are present in recombinant PrP purified and refolded from inclusion bodies expressed in *E. coli*. SDS/PAGE of wild type and helix 1 mutant hamster PrP residues 23-231 in the presence (reduced, lanes 1-4) and absence (oxidized, lanes 5-8) of β-mercaptoethanol. Gels were stained using coomassie blue. Molecular mass markers (kDa) are indicated on the left.
Figure 20a. MALDI-TOF mass spectrometry spectrum of trypsin digested wild type HaPrP (residues 23-231) used in CD spectroscopic studies. The peak at 1534.57 corresponds to a peptide fragment containing Asp144 and Asp147.
Figure 20b. MALDI-TOF mass spectrometry spectrum of trypsin digested D144N HaPrP (residues 23-231) used in CD spectroscopic studies. The peak at 1533.62 amu corresponds to a peptide fragment containing Asn144.
Figure 20c. MALDI-TOF mass spectrometry spectrum of trypsin digested D147N HaPrP (residues 23-231) used in CD spectroscopic studies. The peak at 1533.75 corresponds to a peptide fragment containing Asn147.
Figure 20d. MALDI-TOF mass spectrometry spectrum of trypsin digested D144N/D147N HaPrP (residues 23-231) used in CD spectroscopic studies. The peak at 1532.68 corresponds to a peptide fragment containing Asn144 and Asn147.
for the D144N, D147N, and D144N/D147N mutant protein Trypsin digests (Figure 20b-d) contained peaks from fragments that were 0.95, 0.82 and 1.89 atomic mass units lower respectively than the wild type protein, consistent with the respective theoretical molecular masses, suggesting that the proteins contained the correct mutations.

2. Thermal Denaturation of wild type and mutant HaPrP—The secondary structures and stabilities of the PrP mutants relative to wild type PrP were assessed by CD spectroscopy. Under nondenaturing conditions at 25°C and pH 6.0 (the optimal pH for most PrP-res-induced cell-free conversion reactions), spectra of the wild type and mutant PrP constructs were identical (Figure 21a), each showing negative lobes at 208 and 222 nm indicative of highly α-helical secondary structures as has been seen previously for PrP-sen derived from brain (Pan et al. 1993), mammalian tissue culture cells (Xiong et al. 2001) and recombinant PrP synthesized and refolded from inclusion bodies (Hornemann et al. 1997). These data suggest the Asp residues and their respective salt bridges do not substantially stabilize helix 1. Thermal denaturation was performed to determine if the mutants were destabilized with respect to secondary structure and unfolding transitions. The thermal denaturation curves were sigmoidal for all constructs suggesting that they undergo a two-state unfolding transition (Figure 22). However, none of the thermal unfolding reactions for the PrP constructs were reversible so, thermodynamic values could not be obtained. Transition midpoints and slopes of the unfolding transition regions were used for comparison instead. The transition midpoints (Table 5) for the mutants were within 2.2°C of the wild type protein transition midpoint.
suggesting that the PrP secondary structure is not substantially destabilized by the mutations and the presumed elimination of the helix 1 salt bridges. However, the slopes of the transition regions of the mutant unfolding curves (Table 5) were lower than the slope of the wild type protein-unfolding curve. These data indicate that the mutants unfold differently and less cooperatively than the wild type protein.

To look more closely at the thermal denaturation differences between the wild type and mutant PrP molecules, CD spectra at 62.5°C, 72.5°C, and 25°C after cooling from 72.5°C, were overlaid (Figures 21b-d). The wild type protein changed from a highly α-helical conformation at 25°C (Figure 21a) to a highly β-sheet conformation at 62.5 and 72.5°C as indicated by the negative peak centered at 215 nm (Figures 21b and c). At 62.5°C all three mutants appeared to still have some α-helical content judging by the slight negative dips in ellipticity at 208 and 222 nm (Figure 21b). At 72.5°C the mutant spectra all had negative lobes around 204 nm different from the wild type spectrum, most noticeable in the D144N and D144N/D147N mutant spectra (Figure 21c). This difference indicated that the mutant proteins have different states of denaturation at 72.5°C than the wild type. Negative ellipticities below 200 nm are associated with disordered structure; thus, the mutant PrP conformations appeared to contain more disordered structure than the more β-sheet containing wild type protein. In addition to the conformational differences at 72.5°C, there were differences in the reversibilities of the unfolding induced by this temperature. Thermal denaturation of the wild type protein was highly irreversible, which has been shown previously (Jackson et al. 1999), as can be seen by the fact that the spectra at 72.5 and 25°C after cooling from 72.5°C are shaped similarly.
with main negative lobes for both spectra centered close to 215 nm (Figures 21c and d).
Spectra of the mutant PrP molecules after cooling from 72.5°C to 25°C showed a partial
change back to more α-helical spectra with the D144N and D144N/D147N spectra, in
particular, showing negative lobes at 208 and 222 nm (Figure 21d), which are similar to,
but not identical, to the spectra at 25°C prior to thermal denaturation (Figure 21a). These
data suggest that the mutant proteins unfold to a different denatured state structure at
72.5°C than the wild type protein.

3. GdnHCl Denaturation of Wild Type and Mutant HaPrP—To further investigate the
unfolding of the PrP helix 1 mutants, GdnHCl unfolded samples of wild type D144N,
D147N and D144N/D147N PrP at pH 6.0 were monitored using CD spectroscopy at 222
nm. As shown in Figure 23, the sigmoid shapes of the unfolding curves indicated that all
the constructs underwent a cooperative two-state transition. All unfolding reactions were
found to be reversible (data not shown). Thermodynamic parameters, determined from
the unfolding data assuming a two-state transition, are shown in Table 5. The wild type
protein had a free energy of conformation \( \Delta G(H_2O) \) similar to that for recombinant full
length ovine PrP (Rezaei et al. 2000), but lower than that reported for full-length murine
PrP (Hornemann and Glockshuber 1998). The mutant proteins have lower \( \Delta G(H_2O) \)
values and transition midpoints \( ([\text{GdnHCl}]^{1/2}) \) than wild type PrP. The cooperativities for
the unfolding (m) indicated that the wild type (m = 7.86 kJ/mol/M), D144N (m = 7.43
kJ/mol/M) and D147N (m = 7.85 kJ/mol/M) PrP unfolded more cooperatively than did
the D144N/D147N (m = 6.04 kJ/mol/M) mutant. The \( \Delta G(H_2O) \) and \([\text{GdnHCl}]^{1/2}\) data
Figure 21. Secondary structures and thermal denaturations of wild type PrP and helix 1 PrP mutants. (a) Far-UV CD spectra of samples containing wild type (black), D144N (blue), D147N (red), and D144N/D147N (green) recombinant hamster PrP-sen (23-231) at 25°C in 10 mM sodium phosphate at pH 6.0 are overlaid. To aid comparison of spectral shapes, the spectra were slightly normalized (by < 5%) to the wild type spectrum at 222 nm. The same respective normalization factors were used in the spectral comparisons shown in panels b-d. (b), (c) and (d) Far-UV CD spectra of samples containing wild type, D144N, D147N and D144N/D147N hamster PrP-sen (residues 23-231) at 62.5°C, 72.5°C and 25°C after cooling from 72.5°C respectively. In (d) the wild type PrP spectrum at 25°C (+) is included for comparison. Protein concentrations for all samples were 0.3 mg/mL.
Figure 22. Thermal denaturation curves showing the fraction of protein unfolded determined from the ellipticities at 222 nm for the wild type (black triangles), D144N (blue +), D147N (red squares) and D144N/D147N (green X) PrP. The lines represent the best-fit lines determined by non-linear least squares regression. Transition midpoints are given in Table 5.
Figure 23. GdnHCl denaturation of wild type PrP and helix 1 PrP mutants. The fraction unfolded was determined using the CD ellipticities at 222 nm. Data were fitted and analyzed assuming a two-state transition. Thermodynamic parameters are listed in Table 5. All samples contained 0.1 mg/mL PrP. The samples were incubated at least 1 hour prior to taking the CD spectra. All samples were prepared in 10 mM sodium phosphate buffer at pH 6.0. Data labels are the same as in Figure 21.
<table>
<thead>
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<th>Construct</th>
<th>$T_m$ (°C)</th>
<th>Slope (°C$^{-1}$)</th>
<th>$\Delta G(H_2O)$ (kJ mol$^{-1}$)</th>
<th>$[\text{GdnHCl}]_{1/2}$ (M)</th>
<th>$m$ (kJ mol$^{-1}$ M$^{-1}$)</th>
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<td>Wild type</td>
<td>61.3 ± 0.7</td>
<td>0.174 ± 0.016</td>
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<td>2.02 ± 0.08</td>
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<td>D144N</td>
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<td>1.84 ± 0.02</td>
<td>7.4 ± 1.0</td>
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<tr>
<td>D147N</td>
<td>62.9 ± 0.4</td>
<td>0.139 ± 0.008</td>
<td>15.1 ± 1.6</td>
<td>1.93 ± 0.08</td>
<td>7.9 ± 0.5</td>
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<td>D144N/D147N</td>
<td>61.0 ± 0.5</td>
<td>0.0986 ± 0.005</td>
<td>10.9 ± 1.9</td>
<td>1.80 ± 0.09</td>
<td>6.0 ± 0.8</td>
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</table>

Table 5. Thermodynamic parameters for thermal and GdnHCl denaturation of wild type and helix 1 mutant hamster PrP protein. Transition midpoints ($T_m$) and Slope values (means ± standard deviation) were determined from three individual thermal denaturation experiments for each protein. $\Delta G(H_2O)$, $m$ and $[\text{GdnHCl}]_{1/2}$ values (means ± range) were determined from 2 individual denaturation experiments using GdnHCl for each protein.
support the conclusion from the thermal denaturation experiments that the mutants unfold somewhat differently than the wild type protein. However, while the mutants tended to be slightly more stable against thermal denaturation, they were slightly less stable than the wild type protein to GdnHCl-induced denaturation. Nonetheless, neither the thermal nor the chemical denaturation data suggest that the overall stability of PrP is substantially dependent upon the proposed helix 1 Asp residues and their salt bridges.

4. **Cell-free Conversion Reactions**—It remained to be determined whether the helix 1 aspartic acid residues are involved in the interactions necessary for the conversion of PrP-sen to PrP-res. Specifically, if the critical interaction required for the conversion involved intermolecular ionic bonds between aspartic acid (D144 and D147) and arginine (R148 and R151) residues as proposed (Morrissette and Shakhnovich 1999), one would expect conversion of the mutants lacking the Asp residues to be inhibited. In order to address this question the D144N, D147N and D144N/D147N PrP mutants were expressed in mammalian cells. Transfection and amplification of protein expression using the pSFF retroviral vector with \( \psi 2 \) and PA317 cells and expression of HaPrP by this method has been described elsewhere (Kocisko *et al.* 1994; Robertson *et al.* 1992). The basic process is the \( \psi 2 \) and PA317 cells are transfected with the retroviral vector pSFF containing the desired HaPrP insert in order infect the cells, getting them to incorporate both the retroviral and HaPrP genes into their chromosomes. Once infected the cells express both viral and HaPrP proteins. The viral proteins facilitate retroviral replication within the host cells forming virions containing the retroviral genes, including the inserted HaPrP gene,
which are secreted into the medium and subsequently infect other cells. Viral and HaPrP protein expression, therefore, amplify and persist after transfection. HaPrP expression following transfection was confirmed using immunostaining with the HaPrP specific antibody 3F4. Figure 24 shows the immunostained cells transfected with the retroviral vector pSFF containing gene inserts for wild type, D144N, D147N, or D144N/D147N HaPrP. The red staining for the HaPrP pSFF transfected cells compared to no staining for controls transfected with pSFF lacking a HaPrP gene, confirms the expression of the HaPrP protein constructs. Immunostaining was also performed using an antibody (18-8) directed towards the Gag retroviral capsid protein to confirm expression of viral structural protein. All constructs stained positive for Gag expression indicating the cells were expressing retroviral capsid structural proteins (Figure 25).

After confirming HaPrP expression, cells were radiolabeled using $[^{35}\text{S}]$-methionine and analyzed using the cell-free conversion assay (Kocisko et al. 1994). The assay is performed by “seeding” the conversion of $[^{35}\text{S}]$-labeled PrP-sen using PrP-res from the brains of hamsters infected with 263K scrapie. After an incubation period, newly converted (radiolabeled), partially protease-resistant PrP is detected following digestion with PK by SDS-PAGE and phosphor autoradiography (Caughey et al. 2001; Kocisko et al. 1994). Conversion reactions were limited to PrP-sen that was metabolically radiolabeled in mammalian cells because of difficulties in generating $[^{35}\text{S}]$-methionine labeled PrP-sen with sufficient specific activity when expressed in E. coli. Figure 26 shows the results of conversion reactions performed in the presence of detergents and GdnHCl using purified 263K hamster PrP-res to drive the reaction. Figure
Figure 24. Immunostaining using the anti-PrP antibody 3F4 of ψ2 and PA317 mouse fibroblast cells transfected with the retroviral vector pSFF-containing gene inserts for wild type and mutant hamster PrP lacking the GPI anchor. The red staining in panels a-d, which resulted from the reaction product of antibody bound peroxidase with AEC substrate, indicate that cells transfected with wild type (a), D144N (b), D147N (c), and D144N/D147N (d) HaPrP containing pSFF retroviral vectors express PrP. The negative control (e), containing non-transfected ψ2 and PA317 cells, shows no red staining, suggesting the staining is dependent on the expression of PrP.
Figure 25. Immunostaining using the antibody 18-8 of ψ2 and PA317 mouse fibroblast cells transfected with the retroviral vector pSFF-containing gene inserts for wild type and mutant hamster PrP lacking the GPI anchor. The 18-8 antibody is directed against the retrovirus capsid structural protein Gag. The red staining, which resulted from the reaction product of antibody bound peroxidase with AEC substrate, in panels a-d indicates that cells transfected with the wild type (a), D144N (b), D147N (c), and D144N/D147N (d) HaPrP containing pSFF retroviral vectors express Gag. The negative control (e), containing ψ2 and PA317 cells alone, shows no red staining, suggesting the staining is dependent on transfection with the pSFF retroviral vector and the expression of Gag protein.
26a shows the undigested radiolabeled PrP post incubation without (lanes 1 to 4) and with (lanes 5 to 8) PrP-res. Lanes 9 to 16 show the remaining portion of the same samples after a 1-hour digestion with PK. The lanes incubated without PrP-res had no protease resistant bands. However, samples incubated in the presence of PrP-res showed distinct protease resistant $^{35}$SPrP bands that, like PK-digestion products of brain-derived PrP-res itself, were 6 to 7 kDa lower in molecular mass than full-length precursor $^{35}$SPrP-sen molecules. Quantification of the proportion of the input $^{35}$SPrP-sen converted to PK-resistant bands of 19-26 kDa (i.e. conversion efficiency) is shown in Figure 26b. In this case all three mutants converted (lanes 14 to 16) with statistically indistinguishable efficiencies from the wild type PrP control (lane 13). These data are not consistent with the idea proposed by Morrissey and Shakhnovich (Morrissey and Shakhnovich 1999) that PrP-res is stabilized by intermolecular salt bridges. The data are also inconsistent with the possibility that the previously observed inhibition of conversion of PrP helix 1 deletion mutants (Vorberg et al. 2001) was due to the loss of the putative salt bridge-forming carboxylate groups on the aspartate side chains. Thus, these date suggest that aspartic acid residues 144 and 147 are not essential for the conversion of PrP-sen to PrP-res in the presence of detergents and GdnHCl.

Because the above conversion conditions (especially the presence of GdnHCl) may have blocked the ionic interactions necessary for the presence of the helix 1 salt bridges, conversion reactions were performed without the GdnHCl pretreatment using guanidine-free conversion reaction conditions developed by Horiuchi to better approximate the physiological conditions at which PrP-res formation occurs by removing
<table>
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<th>Component</th>
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<th>GdnHCl-free (mM)</th>
<th>Denaturant-free / Low Detergent (mM)</th>
<th>Urea Pretreatment / Low Detergent (mM)</th>
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Table 6. Buffer conditions used for cell-free conversion experiments.
a) PrP-res

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b) % Conversion Efficiency

- Wildtype
- D144N
- D147N
- D144N/D147N

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Figure 26. Conversion of wild type and mutant PrP-sen molecules using GdnHCl pretreated PrP-res and detergents in the conversion buffer. (a) GdnHCl pretreated, cell-free conversion of wild type (lanes 1, 5, 9, and 13), D144N (lanes 2, 6, 10, and 14), D147N (lanes 3, 7, 11, and 15) and D144N/D147N (lanes 4, 8, 12, and 16) $^{35}$S-PrP-sen into protease resistant forms before and after treatment with PK. Lanes 1-8 contain 10% of the total reaction mixture prior to PK digestion. The remaining 90% of the samples after PK-digestion are shown in lanes 9-16. Molecular mass markers (kDa) are indicated at the left. (b) The means and standard deviations (n = 3) of the conversion efficiencies of the wild type and mutant PrP-sen into PrP-res were determined by comparing and appropriately factoring the integrated intensities of bands from the 20-30-kDa (lanes 13-16) and the 26-37-kDa (lanes 5-8) bands.
Figure 27. Conversion of wild type and mutant PrP-sen molecules without GdnHCl pretreatment of PrP-res. (a) GdnHCl-free cell-free conversion of wild type (lanes 1, 5, 9, and 13), D144N (lanes 2, 6, 10, and 14), D147N (lanes 3, 7, 11, and 15) and D144N/D147N (lanes 4, 8, 12, and 16) \(^{35}\)S-PrP-sen into protease resistant forms before and after treatment with PK. Molecular mass markers (kDa) are indicated at the left. (b) The means and standard deviations (n = 3) of the conversion efficiencies of the wild type and mutant PrP-sen into PrP-res were determined as described in the Experimental Methods and the Figure 25 caption.
the denaturant and lowering the amount of detergent in the reaction buffer (Horiuchi and Caughey 1999; see Table 6 for a summary of all conversion reaction conditions used in this report). Results of the guanidine-free conversions are shown in Figures 27a and 27b. The conversion efficiencies for all constructs were statistically indistinguishable, suggesting the aspartates have no effect on conversion under guanidine-free conditions.

While the guanidine-free conversion reactions lacked the salt-bridge destabilizing cationic denaturant guanidine, reactions still contained a substantial amount of anionic detergent which might have disrupted any stabilizing salt bridges. To test the conversion under conditions which contained a minimal amount of detergents and no ionic denaturants, reactions were performed under denaturant-free/low detergent conditions using conversion buffer containing only a minimal amount of the anionic detergent zwittergent 3-14 and using buffer containing the nonionic denaturant urea and zwittergent 3-14. Zwittergent 3-14 is used as a stabilizer in the storage buffer for purified PrP-res. Its presence in conversion reactions proved to be essential because denaturant and detergent-free conversion reactions performed using purified PrP-res in which the detergent containing storage buffer was replaced with water were unsuccessful (data not shown). The poor results were most likely due to the difficulty in obtaining repeatable pipetting of the PrP-res. Thus, a small amount of zwittergent 3-14 was present for all conversion reactions performed using purified PrP-res. Results of the denaturant-free/low detergent conversion reaction are shown in Figures 28a and 28b. Figure 28a clearly shows that conversion product was obtained for all constructs. The conversion efficiencies for all constructs were, however, statistically indistinguishable (Figure 28b).
Figure 28. Conversion of wild type and mutant PrP-sen molecules using denaturant-free/low detergent conditions. (a) Denaturant-free/low detergent cell-free conversion of wild type (lanes 1, 5, 9, and 13), D144N (lanes 2, 6, 10, and 14), D147N (lanes 3, 7, 11, and 15) and D144N/D147N (lanes 4, 8, 12, and 16) 35S-PrP-sen into protease resistant forms before and after treatment with PK. Molecular mass markers (kDa) are indicated at the left. (b) The means and standard deviations (n = 3) of the conversion efficiencies of the wild type and mutant PrP-sen into PrP-res were determined as described in the Experimental Methods and the Figure 25 caption.
Figure 29. Conversion of wild type and mutant PrP-sen molecules using urea pretreatment of PrP-res and low detergent conditions. (a) Urea and low detergent cell-free conversion of wild type (lanes 1, 5, 9, and 13), D144N (lanes 2, 6, 10, and 14), D147N (lanes 3, 7, 11, and 15) and D144N/D147N (lanes 4, 8, 12, and 16) 35S-PrP-sen into protease resistant forms before and after treatment with PK. Molecular mass markers (kDa) are indicated at the left. (b) The means and standard deviations (n = 3) of the conversion efficiencies of the wild type and mutant PrP-sen into PrP-res were determined as described in the Experimental Methods and the Figure 25 caption.
Conversion reactions performed using urea pretreated purified PrP-res (Figure 29) resulted in no dramatic differences between the conversion efficiencies for the mutant and wild type proteins.

In order to obtain reaction conditions completely lacking salt-bridge interfering ionic detergents and denaturant, conversion reactions were performed using microsomal PrP-res in place of purified PrP-res following a recently described method (Caughey et al. 2001). This method uses a crude scrapie brain microsome fraction containing PrP-res to drive conversion. These conversion reactions more closely mimic conditions found on cell membranes where PrP-sen is located and most likely converted in scrapie-infected cells (Caughey and Raymond 1991; Caughey et al. 1991a). Under these conditions the PrP mutants converted with 2-3 times the conversion efficiency as the wild type PrP (Figure 30a and b). Thus, the replacement of the aspartic acid residues with asparagines at positions 144 and 147 enhanced, rather than prevented, conversion to PrP-res.

The influence of the helix 1 salt bridges on PrP conversion was also tested using [$^{35}$S]-labeled PrP-sen mutants where the aspartic acids at positions 144 and 147 were individually or both replaced with alanines. These replacements should have prevented ion-dipole interactions in addition to ion-ion interactions between the side-chains of the residues involved in the helix 1 salt bridges in wild type PrP. If the increases in conversion efficiencies of the D-to-N HaPrP mutants were solely due to the loss of a salt bridge or any other interaction that stabilizes the native $\alpha$-helical structure from converting to PrP-res then one would expect that the D-to-A mutant proteins would show the same conversion behavior as the respective D-to-N mutants. After subcloning the
desired D to A HaPrP mutants into the pSFF retroviral vector, transfecting y2 and PA317 cells, and confirming PrP and Gag protein expression as described above (data not shown), cells were radiolabeled with $[^{35}\text{S}]-$methionine. Using radiolabeled D144A, D147A and D144A/D147A PrP-sen mutants in the detergent and GdnHCl-free conversion reactions produced the expected conversion products (Figure 31a, compare lanes 9-16 to lanes 1-8). The conversion efficiencies of the D-to-A mutant PrP constructs were roughly 3-4 times as efficient as the conversion efficiency of the wild type PrP (Figure 31b), confirming that mutations expected to disrupt the helix 1 salt bridges promote PrP-res-induced conversion. The fact that D-to-A mutants were even more efficiently converted than the D-to-N mutants suggests that in the latter, some stabilizing interactions between the N144, N147 and the arginines in helix 1 (presumably ion-dipole interactions) still occur.

5. PrP-sen Binding to PrP-res—To determine if there are any PrP-res binding differences between the wild type and mutant PrP-sen, binding experiments were performed using microsomal and purified PrP-res. As mentioned above there are two kinetically separable steps in the interactions of PrP-sen and PrP-res leading to conversion of PrP-sen to the PK-resistant state: the binding of PrP-sen to PrP-res followed by the conversion of PrP-sen to the PK resistant state (DebBurman et al. 1997; Horiuchi and Caughey 1999; Horiuchi et al. 2000). Two different binding experiments were performed to test for differences between PrP-sen and PrP-res in the presence and
Figure 30. D144N and D147N PrP mutants convert more efficiently than wild type PrP-sen without denaturants or detergents. (a) Conversions of wild type (lanes 1, 5, 9, and 13), D144N (lanes 2, 6, 10, and 14), D147N (lanes 3, 7, 11, and 15) and D144N/D147N (lanes 4, 8, 12, and 16) $^{35}$S-PrP-sen into PrP-res performed without the presence of denaturants or detergents before and after treatment with PK. Reactions were performed using microsomal PrP-res derived from crude brain homogenates of uninfected and 263K scrapie-infected hamsters as described in the Experimental Methods. (b) The means and standard deviations (n = 5) of the conversion efficiencies for wild type and mutant PrP's are indicated. Conversion efficiencies were determined as described in the Experimental Methods and Figure 25 caption. The difference between the mean of the wild type and each mutant PrP was assessed with a one-way ANOVA by Dunnett’s multiple comparison test. Statistical significance of the differences is designated by * and ** for P<0.05 and P<0.01 respectively.
a) PrP-res

PK- - - + + +
PK+ - - + + +

100 -
50 -
37 -
25 -
15 -
10 -

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

b) % Conversion Efficiency

Wildtype D144A D147A D144A/D147A

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Figure 31. D144A and D147A PrP mutants convert with increased efficiency relative to wild type PrP without denaturants or detergents. (a) Conversions of wild type (lanes 1, 5, 9, and 13), D144A (lanes 2, 6, 10, and 14), D147A (lanes 3, 7, 11, and 15) and D144A/D147A (lanes 4, 8, 12, and 16) $^{35}$S-PrP-sen into PrP-res performed without the presence of denaturants or detergents before and after treatment with PK. Reactions were performed using microsomal PrP-res derived from crude brain homogenates of uninfected and 263K scrapie-infected hamsters as described in the Experimental Methods. (b) The means and standard deviations ($n = 5$) of the conversion efficiencies for wild type and mutant PrP’s are indicated. Conversion efficiencies were determined as described in the Experimental Methods and Figure 25 caption. The difference between the mean of the wild type and each mutant PrP was assessed the same as described in the Figure 29 caption.
absence of denaturant. Experiments using purified PrP-res were performed in the presence of the detergents sarkosyl and zwittergent 3-14 at physiological salt concentrations using a previously described method (Horiuchi and Caughey 1999). PrP-res was incubated with $[^{35}\text{S}]\text{PrP-sen}$ and then pelleted, along with any bound $[^{35}\text{S}]\text{PrP-sen}$ by centrifugation. Greater than 95% of the PrP-res pellets using this method and therefore any $[^{35}\text{S}]\text{PrP-sen}$ detected in the pellet fraction is indicative of PrP-res bound PrP-sen (Horiuchi and Caughey 1999). For all of the PrP-sen constructs the binding was PrP-res dependent and the average amount of $[^{35}\text{S}]\text{PrP-sen}$ present in the respective pellets ranged from 52 to 66% of the total detectable $[^{35}\text{S}]\text{PrP-sen}$, while the amount present in samples lacking PrP-res ranged from 3 to 11% (Figure 32). The difference between the PrP-res containing and lacking samples is indicative of the PrP-res-dependent binding. The difference values are statistically indistinguishable suggesting that there are no differences in binding. This result is not surprising since the conversion efficiencies resulting from conversion reactions performed under comparable conditions were essentially indistinguishable.

Binding experiments using microsomal PrP-res were performed to determine if the conversion differences seen between the wild type and mutant PrP-sen constructs were due solely to PrP-res binding differences. If this were the case, one would expect to detect twice as much of the asparagine mutant PrP-sen molecules bound to PrP-res as wild type PrP-sen. Results from the microsomal PrP-res binding experiments are shown in Figure 33. In the presence of PrP-res-containing microsomes, between 68 and 69% of the $[^{35}\text{S}]\text{PrP-sen}$ was present in the pellet. Unlike the purified PrP-res binding
Figure 32. Binding of wild type and mutant $^{35}$S-PrP-sen to purified PrP-res The percentages of $^{35}$S-PrP-sen that pelleted with purified PrP-res were determined as described in the Experimental Methods. The graph shows the mean binding percentages and standard deviations (n=3) of wild type and mutant PrP-sen to microsomal 263K PrP-res (PrP-res+) and buffer lacking PrP-res (PrP-res-). The differences between the the PrP-res + and − samples are also shown to indicate the PrP-res-dependent binding. The percentage of the radioactivity in the pellet fraction relative to the total radioactivities which include the supernatant, pellet and tube bound fractions, is indicated on the Y-axis.
Figure 33. Binding of wild type and asparagine mutant $^{35}$S-PrP-sen to microsomal PrP-res The percentages of $^{35}$S-PrP-sen that pelleted with microsomal PrP-res were determined as described in the Experimental Methods. The graph shows the mean binding percentages and standard deviations (n=3) of wild type and mutant PrP-sen to microsomal 263K PrP-res (PrP-res+) and normal brain microsomes lacking PrP-res (PrP-res-). The differences between the the PrP-res + and - samples are also shown to indicate the PrP-res-dependent binding. The percentage of the radioactivity in the pellet fraction relative to the total radioactivities which include the supernatant, pellet and tube bound fractions, is indicated on the Y-axis.
experiments however, there was a significant amount of PrP-res independent binding since pellets of the normal brain microsomes lacking PrP-res contained from 49 to 55% of the $[^{35}S]PrP$-sen. This resulted in the PrP-res binding dependent differences being significantly lower than the differences obtained in the purified PrP-res binding experiments. As a result evaluating the statistical differences between samples was problematic because after propagating the error, the relative standard deviation values were high, ranging from 32% for the D144N/D147N data to 66% for the wild type PrP. Thus, while the data suggest that binding differences are not the cause of the observed conversion efficiency differences between the wild type and helix 1 mutant proteins since the means of the PrP-res dependent binding for all samples were similar, the development of an improved binding assay is needed to generate data with lower relative standard deviations in order to statistically confirm the results.

6. Discussion—This investigation sought to determine the role of the putative Asp-Arg helix 1 salt bridges in the stability of PrP-sen and whether the same residues proposed to be involved in the salt bridges play a role in the conversion of PrP-sen to PrP-res. It appears from the data that helix 1 does not behave as has been previously proposed (Morrissey and Shakhnovich 1999). That is, the data suggest that the helix 1 salt bridges do not substantially stabilize PrP under native conditions and, intermolecular salt bridge formation between the same salt bridge residues is not critical for the conversion of PrP-sen to PrP-res. Instead it appears that the aspartic acid residues affect how the protein unfolds and provide stability against the conversion of PrP-sen to PrP-res.
The contribution of surface salt bridges such as those proposed to exist on helix 1 has generally been considered to contribute little to the overall stability of protein structures. It has been reported that salt bridges buried in the protein interior contribute as much as 20.9 kJ/mol while surface salt bridges contribute only 2.1 to 5.0 kJ/mol to protein free energies of conformation (Matthews 1993; Nakamura 1996). Our data are consistent with this consensus as the Gibbs free energies of conformation of the D144N, D147N, and D144N/D147N mutants were estimated by GdnHCl unfolding to be only 2.3, 0.9 and 5.1 kJ/mol lower respectively than wild type hamster PrP-sen. These data combined with the fact the CD spectra of the mutants are identical to the wild type protein at 25°C suggest that there is no unusual stability gain due to ionic interactions involving the aspartic acid and arginine residues of helix 1 of hamster PrP-sen.

Although the effects of the helix 1 mutations on the overall free energy of PrP are modest, the localized effects on helix 1 stability might be more dramatic. At pHs below 4.0 three-state unfolding transitions have been reported for recombinant human PrP in the presence of GdnHCl and urea with salt (Jackson et al. 1999; Swietnicki et al. 1997). It is tempting to think that the first transition might involve the unfolding of a localized domain containing helix 1 because this helix has been modeled to lose helical structure at low pH (Alonso and Daggett 2001) and has been shown to more easily undergo hydrogen-deuterium exchange than the central portions of helices 2 and 3 (Liu et al. 1999). This suggests a looser, less compact structure that might unfold more readily. Such a transition at low pH might be dependent on the protonation and ionic screening of the aspartic acids disrupting the salt bridges leading to the unfolding of helix 1 prior to
the unfolding of the remaining helices. If such a three-state transition were occurring under the conditions of our experiments (i.e., pH 6), one might expect to have detected it in our unfolding curves monitored at 222 nm since helix 1 makes up 18% of the total helix content of PrP-sen. However, our unfolding curves of both the wild type and helix 1 mutants suggested only two-state processes without any additional inflection points. Nonetheless the helix 1 mutations reduced the slopes of the transition regions for the thermal denaturation curves indicating the mutants unfold less cooperatively than the wild type protein. This effect was especially apparent for the D144N/D147N double mutant. Presumably, replacement of the helix 1 aspartates destabilizes helix 1 to some extent but our data provide no evidence that this allows helix 1 to unfold independently of the other helices under the conditions suitable for the PrP-res-induced conversion reaction. One possibility is that helix 1 is already partially unfolded under these conditions reducing our chances of detecting any independent unfolding in the denaturation curves. In any case, the low structural resolution of CD spectroscopy prevents us from fully discerning the conformational effects of the aspartate replacement and elimination of putative helix 1 salt bridges.

The differences between unfolding cooperativities of the mutant and wild type PrP molecules raise the possibility that the helix 1 aspartic acids are involved with stabilizing interactions other than the intramolecular salt bridges. Eleven different salt bridges involving seven different anionic residues and eight different cationic residues were predicted in molecular dynamic simulations of human and Syrian hamster PrP-sen as reported by Zuegg and Gready (Zuegg and Gready 1999). Five of the eleven proposed
salt bridges involve either D144 or D147 including, in addition to the intrahelix salt bridges investigated here, D144-H140, D144-R208 and D147-H140. The differences in unfolding cooperativities could result from the elimination of any of the five proposed salt bridges.

Protein unfolding cooperativity differences can also result from differences in the stabilities of the denatured state conformations (Matthews 1993; Shortle 1996). The PrP thermal denaturation data show that the denatured states of the mutants at 72.5°C differ from the wild type protein suggesting the mutants unfold via somewhat different conformations. Such alterations in the unfolding pathway, rather than differences in overall thermodynamic stability, could account for the increased conversion efficiencies seen in our cell-free conversion experiments. Consistent with this possibility are observations that decreased thermodynamic stability of PrP-sen is not a common characteristic of mutants associated with familial Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia (Liemann and Glockshuber 1999). Reasons proposed for how mutations associated with inherited forms of spongiform encephalopathies lead to disease include: a) an increase in the stability of PrP-res; b) an increased number of alternative conformations in equilibrium with PrP-sen which may act as precursors to PrP-res; and c) differences in PrP-sen folding kinetics with accumulation of PrP-res-like kinetic folding intermediates (Liemann and Glockshuber 1999). Any of these reasons could apply as reasons for the increase in PrP-res formation seen in the cell-free conversion reactions using microsomal PrP-res and the PrP-sen helix 1 mutants studied herein. Binding differences between the wild type and
mutant PrP-sen molecules with PrP-res could be another reason for the observed conversion differences. However, the binding data, though inconclusive, suggests there are no binding differences.

Thus, the helix 1 mutants may populate intermediate, partially unfolded conformations along the PrP-sen to PrP-res conversion pathway to a greater extent than wild type PrP. Conversely, because the helix 1 mutants do not unfold to the same β-sheet containing denatured structures at 62.5 and 72.5°C as the wild type protein, it is possible that the mutants populate an off-pathway β-sheet conformation to a lesser extent than the wild type protein. The formation of an off-pathway β-oligomer has been previously reported to occur during amyloid fibril formation of recombinant PrP 27-30 (Baskakov et al. 2002). The more disordered conformations seen with the helix 1 mutants might be more convertible to PrP-res than the more β-sheet conformation of wild type PrP during thermal denaturation. However, Rezaei et al. (Rezaei et al. 2002) recently reported that the thermally denatured conformations of sheep PrP variants associated with low susceptibility to scrapie infection contained more disordered structure while the highly susceptible variants contained more β-sheet. The sheep PrP variant data is not consistent with our data because our mutants show more disordered structure but convert with higher efficiencies relative to the wild type protein. Thus, it appears that unfolding to a more disordered conformation is not necessarily associated with increased susceptibility to PrP-res formation. Rather, our data suggest that our D-to-N mutants populate intermediate conformations that contain unspecified secondary structures with less β-sheet that are more susceptible to PrP-res formation than wild type PrP.
Considering all the data, three mechanistic pathways leading to PrP-res formation can be proposed (Figure 34) assuming the conversion is based on the autocatalytic seeded polymerization model (Come et al. 1993; Griffith 1967; Jarrett and Lansbury, Jr. 1993; Lansbury, Jr. and Caughey 1995). Also, assuming PrP-sen is in equilibrium with convertible, intermediate and non-convertible conformations, the pathways vary depending on which conformation binds to PrP-res. In pathway (a), the intermediate PrP-sen conformation binds to PrP-res. After binding, the PrP-sen molecule can undergo a conformational transition to either the convertible conformation or to the off-pathway non-convertible conformation. PrP-res is only formed when the bound PrP-sen molecule attains the convertible conformation. In pathway (b), convertible PrP-sen binds to PrP-res and then can convert directly to PrP-res. In pathway (c), a non-convertible PrP-sen conformer binds to PrP-res and must transform to intermediate and/or convertible PrP-sen prior to converting to PrP-res. Evidence for the existence of bound, but non-convertible states has come from previous studies of interspecies conversion reactions (Horiuchi et al. 2000). In the case of the helix 1 mutants, PrP-sen is proposed to be less likely to adopt the non-convertible conformation resulting in more conversion of the mutant PrP-sen molecules to PrP-res than the wild type PrP-sen.

D. CONCLUSIONS

In summary, these results suggest that PrP-sen is not substantially stabilized by the aspartic acid-arginine salt bridges. However, the helix 1 mutants unfold less
Figure 34. Theoretical mechanistic model for the conversion of PrP-sen to PrP-res. Assuming that conversion follows the autocatalytic seeded polymerization model (Caughey and Lansbury 1995; Come et al. 1993) three pathways leading to conversion are proposed. With all pathways, unbound PrP-sen is in equilibrium with convertible, intermediate and non-convertible conformations. In pathway (a), after binding to PrP-res, intermediate PrP-sen undergoes a conformational transition to either a convertible conformation that can subsequently convert to PrP-res or a non-convertible, off-pathway conformation that cannot convert to PrP-res without returning to the intermediate conformation or unbinding. In pathway (b), the convertible PrP-sen molecule binds to PrP-res where it can then directly convert to PrP-res. In pathway (c), the non-convertible PrP-sen binds to PrP-res in an off pathway manner. PrP-res can only be formed if the bound PrP-sen converts to the convertible PrP-sen conformation by passing through the intermediate conformation or through rebinding in a different conformation. In this model the helix 1 mutant PrP molecules populate the convertible conformation along pathways (a) and (b), avoiding the non-convertible pathway to a greater extent than the wild type protein and, thus, having higher conversion efficiencies in the cell-free conversion assay.
cooperatively and do not attain the same β-sheet denatured state as the wild type protein upon thermal denaturation. Cell-free conversion data suggest the salt bridge residues are not critical for the formation of PrP-res as protease resistant material was generated in all conversion reactions performed with and without the presence of detergents and denaturants. The conversion efficiencies of the mutants in the absence of detergents and denaturants, conditions favorable to salt bridge formation, are higher than the wild type protein, indicating that the elimination of the anionic charge of the aspartic acids increased either the convertibility or the binding of PrP-sen to PrP-res. Results of binding experiments suggest that the conversion differences are not due to binding differences, but rather in the convertibility of the PrP-sen. An increase in convertibility could possibly be explained by the elimination of an off-pathway conformation along the conformational pathway leading to conversion to PrP-res.


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