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Prion diseases are fatal neurodegenerative diseases of mammals. They are characterized by the conversion of normal prion protein (PrP) to a misfolded conformational state that accumulates as plaques in the brain. The diagnosis of prion diseases relies on the ability to differentiate between normal PrP and its misfolded, infectious form. This is difficult to accomplish by traditional testing methods, since it requires discerning between conformational states of a protein that is present in both normal and diseased tissue, rather than identifying the appearance of a new protein associated with infection. We wish to design a reporter PrP substrate that may be monitored by fluorescence spectroscopy. After the conversion of normal-PrP to its infectious state, some amino acid residues of PrP will undergo a change in their local solvent environment. We propose to identify these residues by monitoring the fluorescence emission spectrum of a series of mutant 7-AzaTrp-substituted PrP proteins. The 7-AzaTrp fluorescence emission spectrum is both unique compared with normal Trp and exquisitely sensitive to its local environment. This could lead to the development of a rapid, sensitive, and inexpensive technique to detect infectious PrP, based on its ability to bind 7-AzaTrp-substituted PrP, and convert it to the misfolded form.

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INTRODUCTION

Prion diseases are fatal neurodegenerative diseases of mammals and include Creutzfeld-Jacob disease (humans), scrapie (sheep), chronic wasting disease (elk, deer), and mad cow disease (cattle).(1) The diseases are characterized by the conversion of normal prion protein (PrP) to a misfolded conformational state that aggregates and forms plaques in the brain. Although this conversion process is poorly understood, it is known that the aggregate-prone form has a higher β-sheet content than does normal PrP. To differentiate between normal PrP and its misfolded, infectious form and assist in diagnosis of the diseased state, we wish to design a reporter PrP substrate peptide that will exhibit unique fluorescent properties upon contact with infectious PrP and/or its subsequent conversion. The sequence of the reporter peptide will be based upon experiments conducted with recombinant PrP. We will identify individual PrP residues that undergo changes in their local solvent environment upon conversion by measuring the fluorescence emission spectra of a series of mutant 7-AzaTrp-substituted PrP proteins. The 7-AzaTrp absorbance spectrum is red-shifted compared with Trp, and its fluorescence emission spectrum is exquisitely sensitive to its local environment.(2) Therefore, the conversion of the analog-substituted PrP substrate will be easily detected against a background of Trp-containing protein aggregates.

BODY

Overview

Since receiving the IDEA award in mid-summer 2003, we have made significant progress in accomplishing *Task 1* of the proposal, especially since the arrival of Dr. Hui-Chun Yeh, a post-doctoral scholar, in late January 2004. Dr. Yeh and the PI (Dr. McGuirl) have produced, purified, and characterized five PrP mutants, worked out the methodology for conversion of these from the normal α-form to a soluble oligomeric β-form, and investigated the biophysical properties of the mutants. Our results indicate that the project would be better served by pursuing *Tasks 1* and *2* concurrently rather than sequentially (vide infra). Therefore, Ms. Erin Gray (B.A. in Human Biology) will join the laboratory as of July 1, 2004, for a period of one year. Ms. Gray will focus on *Task 2a* – the development of a bacterial expression system that produces PrP mutant proteins with close to 100% incorporation of 7-AzaTrp in lieu of natural Trp. She will also assist in preparing the PrP mutants proposed for *Task 1*. The PI also involved two volunteer high school students in the project, in partial fulfillment of her university's expectation of community outreach and service by faculty. These students presented their accomplishments at regional and national competitions and received several honors and awards. The US Army Research IDEA Award was acknowledged in presentations by students and the PI.

Accomplishments related to Task 1. **Identify surface residues of the normal PrP form that change conformation and/or participate in binding to aggregated PrP. (Months 1-24)**

1a) Express a series of Trp-substituted mutants of truncated Syrian hamster PrP (90-231) in E. coli.

Expression: We have expressed truncated Syrian golden hamster PrP (residues 90-231) in *E. coli*, as outlined in the Methods Section of our proposal. We have selected the robust pET expression system (Novagen) for expression, since it is proven to work for full-length PrP.(*3*) By using the commercially available Rosetta cell strain from Novagen, we have increased the expression levels of both full-length and truncated PrP to nearly 30 mg/liter of culture. The Rosetta strain contains pRare-lacI, a chloramphenicol-resistant plasmid that supplies tRNAs for seven rare codons, AUA, AGG, AGA, CUA, CCC, GGA, and CGG under the control of their native promoters, along with the lac repressor gene. We have also isolated pRare-lacI to allow for its future use in Trp-auxotrophic *E. coli* hosts.

Mutants Produced: To date, we have produced five of the planned PrP mutants (see Table I), including the wild type truncated PrP. Wild type truncated PrP contains only two Trp residues: W99 and W145. W99 is located in the flexible region, whereas W145 is present within Helix 1, which has been proposed to assume a β-sheet structure in the infectious form.(*4*) We have also prepared mutants that contain a single Trp residue at either 99 or 145. These have proved helpful in understanding the individual contributions of Trp at each sequence position to the overall fluorescence spectrum of the wild type protein. We have also made the Trp-free double mutant W99F/W145Y, for use as a base for other mutants. Finally, we have begun the process of replacing individual residues with Trp with the recent preparation of Y218W in a W99F/W145Y background.

Two additional mutants, Y150W and Y163W, have been added to the list and will receive high priority. Their inclusion is based on the work of Cashman et al,(*5*) who suggest that only upon conversion does a Tyr residue in one of two highly conserved Tyr-Tyr-Arg motifs become suffciently solvent exposed to react with monoclonal antibodies that recognize this motif.

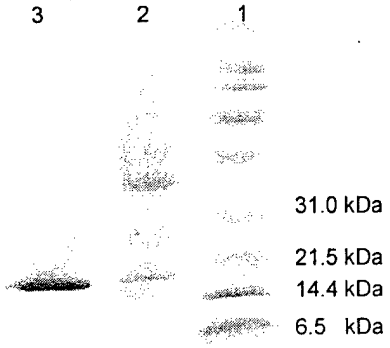
Table I. PrP Mutants: Bold blue font, made and characterized as of 5/14/04; normal font, planned mutants; italicized red font, new proposed mutants.

Trp Mutant	Region of Interest	Rationale for Choice
Wild Type residues 90-231		Control protein for comparison with mutants and with data published by others
Trp99Phe	H1 143-156	Examine Trp fluorescence from W145 in H1
Trp145Tyr	90-120	Examine Trp fluorescence from W99, in conformationally dynamic region (structurally undefined)
W99F/W145Y	Trp-free Base mutant	Remove Trp fluorescence from control protein, Use this for the other mutations listed below
Tyr218Trp W99F/W145Y	200-223	Internal residue in H3 near disulfide bond, contacts H2
Lys106Trp W99F/W145Y	90-120	In conformationally dynamic region (structurally undefined)
Asn108Trp W99F/W145Y	90-120	In conformationally dynamic region (structurally undefined)
Met134Trp W99F/W145Y	119-136	Internal residue in coil region, contacts H3
Ser135Trp W99F/W145Y	119-136	Surface residue in coil region
Asn170Trp W99F/W145Y	166-179	Surface residue in loop near H2 and H3
His177Trp W99F/W145Y	166-179	Surface residue in H2
Gln212Trp W99F/W145Y	200-223	Surface residue in H3
Lys220Trp W99F/W145Y	200-223	Surface residue in H3
Tyr150Trp W99F/W145Y	H1 143-156	Internal residue in H1; suggested to become solvent exposed after conversion(*5*)
Tyr163Trp W99F/W145Y	158-165	Partially exposed residue in loop near H2 and H3 suggested to become more solvent exposed after conversion(*5*)

Purification: We have modified the published purification scheme(*3*) to increase the yield of truncated PrP. Inclusion bodies from 1 liter culture are solubilized in 50 mL 0.1M Na_2HPO_4, 0.01M Tris, pH 8 buffer containing 8M urea. The protein is then batch-bound to a Ni(II)-loaded immobilized metal affinity matrix (IMAC, Pharmacia) in the same buffer. The protein-bound matrix is then loaded into a 5x5 cm column and washed with 5 column volumes of TCB (20mM TrisCl, 150 mM NaCl, 2.5 mM $CaCl_2$ pH 8) + 8 M urea. Next, PrP is re-folded on the column by using a linear urea gradient in TCB (8 → 0 M Urea, 20X column volume). After washing with 5 column volumes of TCB, PrP is eluted with TCB + 60 mM imidazole. The purified protein is then concentrated to about 4 mg/mL and dialyzed into 10 mM TrisCl pH 7.5 for storage. On the rare occasions when PrP is less than >95% pure, a Source-S cation exchange column is used to achieve the desired level of purity, as judged by SDS/PAGE on a Pharmacia Phast System (Figure 1).

Tasks 1 (b-d) For each mutant: (b) determine the stability of the α-structure by CD (circular dichroism) temperature unfolding experiments; (c) determine the solvent exposure of the introduced Trp residue by fluorescence spectroscopy; and (d) determine if conversion to a soluble β-structure changes the solvent exposure by fluorescence spectroscopy.

Figure 1. SDS/PAGE of truncated W99F PrP. Lane 2, inclusion bodies; Lane 3, 60 mM imidazole fraction from IMAC; Lane 1, MW markers.



Determination of the concentrations of PrP variants: The protein concentration of PrP variants is routinely determined using the Bradford Dye Assay (Biorad, BSA standard). This is necessary because the ε_{280} of each mutant will vary with the Trp content and local environment. We have also compared the dye assay results for converted β-PrP before and after unfolding in 10 M urea. No differences were noted, which indicates that the dye is able to fully interact with converted PrP under the assay conditions used.

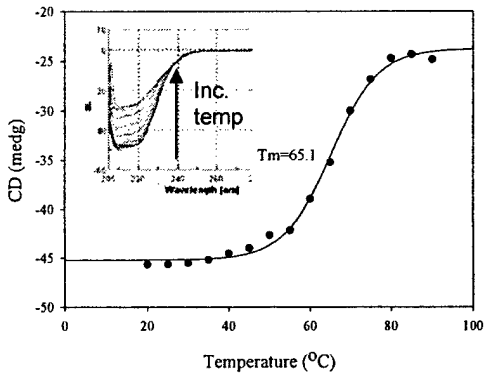
Conversion Method: We have tried several methods to convert α-PrP to an aggregated β-form, based on the method described by Baskakov et al.(*6*) α-PrP is first unfolded in 20 mM Na-Acetate, 0.2M NaCl, pH 3.7 + 10M Urea. The sample is then diluted to 5M urea in the same buffer and incubated at room temperature for 2 days. Afterwards, the urea is removed by dialysis into urea-free buffer (pH 3.7 or 5.5) at 4°C. Using this method, PrP is converted with ~100% efficiency and is stable for weeks. However, if the incubation in 5M urea is performed at pH 6.9 or higher, the efficiency decreases to about 75% (see Figure 2). If the converted protein is dialyzed into a high pH buffer (≥ 6.9), then the β-form partially reverts to α-PrP. This is consistent with published results that claim the α and β forms of PrP are in equilibrium with one another, and that the conversion of α-PrP is facilitated by acidic conditions.(*6*)

Spectroscopic Techniques: The thermal denaturation profile of each mutant was measured, to assess the effects of mutation on the overall structural stability of the α-form. The thermal unfolding was monitored by Circular Dichroism spectroscopy (CD) and the melting temperature (T_M) was determined at 222 nm. The CD instrument is a Jasco 810 fitted with a peltier cell holder and scanning fluorescence detector. All mutants examined thus far exhibit only minor deviation from simple two-state unfolding (Figure 2) and have similar T_M values (Table 2), indicating that the mutations do not affect global stability. The data are consistent with the published T_M for full-length PrP.(*7*) Unless otherwise stated, the conditions for all spectroscopic measurements are 20 mM Na-Acetate, 0.2 M NaCl, pH 5.5 at 25°C. Protein concentrations varied from 1.5 – 3.5 μM and were normalized to 2.3 μM for comparison. CD measurements were collected using 1 nm data pitch, 200 nm/min scan speed, 2 nm band width, 1 sec response, in a 1cm path cuvet. Thermal denaturation curves were determined every 5° from 20 – 90°C at 222 nm (α-helix peak) using a temperature slope of 2°C/minute.

Table 2. Thermal denaturation of α-PrP mutants.

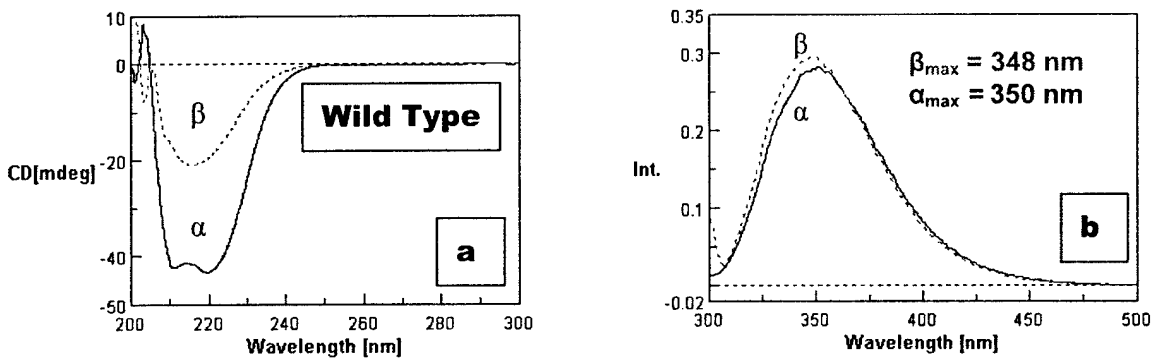
Protein	T_M (± 0.3 °C)
Wild type	65.8
W145Y	65.3
W99F	65.5
W99F/W145Y	65.1
W99F/W145Y/Y218W	67.7
Wild type – with 50% 7-azaTrp	63.7

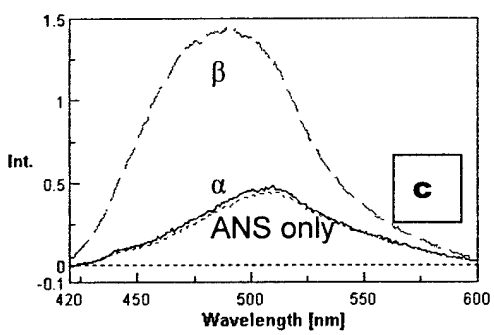
Figure 2. T_M plot and thermal denaturation curves (inset) of W99F/W145Y.



The conversion process has also been confirmed by CD spectroscopy, which detects changes in the α-helical content of proteins. A typical result is shown in Figure 3(a). The monomeric α-helical form displays a CD spectrum with two negative peaks; the bands at 222 and 210 nm indicate a large α-helical content.(*8*) Upon conversion, there is a dramatic change in the CD spectrum. Converted β-PrP displays a single negative band with λ_{max} at 218 nm. This is best understood as representing a structure in with there has been both a decrease in the overall helicity and an increase in β-sheet content. Although it is difficult to quantify the β-sheet content from CD measurements, β-sheets typically have maximal CD peaks near 215 nm. Note the differences in the CD spectra of unfolded, α-PrP (Figure 2, inset) and converted β-PrP (Figure 3); the CD spectrum of unfolded PrP is more consistent with a random coil structure than β-sheet.

Figure 3. Characterization of 2.3 μM wild type truncated PrP in α (blue/solid) and β (green/dashed) forms. (a) CD spectra; (b) Trp fluorescence; (c) ANS fluorescence binding assay - ANS alone is shown in red/dots.





The conversion also been confirmed through by reacting samples with 5-anilino-1-napthalenesulfonic acid (ANS). ANS binds to β-PrP but not to either the α-form or to denatured PrP.(*6*) Binding is measured as an increase and blue shift in fluorescence, as depicted in Figure 3c. We have purchased a scanning fluorescence detector for the CD instrument, using funds provided to the PI by the university. Hence, fluorescence and CD spectra are recorded on samples under identical conditions within minutes, rather than as separate experiments on different days.

The fluorescence emission spectra of the α- and β-forms of each PrP variant were measured after excitation at 295 nm (conditions: 2 or 5 nm bandwidth, 1nm data pitch, 0.25 sec response, 1 cm path length cuvet). The wild type protein, which contains two Trp at 99 and 145, shows little change in fluorescence upon conversion (Figure 3b). This might be interpreted as meaning that neither W99 nor W145 undergo significant changes in solvent exposure after conversion. However, an examination of the single Trp mutants, W145Y in Figure 4 and W99F in Figure 5, shows that the situation is far more complex. Trp99, which resides in the unstructured segment of α-PrP, shows a marked decrease in fluorescence intensity after conversion (Figure 4). In contrast, Trp145, Figure 5 undergoes an increase in intensity and a slight blue shift in fluorescence after conversion. In general, blue-shifted bands (< 340 nm) indicate that Trp is in a hydrophobic pocket, whereas peak maxima near 355 nm suggest that Trp is in a highly solvated, exposed environment. It should be noted that both the quantum yield

and wavelength maximum of Trp fluorescence also depends on local polarization effects caused by water (solvent) and protein dipoles.(*9*) The spectra of the W99F and W145Y mutants suggest that both Trp residues undergo changes in local environment. The similarity of the fluorescence spectra before and after conversion reflects the sum of two opposite effects from the individual Trp residues.

Figure 4. Characterization of 2.3 μM W145Y PrP (Trp at 99) in α (blue/solid) and β (green/dashed) forms. (a) CD spectra; (b) Trp fluorescence.

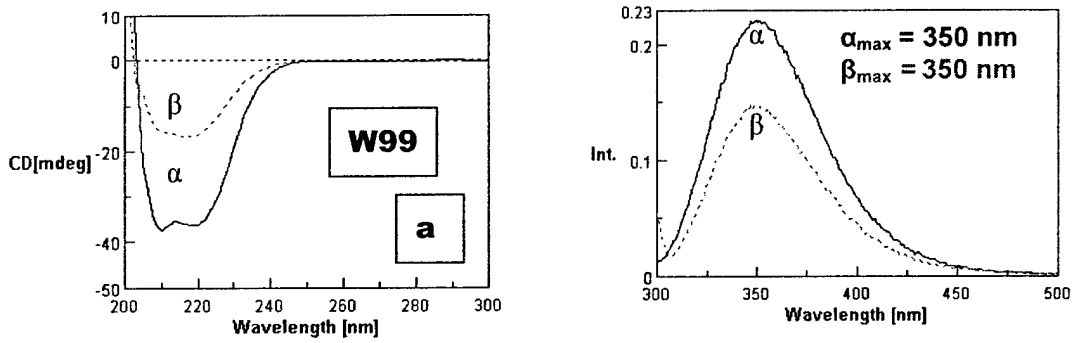
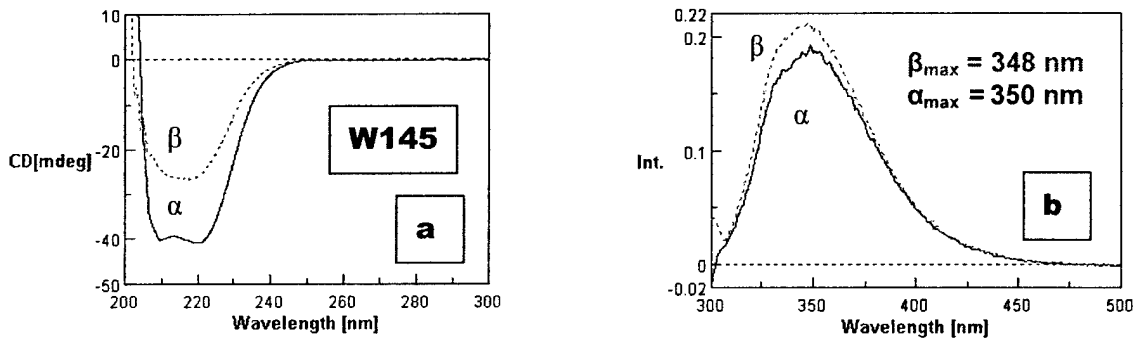


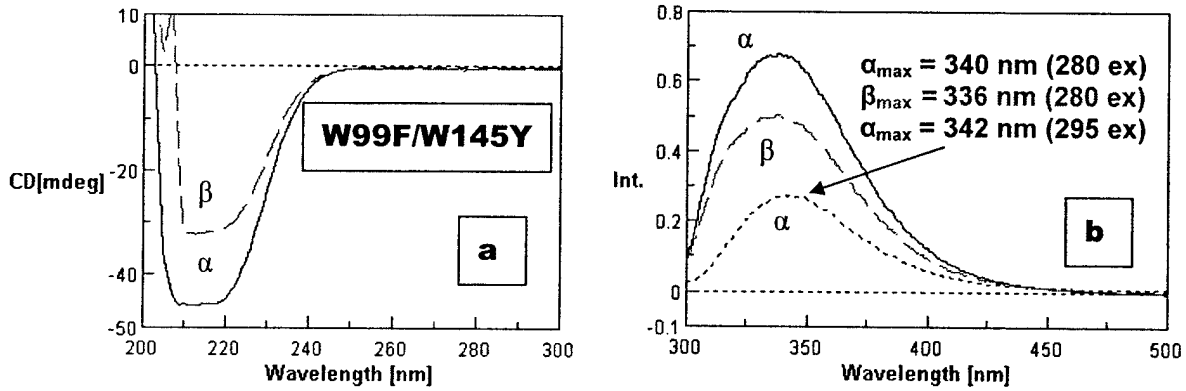
Figure 5. Characterization of 2.3 μM W99F (Trp at 145) PrP in α (blue/solid) and β (green/dashed) forms. (a) CD spectra; (b) Trp fluorescence. Note that the deconvolution analysis of the CD spectra indicates that only ~75% of W145Y has converted to ß-form.



We have used the Trp-free W99F/W145Y double mutant as a control protein. As shown in Figure 6, the high Tyr content (~8%) of truncated PrP makes a significant contribution to the overall fluorescence emission spectrum with 295 nm excitation, even when the bandwidth is decreased from 5 nm to 2 nm. This suggests the need to subtract the tyrosine fluorescence component from the spectra of the Trp mutants, to clarify the fluorescence changes that are attributable to Trp. In cases where a Tyr→Trp mutation occurs, such as W99F/W145Y/**Y218W**, the most rigorous control possible would be a mutant containing the analogous Tyr→Ala mutation, e.g., W99F/W145Y/**Y218A**. These control mutants will be constructed if future experiments warrant it.

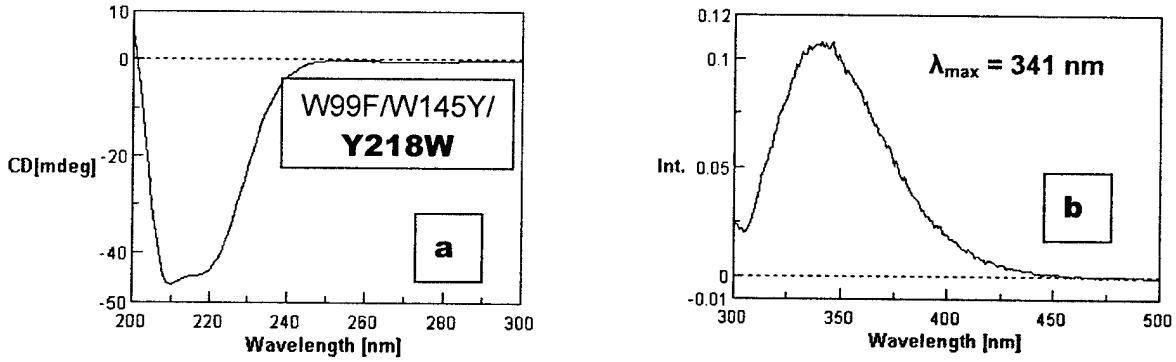
The finding that Tyr fluorescence substantially contributes to the 295 nm-excitation emission spectrum of PrP motivated us to pursue analog incorporation sooner than we had planned. The red-shifted absorbance spectrum of 7-AzaTrp allows selective excitation of this analog. The fluorescence emission spectrum of wild type PrP containing 7-AzaTrp (310 nm excitation) is discussed in detail under the section entitled: ***Accomplishments related to Task 2.***

Figure 6. Characterization of Trp-free 2.3 μM W99F/W145Y PrP in α (blue) and β (green) forms. Note that the deconvolution analysis of the CD spectra indicates that only ~75% has converted to β-form. (a) CD spectra; (b) Fluorescence emission spectra with excitation at 280 nm (maximal excitation of Tyr) and 295 nm (control experiment); α/280 nm excitation, solid blue; α/295 nm excitation, dotted blue; β/280 nm excitation, dashed green.



We have also characterized the α-form of PrP containing a single Trp at 218 (W99F/W145Y/Y218W); the CD and fluorescence spectra are shown in Figure 7. The published structure of α-PrP places Y218 in a hydrophobic environment, which agrees with the blue shifted Trp fluorescence band of the Y218W mutant. We plan to convert this mutant to the β-form in the near future.

Figure7. Characterization of 2.3 μM W99F/W145Y/Y218W PrP in α form. (a) CD spectra; (b) Trp fluorescence.



We would like to more thoroughly characterize the aggregated β-forms produced by our conversion method. In the future, we plan to use size exclusion chromatography, fluorescence anisotropy, and ultracentrifugation techniques in conjunction with wet biochemistry to assess the dimensions, oligomeric state, and total β-content of converted PrP. We also plan to examine the samples by infrared spectroscopy, which will allow us to more accurately assess the β-sheet content of the samples.(*10*) These techniques are available in the PI lab or through collaboration with members of the newly formed Center for Biomolecular Structure and Dynamics at the University of Montana. The PI is a founding member of this center.

Task 1e. For each mutant, determine if copper or manganese binding influences fluorescent spectrum of either the soluble α-form or soluble β-form.

We will begin these experiments in Year 2 (May 15, 2004 – May 14, 2005).

Accomplishments related to Task 2. **Create a 7-AzaTrp-substituted PrP protein as a reporter protein that may be used to detect infectious PrP in the field. (Months 25-36)**

Although the Statement of Work indicates that these experiments will be done in Year 3, we felt the project would be enhanced if we began parts **a** and **b** of this task sooner than planned. Through characterization of the Trp-free double mutant W99F/W135Y, we have shown that tyrosine residues of PrP contribute substantially to the fluorescence emission spectrum when the sample is excited at 295 nm. Hence, any spectral changes that occur upon conversion will reflect structural changes in Tyr as well as Trp residues. To elucidate the Trp contribution, we decided to incorporate 7-azaTrp into PrP in lieu of normal Trp. This amino acid analog has a red-shifted absorption spectrum compared with Trp. Excitation at 310 nm allows 7-AzaTrp to be selectively excited, with little to no contribution from natural amino acids. Moreover, 7-AzaTrp is exquisitely sensitive to its local solvent environment (Table 3) and exhibits long lifetimes.(2)

2a) Based on the results of Task 1, one or more Trp-substituted sequence positions of the truncated PrP will be expressed in a Trp auxotroph strain of *E. coli*, to incorporate 7-aza-tryptophan in lieu of the normal amino acid.

The yield of Trp analog incorporation into recombinant proteins depends on the use of Trp-auxotrophic *E. coli* strains. We expressed wild type truncated PrP using the pET expression vector and CT19(DE3) cells (genotype *aspC, ilvE, tyrB, avtA, trpB*; auxotrophic for Asp, Ile, Leu,Phe, Tyr, and Trp, provided by DS Waugh). The pRARE plasmid from Novagen was also included, to eliminate codon bias. Cells were grown to OD_{600} = 2.0 in media containing CAS amino acids supplemented with 50 mg/L L-tryptophan. The cells were then centrifuged and washed twice with fresh media contaiing 7-azaTrp in place of Trp. The culture was induced with IPTG, which induces the production of T7 RNA polymerase in the host. This polymerase then transcribes the *prp* gene on the expression plasmid.

Protein expression levels were much lower than for the normal BL21(DE3) host strain – only ~ 2 mg/L of culture. After purification, a trypsin digest of the protein was analyzed by electrospray mass spectrometry. The results indicated a 50/50 mixture of normal peptides and analog-substituted peptides. (7-AzaTrp is +2 mass units larger than Trp at acidic pH). The analog was evenly distributed between the W99 and W145 tryptic peptides.

The most likely source of the low levels of overall expression and analog incorporation is the use of the pET-based T7 promoter system. In the pET system, the genomic copy of T7 RNA polymerase is controlled by a *lac* promoter and is primarily expressed when IPTG is added, although some leaky expression occurs during cell growth. The T7 RNA polymerase then transcribes the plasmid-borne *prp,* which is regulated by the T7 promoter. Prior to induction, any "leaked" T7 RNA polymerase present would produce a small amount PrP containing natural Trp. At the induction stage, the media contains 100% 7-AzaTrp and no natural Trp, so any PrP that is made post-induction will contain 7-AzaTrp. Unfortunately, all RNA polymerases are severely impaired when their own Trp residues are replaced with this analog.(2) Therefore, any T7 RNA polymerase expressed upon induction is non-functional. The amount of analog-containing PrP produced after induction and media swapping is limited to that attributable to the action of the normal T7 RNA polymerase leaked by the cell during the pre-induction phase.

Other expression systems are based on directly inducible promoters such as the *lac, tac,* and *trc* promoters, or the *T5/lac operator* promoter. These are inducible with IPTG, but do not rely on the induction of a specific RNA polymerase for transcription. We opted to try the *T5/lac operator*-based pQE60 expression vector (Qiagen). Although no problems were encountered in the cloning techniques used, the plasmid did not express PrP in any of the host cell strains used. We plan to clone *prp* into a vector using a *lac*-based promoter later this summer. We are optimistic that the proper choice of expression vector will achieve high levels of protein expression (> 5 mg/L) containing >90% analog incorporation. However, the 50% incorporation

level achieved with the pET system and *E. coli* host CT19 are sufficient to allow us to accomplish all of the planned work.

2b) The fluorescence spectrum of this substrate protein will be determined in its soluble α- and β-forms.

Although we have not yet created a substrate reported protein, we have investigated the spectral properties of wild type PrP doped with 7-AzaTrp. The fluorescence emission spectra of wild type α-PrP containing 0% and 50% 7-AzaTrp are displayed in Figure 8. Excitation with 295 nm light (solid lines) yields large signals for both samples. In contrast, excitation at 310 nm (dashed lines) allow the selective excitation of 7-AzaTrp (7AW), since neither Trp nor Tyr absorb at 310 nm. A comparison of the α- and β-forms of PrP containing 7-AzaTrp (310 nm excitation) is shown in Figure 9. Here the change in the local solvent environments of 7-AzaTrp (either 7AW99, 7AW145, or both) after conversion is much more evident. The emission peak maxima shifts only slightly from 378 nm (α) to 380 nm (β), but the quantum yield drops about 40%. The peak maxima suggest that the average 7-AzaTrp environments remain quite solvent exposed (see Table 3). Additional data were collected on the pH dependence of the β-form (Figure 10). The data are consistent with the N-7 of 7-AzaTrp becoming protonated upon conversion, for at least one of the residues. The characterization of the single-Trp mutants (W99F, W145Y, and other proposed mutants in Table 1) containing 7-AzaTrp will be necessary to interpret spectral changes with regards to unique sequence positions.

Figure 8. Fluorescence emission spectra of 2.3 μM α-PrP. Solid lines are from excitation at 295 nm; dashed lines are from excitation at 310 nm. Red lines are PrP containing normal Trp; blue lines are PrP containing ~50% 7AzaTrp.

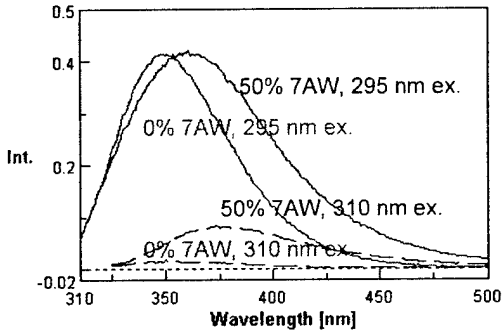


Figure 9. Fluorescence emission spectra (310 nm excitation) of 2.3 μM α-PrP (solid blue) and β-PrP (dashed green) containing 50% 7-AzaTrp.

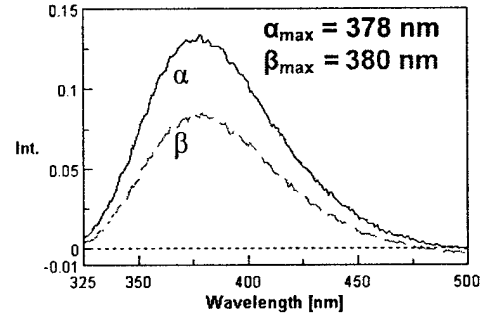
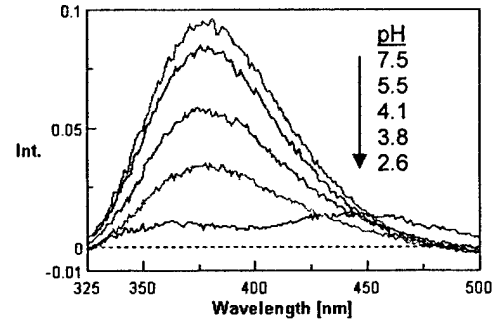


Table 3. Fluorescence of 7-AzaTrp in different solvents, from (*2*). The anomalous result for ethanol is believed to be a mixture of 7-AzaTrp and its tautomer, which is catalyzed by protonation and/or hydrogen bonding of solvent to the two indole nitrogens.

Solvent	λ_{max} (nm)	Quantum Yield
Water pH 7	412	0.01
Ethanol	370	0.004
Acetonitrile	370	0.34
Dioxane	360	0.06

Figure 10. The pH dependence of β-PrP containing 50% 7-AzaTrp at W99 and W145.



These preliminary data show the utility of 7-AzaTrp incorporation for following structural changes associated with conversion and/or aggregation of PrP. The red-shifted absorbance spectrum of this analog allows it to be selectively excited in the presence of natural amino acids. Additionally, the emission spectrum of 7-AzaTrp exhibits large spectral and quantum yield shifts, depending on the polarity and pH of its local environment.

The solvent environment of a particular residue may differ among the monomers within the ~9-mer β-aggregates that we produce *in vitro* for fluorescence experiments. We will address the nature of the β-aggregate by characterizing a series of β-PrP samples made with varying ratios of analog-containing PrP and wild type PrP. These experiments are precursors to our use of FRET (fluorescence resonance energy transfer) techniques planned for future years.

2c, 2d) The substrate protein will be exposed to an aggregated, insoluble β-form of PrP (PrP-res) produced by *in vitro* techniques. This will mimic the conversion of the substrate protein by infectious PrP. The overall yield and time course of conformational changes in the substrate protein will be determined by monitoring changes in the 7-azatryptophan fluorescence.

No work has yet been done on these aspects of Task 2.

KEY RESEARCH ACCOMPLISHMENTS

- Produced, purified, and characterized five mutants of Syrian hamster prion protein
- Measured the fluorescence emission spectra of these mutants in their normal monomeric α-form
- Modified a published procedure for conversion of α-prion protein to a soluble β-form aggregate, to be used as a model of infectious prion protein
- Determined the fluorescence emission spectra of 4 mutants after conversion to the β-form
- Determined that Trp at positions 99 and 145 contribute differently to the overall fluorescence changes observed upon conversion.
- Produced prion protein doped with 7-AzaTrp-doped at ~50% incorporation
- Determined the fluorescence spectra
- Demonstrated the utility of 7-AzaTrp as a reporter of conformational change during PrP conversion

REPORTABLE OUTCOMES:

Poster Presentation, **International Conference on Prion Diseases,** Munich Germany. October 2003. *Characterization of Truncated Prion Protein Mutants.* **Michele McGuirl,** Sam Chelmo, and Shiloh Small.

Oral Presentation, The **University of Montana CAS Graduate Student Conference**, Missoula, MT. April 2003. Prion Diseases: Protein Wrecks on the Highway of Life. **Michele McGuirl**

One oral and two poster presentations at various state, regional, and national high school scientific conferences. Prion Transformers: from Normal Proteins to Deadly Disease. **Alex Hughson, Jay Rutherford**, Jim Harkins, and Michele McGuirl.

CONCLUSIONS:

We have made substantial progress toward determining key residues of the prion protein that undergo structural perturbations upon conversion to the disease-causing conformation. Except for lifetime and FRET measurements, all necessary methodology for future experiments has been developed. We hope to improve the level of analog incorporation into prion protein, to facilitate future experiments. To accomplish this, we have added another researcher to the laboratory. Ms. Gray is experienced in site-directed mutagenesis and recombinant protein expression in bacteria and insect cells.

Once we have completed the work and have elucidated the key residues, we will design substrate peptide that contains 7-AzaTrp. Through fluorescence changes that occur upon conversion of the substrate by diseased but not normal prion protein, the substrate may be used to detect the presence of disease. The work will also give much-needed structural information about the conformational changes that occur upon conversion and may help design drugs to prevent the disease from propagating.

REFERENCES:

1. B. Caughey *et al.*, *Adv Protein Chem* **57**, 139 (2001).
2. J. B. Ross, A. G. Szabo, C. W. Hogue, *Methods Enzymol* **278**, 151 (1997).
3. R. Zahn, C. von Schroetter, K. Wuthrich, *FEBS Lett* **417**, 400 (Nov 17, 1997).
4. M. P. Morrissey, E. I. Shakhnovich, *Proc Natl Acad Sci U S A* **96**, 11293 (Sep 28, 1999).
5. E. Paramithiotis *et al.*, *Nat Med* **9**, 893 (Jul, 2003).
6. I. V. Baskakov, G. Legname, M. A. Baldwin, S. B. Prusiner, F. E. Cohen, *J Biol Chem* **277**, 21140 (Jun 14, 2002).
7. J. O. Speare, T. S. Rush, 3rd, M. E. Bloom, B. Caughey, *J Biol Chem* **278**, 12522 (Apr 4, 2003).
8. P. Bayley, in *An Introduction to Spectroscopy for Biochemists* S. B. Brown, Ed. (Academic Press, New York, 1980) pp. 148-234.
9. J. T. Vivian, P. R. Callis, *Biophys J* **80**, 2093 (May, 2001).
10. B. W. Caughey *et al.*, *Biochemistry* **30**, 7672 (Aug 6, 1991).

MANUSCRIPTS/REPRINTS, ABSTRACTS

none

APPENDICES

none