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Detection and Discrimination of PrP^{Sc} by Multi-spectral Ultraviolet Fluorescence

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Prion diseases are progressive degenerative disorders of the central nervous system. The transmissibility and fatal nature of these diseases necessitate their rapid and accurate diagnosis. The hallmark of these diseases is the accumulation of PrP^{Sc}, a protease-resistant form of a host-coded glycoprotein. We have been evaluating the use of multi-spectral ultraviolet fluorescent spectroscopy as a means of detecting and distinguishing between different forms of PrP^{Sc}. Spectroscopic measurements of fluorescence from untreated and proteinase K (PK)-treated PrP^{Sc}, purified from 263K scrapie strain-infected hamster brains and ME7 scrapie strain-infected mouse brains, were performed. Spectra of untreated and PK-treated PrP^{Sc} samples for 263K and ME7 appeared qualitatively different. The identification and discrimination of PrP^{Sc} were possible based on these spectral signatures, calculations of their fluorescence cross sections, and determination of the orthogonal differences. This technique has the potential not only for the sensitive, specific, and direct detection of PrP^{Sc}, but also for the ability to distinguish between different forms of the prion protein.

Prion diseases, which includes Creutzfeldt-Jakob disease (CJD) in humans, sheep scrapie, and bovine spongiform encephalopathy of cattle, are transmissible neurodegenerative diseases characterized by the accumulation in the central nervous system of the protease resistant isoform (PrP^{Sc}) of a host-coded membrane glycoprotein (PrP^{C}). The formation of PrP^{Sc} involves a conformational change in PrP^{C} from an α-helix to a β-pleated sheet dominated structure. The existence of agent strains is a well established phenomena. Strain differences in clinical manifestations and histopathological changes have been demonstrated in the natural hosts, sheep and goats (1). In addition, using laboratory animals, numerous scrapie strains have been identified which can be distinguished biologically by their species tropism, incubation periods, histopathological changes, clinical characteristics, and PrP^{Sc} distribution in the brain tissue (2, 3, 4). In some cases, these agent strains differ with respect to their PrP^{Sc} properties. Strain differences in PrP^{Sc} have been described for scrapie (5), CJD (6), and hamster adapted transmissible mink encephalopathy (7). There exists a variety of PrP-related features which have been reported as specific markers for individual scrapie strains. These markers include Western blot profile, proteinase K (PK) sensitivity and lectin binding (5,8). Conformational differences in PrP^{Sc} associated with the different scrapie strains strongly influences the properties of the protein (8). However, although these parameters have been useful in distinguishing between a variety of different agent strains, numerous strains cannot be identified by these criteria, despite wide variations in their biological properties (8).

The availability of polyclonal and monoclonal antibodies to PrP^{Sc} has been very useful for the immunodiagnosis of prion diseases (9, 10). The majority of PrP^{Sc} antigenic sites are species-directed and involve non-self sites. Furthermore, the availability of these antigenic sites for antibody binding is restricted by the conformation of PrP. In addition to these factors which limit epitope availability, the antibodies to PrP react with both PrP^{Sc} and PrP^{C}. However, a recent report has suggested the possibility of generating antibodies which are specific to PrP^{Sc} (11).

The development of non-immunologic techniques for PrP detection might eliminate some of the limitations associated with immunodiagnostic procedures. Spectroscopic methods have been used for the rapid detection and analysis of biological samples under mild conditions (12). The optical sensing of samples using more than one wavelength of light to excite the samples, which for these studies is in the ultraviolet range, is known as multi-spectral ultraviolet fluorescence spectroscopy (MUFS). MUFS may be a useful tool not only for the detection of, but also the differentiation be-

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tween, PrP^{Sc} from different scrapie strains. With MUFS, a sample is sequentially illuminated with monochromatic light at chosen wavelengths, λ_{exc}, in the ultraviolet spectrum, and then the fluorescence is measured over a finite bandwidth, λ_{flr}, thus building up a two dimensional spectral signature. With this technique, spectra from unknown samples can be analyzed by multivariate techniques to determine the presence and concentration of chemicals and proteins for which known database spectra have been acquired (13). For proteins, the UV fluorescence signal is mainly influenced by the presence of aromatic amino acid residues (tryptophan, phenylalanine, tyrosine), with the fine structure of the signature due to the relative amounts of these residues and their local environment (14) (i.e. nearest neighbor effects of the residues, and exposure of the residues to water through folding of the protein).

There are two issues that must be addressed to allow one to determine whether detectability of a chemical or protein from its UV fluorescence signature will be feasible. First, the fluorescence cross section for the protein must be sufficiently large. This quantity which indicates how large the returned fluorescence signal (in number of photons) will be for a given flux of excitation light, of a given wavelength, is a fundamental property of the protein. Second, differences in the shape of the spectral signatures must be sufficiently large to allow them to be distinguishable. As noted above, the signatures from proteins tend to be broad and diffuse. As a result, signatures from different molecules tend to have features which are shared and overlap. Thus, only a fraction of the fluorescence signal may contain information which can be exploited to detect or identify a molecule in a mixture or against its natural background.

Our data are consistent with the hypothesis that optical sensing can be used not only as a rapid and sensitive method for detecting PrP^{Sc}, but also for distinguishing between this protein from different species.

MATERIALS AND METHODS

Sample preparations. The preparation of inoculum, injection, scoring, and sacrificing of animals were performed as previously described (15, 16). Partially purified fractions of PrP^{Sc} were isolated from the brains of 263K scrapie strain infected hamsters or ME7 scrapie strain infected mice, using a modification (17) of the procedure originally described by Hilmert and Diringer (18). In brief, a 10% homogenate of 12 gm of brain tissue was prepared with 10% sarcosyl in TBS (10 mM Tris-HCl, 133 mM NaCl, pH 7.4). The supernatant was centrifuged at 22,000 ×g for 30 min. at 20° C, followed by ultracentrifugation (200,000 × g for 2 hrs. at 4°C). The resulting pellet was resuspended in TBS/10% Sulfobetaine 3-14 (SB3-14) and centrifuged through a 20% sucrose cushion (210,000 × g for 2 hrs. at 4°C). The resulting pellet was resuspended in TBS/0.1% sarcosyl and incubated with either PK (100 μg/ml) or TBS for 2 hours at 37°C. Following the addition of phenylmethylsulfonyl fluoride to a final concentration of 10 mM, the sample was layered over a 20% sucrose cushion and centrifuged in a Beckman table top ultracentrifuge at 250,000 × g for 2 hours. The final pellet was resuspended in 20 μl of TBS/0.1% SB3-14 per gram of original brain material. The PrP^{Sc} preparations were analyzed and quantitated by SDS-polyacrylamide gel electrophoresis and Western blotting as previously described (19). The PrP^{Sc} concentration in each sample prior to MUFS was approximately 3 μg/ml.

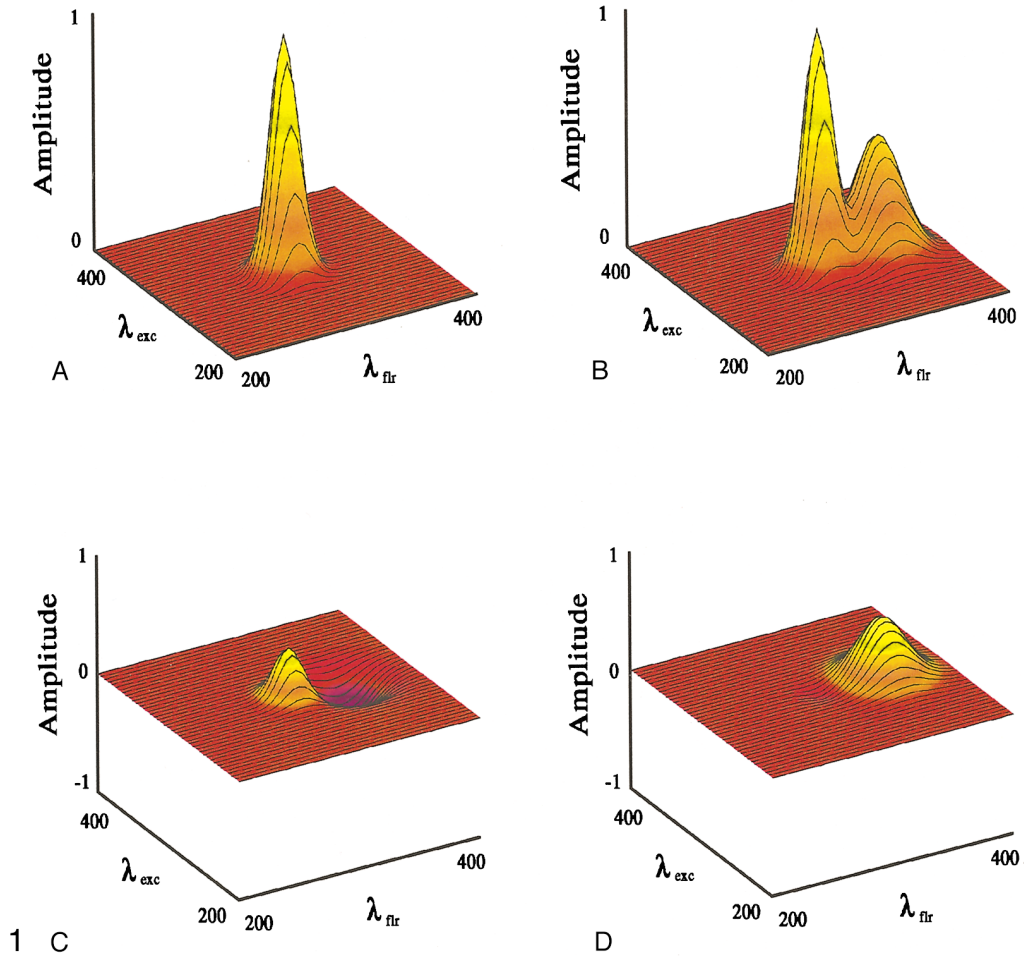
Fluorometer. The excitation source for the system was a 150 watt high pressure Xe arc lamp (Oriel Model 68805 arc lamp with Model 6254 Xenon bulb). The white light from the lamp was focused into an F/4, 1/8 meter double monochromator (CVI Model 120, 200-400 nm range, 5 nm steps, 7 nm bandwidth) in order to reduce the scattered light outside of the pass band of the monochromator. The light from the monochromator was then imaged into the center of a RF-3010-T FUV ultraviolet quartz sample cell (Spectrocell, Oreland, PA). The scattered light and the fluorescence from the material in the cell was detected at 90 degrees to the incident excitation source. The light from the excitation region was imaged into the entrance slit of an F/4, 1/8 meter imaging spectrometer (200-700 nm range, 5 nm resolution) (ISA Jobin Yvon, Edison, NJ). At the image plane the light was detected by a 1024 element intensified reticon array (Model 1420, EG&G Princeton Applied Research, Trenton, NJ). The resolution of the detection spectrometer was 5 nm. The response of the detector system in the spectral range of 200 to 400 nm was determined by using a calibrated D_2 lamp. The spectrum of this lamp was calibrated by the manufacturer with reference to NIST standards. In addition, the relative output of the Xe lamp through the monochromator was measured using an Ophir Model PD-300-UV calorimeter. Based on the sensitivity of the test apparatus we are currently using and the number of aromatic amino acid residues in PrP (18), detection should be possible by UV fluorescence techniques coupled with multivariate analysis.

Calculations of fluorescence cross sections. The water Raman line in the TBS control spectrum was a useful standard to monitor the operation of the system. The absolute cross section of the protein was estimated by comparing the measured spectrum, corrected for excitation intensity and detector response, with the water Raman scattering signal calibrated to measured values for the Raman cross section (20, S. Christesen-personal communication).

Calculations of orthogonal differences. Orthogonal analysis was performed as described (12). The data from the two spectra under consideration were masked to include the fluorescence lying between the first and second order elastically scattered light so that differences computed represent the information in the fluorescence and Raman scatter alone.

RESULTS AND DISCUSSION

A central issue to the spectroscopic detection of chemical compounds is the determination of the amount of information carried by an individual compounds spectrum, and the degree to which it is unique to that compound. This information determines how much of the spectral signature can be used to detect the compound unambiguously in a sample. It also gives a true measure of the signal to noise ratio (SN) for a particular instrument. The SN for an instrument is usually quoted in terms of the signal above the fluctuation noise for the instrument. However, if only a fraction of the spectral information is unique to the compound of interest, then the SN should be multiplied by this factor and the effective sensitivity for the detection of the compound can be orders of magnitude lower than expected from quoted SN. Our approach in dealing with



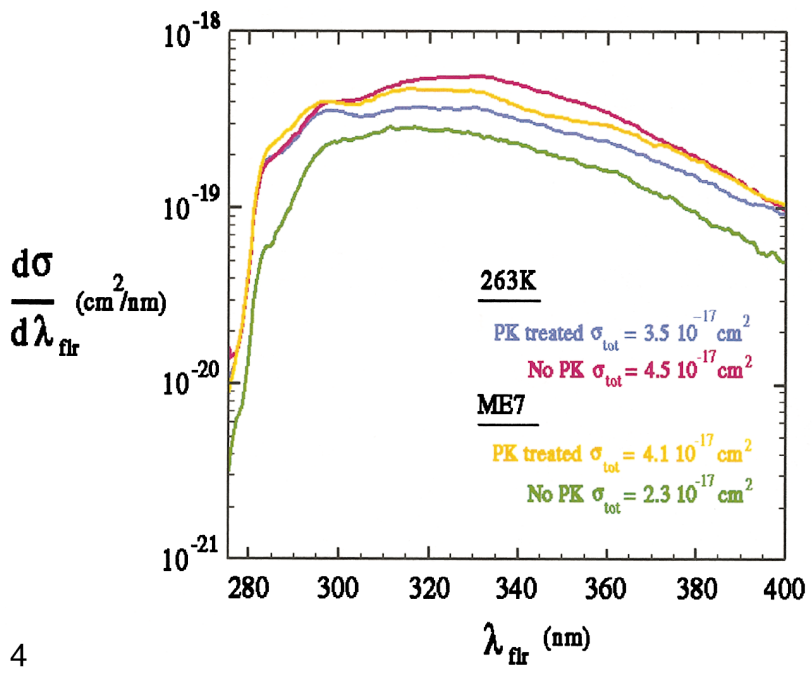
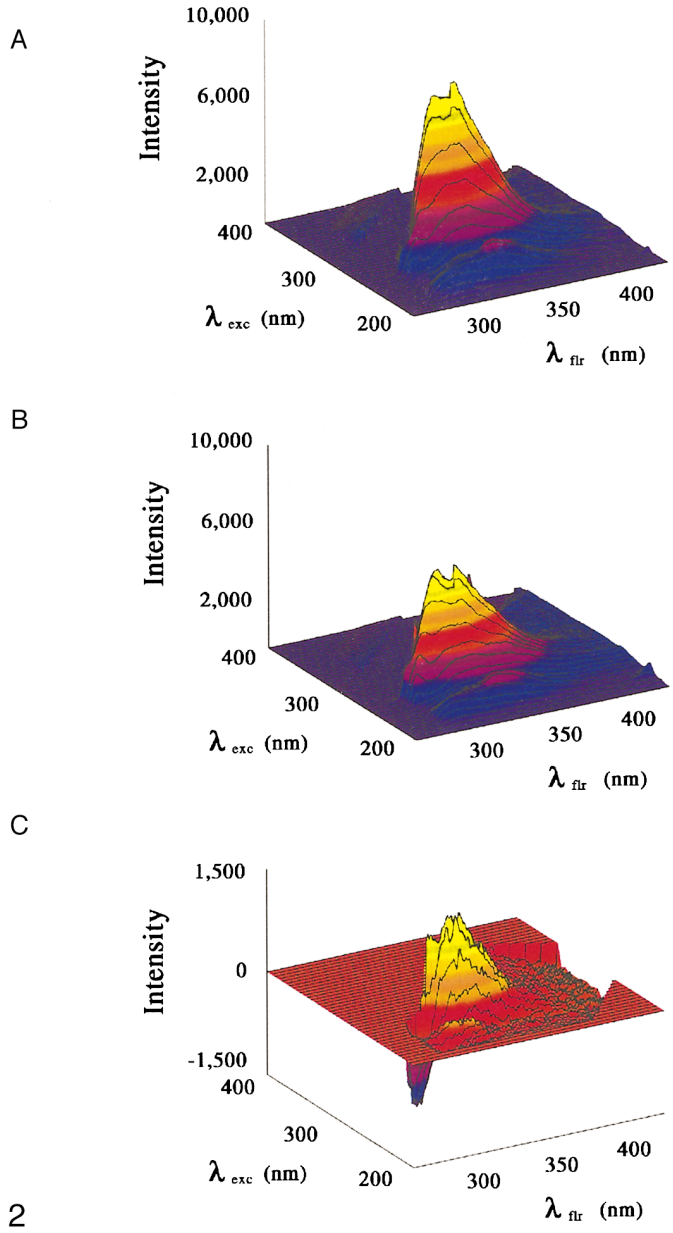


FIG. 1. Orthogonal differences computed for synthetic spectra consisting of a single Gaussian (A), the sum of two Gaussian (a second added to A) (B), and the differences computed for A in terms of B (C) and B in terms of A (D).

FIG. 4. Differential and total fluorescence cross section (s_{fl}) for untreated and PK-treated PrP^{Sc} from 263K-infected hamsters and ME7-infected mice calculated at an excitation wavelength of 290 nm.



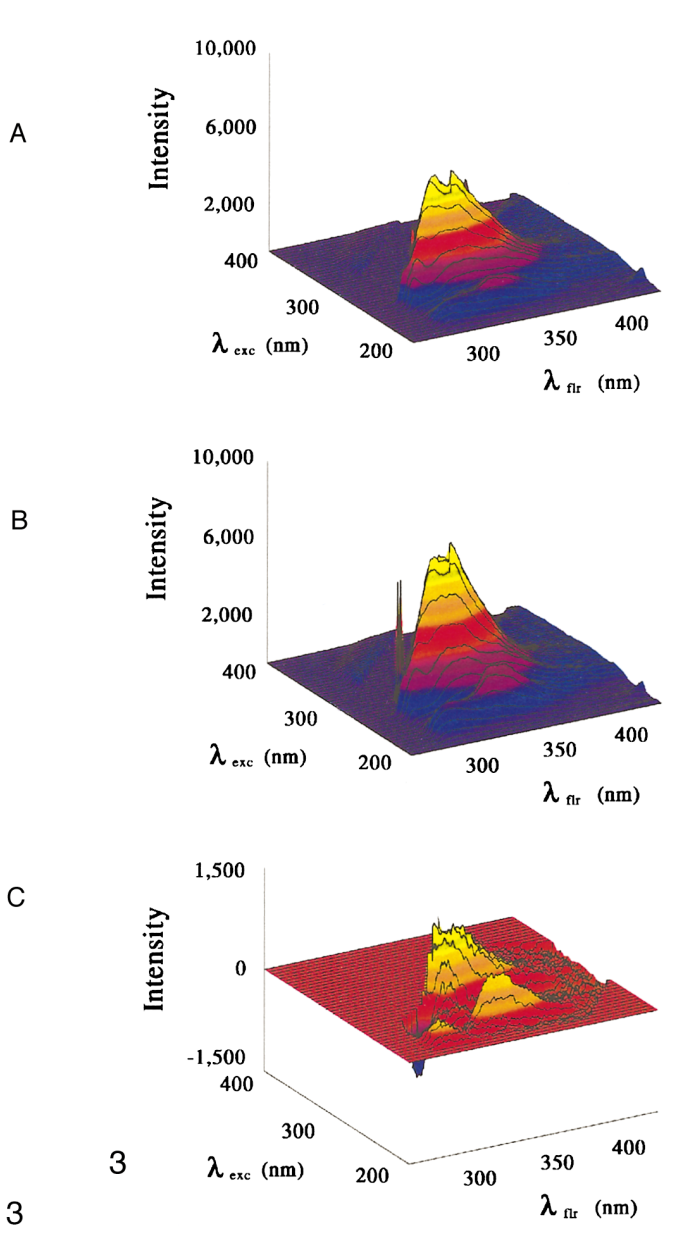


FIG. 2. Spectra of PrP^{Sc} from 263K-infected hamsters in the absence (A) or presence (B) of PK treatment, and the orthogonal differences calculated between the two spectra (C).

FIG. 3. Spectra of PK-treated PrP^{Sc} from 263K-infected hamsters (A), ME7-infected mice (B), and the orthogonal differences calculated between the two spectra (C).

this issue is to compare, on a component by component basis, the relative orthogonal differences between spectra. The orthogonal difference can be thought of as the minimum residual from a multi-dimensional least squares fitting of the spectrum of one sample to another. The information it conveys is the location of the differences in the shape of the spectra, and the relative amount of signal available to distinguish one spectrum for the other (a true measure of the SN for nonorthogonal signatures). The computation of these differences follows directly from the sort of least squares analysis one would apply to computing estimates of concentrations for unknown samples. If we have a known spectrum S_A and an unknown spectrum S_B we can estimate the amount of S_A in the unknown as

$$S_B = C_A S_A + \delta_{BA} \quad [1]$$

where the concentration C_A, and the difference δ_{BA} are

computed in a least squares sense. Rewriting (1) in terms of the square of δ_{BA} and differentiating with respect to C_A gives us the least squares estimate for C_A which minimizes

$$\delta_{BA}:\partial/\partial C_A\ (\delta_{BA}^2) = \partial/\partial C_A\ (\ S_B - C_A S_A)^2 = 0$$
$$C_A = (\Sigma\ S_A S_B)/(\Sigma S_A^2)\ .$$

Figure 1 shows this approach applied to two synthetic spectra, one a single Gaussian of unit amplitude, and a second composed of the first spectrum with a second Gaussian added. The bottom frames in Figure 1 show the differences computed for S_A in terms of S_B and visa versa. There are two things to note from Figure 1, first that the differences, unlike the spectra themselves, are not positive definite, and second that the difference operation as a whole does not commute (i.e., $\delta_{AB} \neq \delta_{BA}$). In least squares analysis this would be seen as a least squares fit of S_A to S_B, and the difference δ_{BA} would be considered an estimate of the error. However, in this study where we know the relation of the two spectra (S_A and S_B being the spectra from ME7 and 263K), δ_{BA} gives a measure of the difference in spectral shape between the two. The measure of this difference, which is significantly above the instrument noise, results in our estimates for the level of detectability (see below).

Two criteria must be met in order to detect PrP^{Sc} by MUFS. First, the fluorescence cross section for the protein must be of a magnitude to produce sufficient signal. Second, the spectral signature from the protein must be sufficiently distinctive. Consequently, our initial goals in measuring the fluorescence spectrum of the protein were to calculate fluorescence cross sections and determine whether there were qualitative differences in signatures between the untreated and the PK-treated protein, as well as differences between PK-treated PrP^{Sc} from hamster and mouse. Quantitative estimates of the differences between spectral signatures were obtained by calculating the orthogonal differences between pairs of spectra (12). Figure 2 shows the spectra from untreated, and PK treated samples of PrP^{Sc} from 263K scrapie strain infected hamsters and the orthogonal difference between the two spectra shown. Figure 3 shows the spectra for PK-treated PrP^{Sc} from mouse (ME7) and hamster (263K), and the orthogonal difference calculated from the two spectra shown. Figures 2 and 3 are surface plots of the spectra scaled to the same peak intensity of 10^4 counts (relative), along with the orthogonal differences calculated for the pairs of spectra shown. The data shown in these figures are neither corrected for excitation lamp intensity nor detector response. The data is displayed in this manner because normalization for excitation intensity increases the background noise along with the signal, and hence no additional information is conveyed. For the analysis of the data, normalization only becomes an issue when data from more than one instrument is being used (and hence is not an issue for these studies). However, this also means that the differences exhibited at low excitation wavelength (below 250 nm), where the excitation intensity is lower than its peak by an order of magnitude, are underrated. In the spectra and orthogonal differences shown, the first order scattered light (where the signal obeys $\lambda_{flr} = \lambda_{exc}$) has been removed in order to display the information in the fluorescence alone. There are two important points to note in both Figures 2 and 3. First, that there is a significant amount of fluorescence from the protein. Second, in both cases the orthogonal differences were ~10% of the overall signal strength, which is more than enough to allow one to distinguish between signals by standard multivariate methods (11, 21, 22). The spectral analysis was performed using only 1 μl of the ~3 μg/ml PrP^{Sc} preparations. Based on the relatively high fluorescence cross section measurements using ~3 ng of partially purified PrP^{Sc}, the ability to detect the protein at, or below, the picogram level is being explored. There are several ways to improve sensitivity. First, we note that multivariate analysis would be effective even with a signal that is an order of magnitude less than that shown in Figures 2 and 3 (1000 counts corresponds to a 3% fluctuation noise in the signal). Secondly, the signal obtained is proportional to the integration time. Since the data shown in Figures 2 and 3 were obtained by integrating for 410 sec., increasing the integration time to an hour will improve sensitivity by at least another order of magnitude. Third, charged coupled device detectors currently available are a factor of two more sensitive relative to the detector used in these studies, due to their decreased background noise and increased quantum efficiency. Taken together, this indicates that detection is possible in samples that are 200-fold more dilute than the samples used for Figures 2 and 3. In addition, by changing from a white light source to a laser, thereby increasing the amount of light used to excite the sample, a 10- to 100-fold increase in sensitivity could be obtained.

The fluorescence cross section for the protein establishes the limits of detectability for a given instrument. We have performed calculations to estimate the fluorescence cross section for PrP^{Sc} using the signatures from all four samples. Spectra were corrected for excitation intensity, and detector response so that an estimate of the fluorescence cross section for the protein at an excitation wavelength of 290 nm (near the peak fluorescence for tryptophan) could be calculated numerically. The value obtained for the integrated fluorescence cross section at 290 nm excitation is $\sim 10^{-17}$ cm^2/molecule. Figure 4 shows the differential fluorescence cross sections at 290 nm excitation as a function of fluorescence wavelength. These values are very similar (differing only by a factor of two) indicating that the fluorescence cross section is determined by properties

common to all the samples. This is most likely their amino acid sequence homology. However the calculated cross sections should only be taken as an order of magnitude estimate since it is limited by the accuracy of the estimate of protein concentration in the samples. The method of purification of PrP^{Sc} takes advantage of the physical properties of the abnormal form of the protein, in particular its tendency to aggregate. As a result, the samples in the absence of PK treatment contain PrP^{Sc} and extraneous material which co-purifies with the protein. The differences in spectral shape observed between untreated and PK-treated samples in Figure 2 may be due to: the elimination of the extraneous material following digestion with PK, a shift in signature of PrP^{Sc} due to cleavage of amino acid residues by PK, and/or a shift in fluorescence due to the association of the PrP^{Sc} with the extraneous material.

The fluorescence cross section values are an order of magnitude larger than what could be accounted for from the aromatic residues alone. Typically, the molecular fluorescence cross section for molecules such as tryptophan are 10^{-21} to 10^{-19} cm^2/molecule (23). Therefore, mouse and hamster PrP^{Sc}, which have 30 aromatic residues per molecule (19), might be expected to have a cross section as high as 10^{-18} cm^2/molecule. The 10-fold greater fluorescence values we have obtained can be accounted for by the presence of non-aromatic residues. Furthermore, the orthogonal difference observed between PK-treated PrP^{Sc} samples from hamster and mouse (Figure 3) are of interest with regard to protein conformations. There are relatively small differences in the PrP sequence between these two species. Sequence comparisons (19) shows differences in 16 out of the 254 residues or approximately 94% homology, with only a minority involving aromatic amino acids (only 2 of the 16) which are the dominant source of the fluorescence. These results suggest that conformational differences between these proteins, along with spatial interactions between specific aromatic and non-aromatic amino acids, plays an influential role in contributing to both the significant amount of fluorescence detected and the distinctive spectral signatures.

We have demonstrated that optical sensing technology, a novel nondestructive method for the analysis of biological samples, can be utilized for the rapid, specific, and direct detection of PrP^{Sc}. In addition, it may be possible to use this technique to distinguish the PrP^{Sc} isolated from a single host infected with various scrapie strains, and to discriminate between the PrP^{Sc} and PrP^{C} isoforms within the same host. Furthermore, the ability to make direct measurements for the presence of PrP^{Sc} within an optically dense background (i.e. blood or spinal fluid) is being investigated. Thus, MUFS shows promise as both a scientific tool, and a diagnostic method for the study of prion diseases. The cross sections of PrP^{Sc}, and their distinct spectral signatures, suggests that this method can be developed for use as an alternative, and perhaps more sensitive, assay than the current immunological methods of detection for PrP.

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