Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Membrane Proteins: The Scrapie Prion Protein

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I. Introduction

The role of mass spectrometry in protein analysis has been transformed by the development of new ionization techniques such as matrix assisted laser desorption/ionization (MALDI) (1-3). This is capable of ionizing intact proteins and providing molecular masses with a high degree of accuracy, in favorable cases to within 0.01%, sufficient to identify protein mutations and post-translational modifications. Although MALDI is a solid phase technique in which the ions are ablated from a dried target, it is dependent on protein solubility as the sample must co-crystallize with a UV-absorbing matrix compound that is mixed in large excess with the analyte (10^4:1). The sample and matrix must dissolve in a common solvent that encourages crystal growth as it evaporates. Solvents drying to leave glassy, oily or amorphous solids usually result in poor quality spectra.

The first matrix employed was nicotinic acid and although other compounds have subsequently been found to give superior results, this is typical of the general class of compound in that it is a substituted aromatic acid. Sinapinic acid (SA) (4) and 4-hydroxy-α-cyanocinnamic acid (4-HCCA) (5) are more recent examples, both of which are soluble in aqueous buffers and are normally prepared in 1:2 (v/v) acetonitrile, 0.1% trifluoroacetic acid at a concentration of 10 mg/mL for SA (approx. 50 mM) or 5 mg/mL for 4-HCCA.

The scrapie prion protein (PrP^{Sc}) is a GPI-anchored membrane sialoglycoprotein encoded by a chromosomal gene and is implicated in the development of a unique group of fatal neurodegenerative diseases including several human diseases, scrapie of sheep and bovine spongiform encephelopathy of cattle (6). PrP^{Sc} is derived from a normal cellular isoform (PrP^{C}) by an unidentified posttranslational event which causes physical changes and alters the location of PrP in the cell (7). PrP^{Sc} is more resistant to proteolysis and after limited digestion by proteinase-K, an N-terminally truncated form accumulates (PrP 27-30). We have carried out extensive peptide mapping of PrP^{Sc} using enzyme digestion and HPLC separation of the resulting peptides followed by

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amino acid analysis, Edman sequencing and mass spectrometry (8) (Stahl *et al*, submitted).

MALDI has the capability of revealing whether any labile posttranslational modifications have been lost or overlooked in the peptide mapping experiments. Furthermore, as a step toward determining the biochemical mechanisms involved in the transmission and development of prion diseases it is necessary to determine whether there are any covalent differences between PrP^{Sc} from different strains of the disease and also between PrP^{C} and PrP^{Sc}. MALDI requires very little material and has the potential to reveal whether the two forms differ in the nature of a posttranslational modification that is manifested by a mass difference. However, membrane proteins are insoluble in aqueous buffers in the absence of detergents such as sodium dodecylsulfate (SDS) which is incompatible with MALDI, or denaturants such as guanidinium hydrochloride (GuHCl) which inhibits ionization if used at concentrations in excess of 1 M, even if this is then diluted tenfold in the matrix. Formic acid (70-90%) has been used as a solvent and some preliminary data were obtained for both PrP 27-30 and PrP^{Sc} (8, 9), but it was found to partially suppress the signal, even for a water soluble protein such as myoglobin, and it is liable to introduce mass errors by formylation of basic sites. Furthermore, treatment with formic acid could remove acid-sensitive modifications. We therefore adapted a method that has been reported for electrospray ionization MS analysis of hydrophobic proteins, in which hexafluoroisopropanol was used as the solvent for the protein and was injected into a flowing stream of 2:5:2 chloroform, methanol, water (10).

II. Materials and Methods

Protein preparation

Bacteriorhodopsin was supplied by Sigma Chemical Co. PrP^{Sc} was isolated from the brains of scrapie-infected Syrian hamsters and was purified by ultrafiltration. After reduction and carboxymethylation, N-linked sugars were removed with PNGase F. The protein was separated by SDS PAGE, eluted from the gel with 10 mM ammonium bicarbonate/0.1% SDS, filtered, precipitated with ethanol and pelleted by ultracentrifugation. Any remaining SDS was removed by Konigsberg precipitation (11). In some experiments the lipid portion of the GPI anchor was removed by treatment with PIPLC and selected fractions were treated overnight with 50% aqueous HF at 4°C to remove the GPI anchor.

Mass spectrometry

Mass spectra were obtained on a laser desorption time-of-flight mass spectrometer constructed at the Rockefeller University and described previously (12, 13). Pulses of 10 ns duration of 355 nm radiation from a Nd(YAG) laser were directed at the solid sample/matrix mixture. The resulting ions were accelerated through a potential difference of 30 kV and detected at the end of a 2 m long flight tube by a hybrid microchannel plate/gridded discrete dynode electron multiplier detector. The laser fluence and spot position were varied manually during data acquisition. Spectra were recorded in a LeCroy Model

8828D transient digitizer (200 MHz sampling rate) at a repetition rate of 2.5 Hz. They were subjected to a time-to-mass conversion but no smoothing, background subtraction or other data manipulation was employed.

III. Results and Discussion

Experiments with various solvent/matrix systems

In earlier MALDI experiments with PrP^{Sc} we employed SA as the matrix and 70% formic acid as the solvent. We also used 70% formic acid as the solvent with the protein and mixed this 1:1 with SA in 1:2 acetonitrile, 0.1% TFA. The results were inconsistent and we frequently obtained no protein signal. The GPI anchor for PrP^{Sc} is known to be heterogeneous (14) and it was anticipated that the MALDI peaks would be broad but when protein spectra were obtained the width of the molecular ion peak was substantially greater than calculated, probably due to, (a) multiple and variable formylation by the solvent, and (b) the well-known laser-induced covalent addition of a 206 Da photodehydration product of SA. The molecular mass of deglycosylated but not delipidated PrPSc was measured as 25,540 Da, approximately 150-200 Da higher than anticipated. It was decided to use 4-HCCA as the matrix as this does not give rise to photo-adducts although it can lead to adduction of contaminating copper and we found it to give stronger peaks. We also established that the signal due to myoglobin was significantly suppressed when using 70% formic acid as the solvent. Thus it was decided to explore the use of an organic solvent hexafluoroisopropanol (HFIP) that is known to be an effective solvent for membrane proteins. We have established that HFIP dissolves native PrP^{Sc}, which becomes denatured and loses its resistance to proteolysis (unpublished work).

Due to the limited amount of PrP available it was decided to employ bacteriorhodopsin as a model membrane protein. Although this is extremely hydrophobic its properties are more amenable to analysis than PrP as it readily forms well-defined crystals whereas PrP aggregates to give an amorphous gum. Attempts to obtain MALDI spectra either of myoglobin or bacteriorhodopsin with SA or 4-HCCA directly in HFIP were unsuccessful. Various solvents containing 4-HCCA were then mixed 1:1 with HFIP containing the protein. The following solvents were investigated:

A	1:2	Acetonitrile, 0.1% TFA/water
B	1:1:1	Acetonitrile, isopropanol, 0.1% TFA/water
C	1:2:3	Formic acid, isopropanol, 0.1% TFA/water
D	1:2:3	Formic acid, tetrahydrofuran, 0.1% TFA/water
E	2:5:2	Chloroform, methanol, 0.1% TFA/water

For completeness we also investigated bacteriorhodopsin dissolved in 70% formic acid and mixed with solvent A.

All of the above combinations gave spectra but they varied significantly in quality. The least satisfactory was solvent D which was slow to dry and gave very unstable signals. In general the mixtures containing less water were the least effective solvents for 4-HCCA but they were more readily miscible with HFIP and gave the best quality data. Thus solvent A which is the "standard"

matrix solvent was immiscible with HFIP, causing the matrix and analyte to partition to different regions of the target on drying with very little mixing. This phenomenon was evident to some to degree for all of the solvents tested but solvent E which contained the lowest amount of water was the least problematical. 4-HCCA readily dissolved in solvent E at about 5 mg/mL, the concentration normally employed in MALDI. To evaluate HFIP compared with formic acid, the signal strength obtained for bacteriorhodopsin with 1:1 solvent E and HFIP was both more intense and more stable than that given by 70% formic acid 1:1 with solvent A.

Analysis of PrP^{Sc}

In an initial experiment, 10 µL 70% formic acid was added to an Eppendorf tube that was estimated to contain ~100 pmol PrP^{Sc}. Several aliquots of 0.5-1 µL were removed and mixed with various matrices but no protein signals were observed by MALDI. The formic acid was then removed on a Speedvac and HFIP was added. An aliquot was mixed 1:1 with 4-HCCA in solvent A and a stong spectrum was obtained, thus vindicating the decision to employ HFIP rather than formic acid. Furthermore, after being immersed in formic acid for two hours this sample also gave a molecular mass ~200 Da higher than anticipated. Subsequent experiments were carried out with HFIP and solvent E.

Fresh samples of PNGase-treated PrP^{Sc} were analysed. Spectra were obtained displaying ions ranging from $M+H^+$ to $M+4H^+$, multiply charged species being more prevalent with 4-HCCA than with SA (5). The peaks were substantially narrower than had been observed on previous occasions using SA and formic acid (9) and were also narrower than those in the 4-HCCA spectrum of the sample which had been treated with formic acid. In a separate experiment myoglobin was added to the matrix solution as an internal mass standard, giving a molecular mass for PrP of 25,394 Da, approximately 150 mass units less than had been measured after immersion in formic acid and close to the arithmetic mean of the predicted molecular masses for the four major species of 25,412 Da. This latter value is probably high as the calculation should be weighted to favor the two lowest mass forms which are present in higher abundance. Other minor peaks observed in the spectrum were attributable to a singly glycosylated protein resulting from incomplete PNGase digestion, a small amount of delipidated protein which the PIPLC had digested successfully, and a non-GPI-containing form truncated at Gly-228 which was identified in our earlier mapping experiments (15).

A reduction in molecular mass was observed after treatment with 50% aqueous HF, a procedure which should eliminate the GPI anchor by cleavage at the ethanolamine-phosphodiester bond which is the attachment site to the C-terminus of the protein, thereby removing the remaining source of heterogeneity and giving a single sharp peak. Despite the shift to lower mass the reaction was incomplete and resulted in broader rather than narrower peaks. It may be possible to incubate the samples with HF for a longer period but there is evidence that the amino acid chain can be cleaved by this procedure.

IV. Conclusions

We have established that MALDI mass spectra can be obtained for highly aggregated and hydrophobic membrane proteins using a new approach that gives enhanced sensitivity and does not cause the mass artefacts associated with solvation by formic acid. The data obtained to date indicate that Syrian hamster PrPSc after deglycosylation has a molecular mass very close to that predicted from a knowledge of the gene sequence and the structure of the GPI.

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