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Production of Artificially Doubly Glycosylated, ^{15}N Labeled Prion Protein for NMR Studies Using a pH-Scanning Volatile Buffer System

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Abstract

Bacterially expressed proteins used in NMR studies lack glycans, and proteins from other organisms are neither ^{15}N labeled nor glycosylated homogeneously. Here, we add two artificial glycans to uniformly ^{15}N labeled prion protein using a buffer system that evolves over a pH range to accommodate the conflicting pH requirements of the substrate and enzymes without the need to fine-tune buffer conditions. NMR and CD spectroscopy of the protein indicates that the glycans do not influence its fold.

Graphical Abstract

The transmissible spongiform encephalopathies (TSEs), commonly referred to as prion diseases, are caused when the cellular prion protein (PrP^{C}) misfolds into a β -sheet-rich “scrapie” structure (PrP^{Sc}) in a template mediated manner, forming toxic aggregates.¹ PrP^{C} contains two N-linked glycosylation sites proximal to the location of many of its pathological mutations.² We wished to investigate the effect that these glycans have on the

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.9b02430. LC, MS, NMR, and CD spectra for glycosylated PrP and table and graph of buffer pH over time (PDF)

The authors declare no competing financial interest.

structure of the protein using nuclear magnetic resonance (NMR). However, uniformly ^{15}N labeled protein produced for NMR by recombinant expression in *E. coli* does not contain glycans, and other methods such as mammalian or insect expression are neither easily ^{15}N labeled nor glycosylated homogeneously, making NMR investigations difficult. Hornemann et al. have previously shown that the structure of glycosylated bovine PrP extracted from healthy calf brains is essentially identical to that of unglycosylated recombinant PrP using circular dichroism and one-dimensional ^1H NMR spectroscopy but were unable to perform two-dimensional NMR due to low protein concentration and lack of isotopic labeling.³

Recently, advances in bio-orthogonal organic chemistry, coupled with the ability to incorporate unnatural amino acids in expressed proteins, have led to the ability to attach small molecules selectively to specific positions in proteins. Methods to prepare and attach artificial glycans have been published, but these methods have not to our knowledge been applied to ^{15}N labeled expressed protein or to create multiple homogeneous glycans per protein.^{4,5}

We initially attempted the procedure from the Schultz laboratory that couples aminoxy sugars onto the ketone of *p*-acetyl-phenylalanine and extends them into longer glycans using glycosyltransferases,⁴ but we encountered conflicting pH requirements between the reactants. Recombinant prion protein is soluble at low-medium pH, and the ketone to aminoxy coupling works best at low pH, thus achieving the first synthetic step. However, the glycosyltransferases have published activities near physiological pH. Thus, when the glycan extension reactions were performed in buffer systems typically used for enzymatic ligation, the prion protein precipitated, and when performed in the low salt, low pH buffers that are compatible with the prion protein, the glycosyltransferases were not active. We attempted the reactions at a few select intermediate pH values but were not able to identify solution conditions. We determined that if there *was* an intermediate pH where the prion protein was soluble and the enzymes were active, that the best way to find this, without an exhaustive and reagent-consuming test of many pH values, was to attempt the reaction at all pH values within the reasonable pH range in one shot. If the reaction buffer could slowly change in pH over time, the reaction would then proceed when both the substrate solubility and enzyme activity pH needs are met.

Carbon dioxide reduces the pH of aqueous solutions through the transient formation of carbonic acid, which dissociates to H^+ and bicarbonate anion. We reasoned, therefore, that an acidified solution prepared with a bicarbonate buffer would slowly drift up in pH due to transfer of protons to bicarbonate base, followed by loss of carbon dioxide. We prepared a mixture of ammonium bicarbonate and acetic acid at pH 6.5. All ions in this mixture are compatible with analysis by mass spectrometry. As carbon dioxide evolves out of solution, the pH rises until an equilibrium is reached among the remaining weak bases (Figures 1, 2). The starting and ending pH are determined by the concentration of buffer components, and the rate of pH increase is affected by stir rate and atmospheric pressure (Figures S7, S8). Through variation of the stir rate and pressure, the duration of pH scanning can be controlled from 3 h to indefinitely. Tightly capping the reaction with limited headspace slows pH drift, and application of a vacuum quickens pH drift. For proteins sensitive to oxygen exposure, this process can be done under an inert atmosphere. We performed both of the enzymatic

coupling steps in this buffer. Termination of the reactions was indicated by protein precipitate. Lowering the pH of this solution solubilized the products and analysis by mass spectrometry revealed homogeneously glycosylated protein.

The synthetic scheme for the glycosylated PrP is shown in Figure 3. Although lower in molecular weight than typical branched PrP^C glycans, we sought to retain fundamental chemical features by terminating with a sialic acid, typical of PrP^C and other proteins of the central nervous system. The sialylation status of PrP^C controls its ability to form toxic misfolded aggregates in *in vitro* protein misfolding cyclic amplification (PMCA) assays, alters the infectivity of these aggregates, and controls the species barrier for transmission of prion disease.^{6–9}

After successful preparation of the artificially glycosylated prion protein, we performed ¹H–¹⁵N HSQC NMR and circular dichroism spectroscopy to look for any structural effects that the glycans may have on the protein. The NMR spectrum overlapped closely with nonglycosylated prion protein with the exception of amino acids in close proximity to the glycans, and the circular dichroism spectra overlapped closely with each other as well as with literature spectra,¹¹ indicating that the glycans had not misfolded the protein (Supporting Information, Figures S5, S6).

In conclusion, we report the artificial glycosylation of ¹⁵N labeled prion protein for NMR studies, adding two homogeneous trisaccharides at the protein's glycosylation sites, and the verification by NMR and CD that these glycans did not disturb the overall fold of the protein. The required reactions were made possible by a buffer that scans over a pH range, allowing the reactions to proceed when a pH is reached that balances substrate protein solubility with enzyme activity. In addition, this buffer is volatile, allowing direct use of mass spectrometry during the reactions without the need for liquid chromatography.

EXPERIMENTAL SECTION

To prepare murine PrP with *p*-acetyl-phenylalanine at its two glycosylation sites, we mutated the corresponding amino acid codons (N180 and N196) to the amber stop codon TAG using site-directed mutagenesis PCR. The resulting sequence was murine Met-PrP(23–230, Asn180pAcPhe, Asn196pAcPhe). The *E. coli* strain B-95.AAAfabR, which has many of its TAG codons replaced,¹² was cotransformed with this plasmid and with the pEVOL-pAcF plasmid from the Schultz lab, which codes for the aminoacyl-tRNA synthetase (aaRS) and transfer ribonucleic acid (tRNA) corresponding to *p*-acetyl-phenylalanine.¹³ The protein was expressed in 1.0 L of M9 minimal media made with 1.0 g of ¹⁵N NH₄Cl and 400 mg of *p*-acetyl-phenylalanine. The protein was incubated at 37 °C with shaking. At an OD₆₀₀ of 0.2, arabinose was added to 1.0 mM to induce expression of the aaRS and tRNA, and at an OD₆₀₀ of 0.8, isopropyl- β -D-thiogalactoside (IPTG) was added to 1.0 mM to induce expression of PrP, and the culture was incubated overnight at 25 °C. The cells were pelleted and lysed, and inclusion bodies were washed with 1 M urea before being extracted with 8 M guanidine hydrochloride and purified by nickel immobilized metal ion chromatography. The protein was brought to pH 8.0 for 1 day to allow its disulfide bond to form and then further purified by HPLC on C4 resin using water and acetonitrile with 0.1% trifluoroacetic acid,

yielding 6 mg. The protein mass was checked by mass spectrometry (23 516 g/mol measured, 23 519 g/mol expected) and lyophilized and stored at -80°C (Figure S1). The Advion Expression-L used has mass inaccuracy up to 0.2%.

To prepare the aminooxy *N*-acetylglucosamine, the procedure outlined by Liu et al. was followed, which itself references the more detailed protocol in Cao et al.^{4,14} (Figure 3). We found the purity of the 2-acetamido-2-deoxy- α -D-glucopyranosyl chloride 3,4,6-triacetate to be highly vendor and batch dependent, and had success with material from Combi-Blocks, which was received as a white powder.

To couple the sugar to the mutant PrP, 6 mg of the PrP was dissolved in 200 mM acetic acid, 100 mM ammonium bicarbonate, pH 4.5, and 10 mg aminooxy-*N*-acetylglucosamine. The reaction was left overnight, and the protein was purified by HPLC on C4 resin using water and acetonitrile with 0.1% trifluoroacetic acid, yielding 3 mg. The protein mass was checked by mass spectrometry (23 937 g/mol measured, 23 957 g/mol expected), and lyophilized (Figure S2).

To extend the two single sugars into two disaccharides, 3 mg of the mutant PrP was dissolved in 100 mM acetic acid, 200 mM ammonium bicarbonate, pH 6.5, with 5 mg of UDP- α -D-galactose, 5 μg of β -1,4-galactosyltransferase (R&D Systems 3609-GT-010), and 1 U of alkaline phosphatase (Sigma-Aldrich 10713023001). The reaction was left overnight and found to be at pH 7.9 the next day, with the PrP having precipitated. The PrP was resuspended with the addition of glacial acetic acid and purified by HPLC on C4 resin using water and acetonitrile with 0.1% trifluoroacetic acid, yielding 2 mg. The protein mass was checked by mass spectrometry (24257 g/mol measured, 24281 g/mol expected), and lyophilized (Figure S3).

To extend the two disaccharides into two trisaccharides, 2 mg of the mutant PrP was dissolved in 100 mM acetic acid, 200 mM ammonium bicarbonate, pH 6.5, with 2.5 mg of cytidine-5'-monophospho-*N*-acetylneuraminic acid (CMP-Sialic acid), 0.5 U of α -2,3-sialyltransferase (Sigma-Aldrich S1951-1UN), and 1 U of alkaline phosphatase. The reaction was left overnight and found to be at pH 7.9 the next day with the PrP having precipitated. The PrP was resuspended with the addition of glacial acetic acid and purified by HPLC on C4 resin using water and acetonitrile with 0.1% trifluoroacetic acid, yielding 1 mg. The protein mass was checked by mass spectrometry (24 868 g/mol measured, 24 863 g/mol expected) and lyophilized (Figure S4).

^1H - ^{15}N HSQC NMR samples were made to pH 6.0 in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer using potassium as a counterion and containing 10% D_2O . For all samples, the protein was added to a concentration of 100 μM . Spectra were recorded at 37°C on a Bruker 800 MHz spectrometer at UCSC NMR facility (Santa Cruz, CA). NMR spectra were analyzed with NMR Pipe and Sparky using assignments transferred from previous experiments by visual inspection. Circular dichroism spectroscopy was performed on a Jasco J-1500 instrument, and the data were analyzed on the CAPITO web server.¹⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Carbonic acid leaves the system as carbon dioxide, raising the pH of the solution.

Figure 2.

Change in pH over time of 100 mM acetic acid and 200 mM ammonium bicarbonate with vigorous stirring. Other concentrations that scan over different pH ranges at different rates are presented in the Supporting Information (Figures S7, S8). The increase in slope at 210 min (arrow) is from application of a vacuum to the sample cell.

Figure 3. Synthesis scheme for glycosylated PrP. The base structure for this image is PDB 1XYX, which is an NMR structure of the C-terminus of mouse PrP^C.¹⁰