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In Vitro Glycosylation of Membrane Proteins Using *N*-Glycosyltransferase

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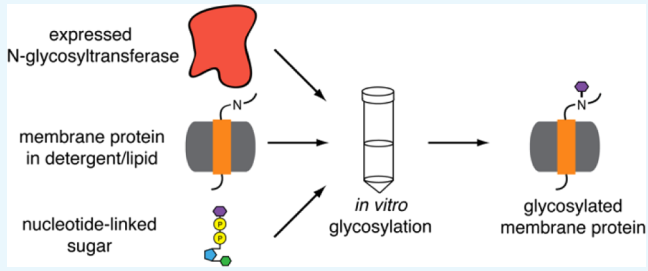
ABSTRACT: Glycoproteins are post-translationally modified proteins that take part in nearly every biological process and make up a large percent of the proteome. N-Linked glycosylation can be performed by *N*-glycosyltransferase (NGT), which recognizes the consensus amino acid sequence, -Asn-X-Ser/Thr- (NXT), within the protein. The enzyme catalyzes glycosidic bond formation between the oligosaccharide donor, containing nucleoside phosphatase, and the amide nitrogen of the asparagine residue. The attachment of the sugar moiety can influence physiological and biological properties of the protein by affecting their folding, modulating interactions with other biomolecules, and modifying their functions at the cellular level. We are specifically interested in the properties of membrane glycoproteins, which are key components in a number of different disease states. Therefore, the use of *in vitro* protein glycosylation can help further evaluate the effects of the properties for these important macromolecules. *In vitro* studies of N-linked glycosylation were done in a stepwise fashion in a membrane-mimetic environment to confirm that the methods for glycosylating soluble proteins could be applicable to membrane proteins. Detergent and lipid systems were used since hydrophobic peptides and membrane proteins are insoluble in aqueous solvents. The stepwise method consisted of the glycosylation of a soluble 7-residue peptide, a hydrophobic WALP-NVT peptide, and a γ-sarcoglycan membrane protein, all of which contained the glycosylation site Asn-Val-Thr (NVT). Glycosylation of the samples was performed using *Escherichia coli*-expressed NGT from the *Actinobacillus pleuropneumoniae* genome, and a single sugar moiety of glucose, provided from a nucleotide-linked donor, was added to the glycosylation site. Gel electrophoresis, mass spectrometry, and NMR studies were used for the detection of glycosyltransferase activity and to show the attachment of a single glucose molecule. Our experiments demonstrated that small or large membrane proteins that contain an N-glycosylation consensus sequence can be glycosylated by NGT in membrane-mimetic environments.



INTRODUCTION

Glycosylation is a very abundant and highly diverse protein post-translational modification found in eukaryotes[1] and prokaryotes, including all archaea[2] and some bacteria.[3] It is estimated that approximately half of all eukaryotic proteins expressed in the cell undergo glycosylation resulting in glycoproteins.[4] Glycosylated proteins possess the same protein backbone but different oligosaccharide components and sites of glycosylation, which give rise to greater variation and diversity. N-Linked glycosylation involves the attachment of an oligosaccharide from a donor molecule to the side-chain amide nitrogen of an asparagine residue within the consensus sequence Asn-X-Ser/Thr (NXS/T) in the polypeptide chain. This reaction can be catalyzed in eukaryotes by the membrane-bound enzyme oligosaccharyltransferase (OST), which uses lipid-linked oligosaccharides as the sugar donor.[5] It has also been found that a cytosolic bacterial *N*-glycosyltransferase (NGT) can also perform glycosylation in a similar manner using the same consensus sequence.[6] These Asn-glycosylation modifications exhibit enormous biological significance and are involved in nearly every process in cells including protein folding, signal transduction, secretion, and cellular recognition.[1,7−9]

Greater variation in oligosaccharide structure and its combination with proteins have caused a serious challenge in structure determination and obtaining a detailed mechanism of glycosylation. This has spurred research focused on designing methodologies to synthesize well-defined glycopeptides and glycoproteins, which are difficult to obtain from existing natural sources. As part of these efforts, several research groups have synthesized homogeneous glycopeptides and glycoproteins *via* chemical, enzymatic, and chemoenzymatic methods. Because it is difficult to make the glycosidic linkage between oligosaccharides and proteins, many glycoprotein analogues

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are prepared *in vitro* using unnatural linkages between them. These conjugates are called neoglycoproteins.[10] One straightforward approach to the synthesis of neoglycoproteins involves direct chemical conjugation of glycans with polypeptides using reactive amino acid side chains already present in the protein.[11] This simple strategy has been used to make a majority of neoglycoproteins reported in the literature.[12−14] For example, glycoconjugates of bovine serum albumin were synthesized using the Amadori reaction with dextran.[15] Simple saccharides have also been conjugated with bovine serum albumin using commercially available linkers.[16] Similarly, ribonuclease A was conjugated with a naturally derived mannopentose *via* reductive amination of the oligosaccharide followed by coupling using an azide heterobifunctional reagent.[17] As an alternative approach, Yuan and co-workers have developed a convergent chemical method to develop a stable acyl donor to enable glycopeptide−glycopeptide ligation.[18] However, the chemical synthesis of glycoproteins with complex oligosaccharides generates several challenges because they require strictly anhydrous conditions, which are incompatible with most proteins, and require an unfeasibly complex series of protection/deprotection steps of both carbohydrates and polypeptide side chains.[13] Thus, the development of chemoenzymatic methods has attracted greater interest for the synthesis of oligosaccharide precursors modified by a range of glycosyltransferases. Wang and co-workers have developed a convergent chemoenzymatic method, which performs site-specific enzymatic ligation between an activated glycan oxazoline and *N*-acetylglucosamine (GlcNAc)-peptide/protein to synthesize homogeneous glycopeptides/glycoproteins with asymmetrically branched N-linked glycans attached.[19] This chemoenzymatic method is based on the transglycosylation activity of endoglycosidases, which sequentially removes the *N*-glycans from glycoproteins and ligates large intact oligosaccharides from a diverse set of glycosyl donors in a single step. These endoglycosidases strictly require an Asn-linked donor for transglycosylation and have been employed in the construction of complex glycopeptides in low yields.[20,21] Moreover, an elongation of glycan structures from initiating sugars is achieved by different glycosyltransferases, the so-called Leloir transferases. These enzymes transfer a given carbohydrate from the corresponding sugar nucleotide donor−substrate to a specific hydroxyl group of the acceptor protein.[22] Due to the unique selectivity of glycosyltransferases, it is possible to design and synthesize complex glycans in a predetermined manner.[23,24]

Selective protein modification and formation of homogeneous glycoconjugate can be achieved, albeit rarely, if the protein has a single reactive amino acid residue targeted by the modifying event.[11] To synthesize homogeneous glycoconjugates, a number of different strategies have been developed over the last decade.[21,24] The basis of these strategies is the introduction of a specific glycan ligation site in the polypeptide chain, which can be used for selective glycan attachment. For instance, it has been reported that the *Haemophilus influenzae* HMW1 adhesin can be glycosylated at multiple asparagines, in all but one case, within the consensus sequence of N-glycosylation.[25] The interesting feature here was that the modifying glycans at these sites are hexoses and dihexoses, instead of N-acetylated sugars, revealing a novel enzymatic activity capable of attaching hexose moieties to asparagine residues in the polypeptide chain.[25] Subsequent work done by St. Geme and co-workers showed that the enzyme responsible for the modification of asparagine residues of HMW1 with a single glucose or a single galactose is the HMW1C protein.[25,26] A recent study performed by Aebi and co-workers demonstrated that a HMW1C homologue, from *Actinobacillus pleuropneumoniae*, a soluble *N*-glycosyltransferase (NGT), is capable of modifying peptides or proteins having the consensus sequence Asn-X-Ser/Thr, with the simple hexose monosaccharides from a nucleotide-activated sugar donor as a substrate.[6] In that study, they established an assay to perform *in vitro* glycosylation for short soluble peptides, suggesting that NGT shows novel enzymatic activity in modifying peptides.

Although N-glycosylation occurs on both secreted and membrane-bound proteins, much of the research on *in vitro* N-glycosylation has been achieved with several soluble proteins but has yet to be shown successful with membrane proteins. Membrane proteins represent between 20 and 30% of proteomes of most organisms, and a majority of these are glycoproteins.[27] Studying glycosylation of membrane proteins is absolutely essential since membrane glycoproteins are key components in many disease states and are important as pharmaceutical targets. However, investigating N-glycosylation of membrane proteins is complicated by the fact that membrane proteins have partially hydrophobic surfaces, flexibility, and lack of stability.[28] Due to these difficulties, the structures and exact structure−activity relationships (SARs) of membrane glycoproteins have been difficult to evaluate. Therefore, there is an urgent need to develop methodologies to synthesize homogeneous, perhaps synthetic, membrane glycoproteins to study SAR of biologically relevant glycoproteins.

The main purpose of the experiments reported here was to confirm, in a stepwise fashion, that methods developed for soluble proteins could be applicable to membrane proteins. This will lay the groundwork for studying how glycan attachment to membrane proteins affects their structure, function, and dynamics and perhaps provide an understanding of how it is involved in regulating fundamental biological processes in cells. In the present study, we performed a similar *in vitro* assay for membrane proteins like the one performed by the Aebi group for soluble proteins. This research investigated the glycosylation of a simplified hydrophobic peptide and a full-length membrane protein that each have a single N-glycosylation site. This study was guided by the question: what conditions can be used for the *in vitro* assays that allow solubilization of the membrane proteins and also allow the enzyme activity of NGT to be retained? To assess whether membrane proteins can be glycosylated by the NGT enzyme, we focused on the incorporation of these proteins into a wide range of detergents and lipid assemblies such as micelles and isotropic bicelles. Careful selection of a detergent/lipid system is crucial for the *in vitro* studies. In this work, we needed to consider sample conditions that allow the water-soluble *N*-glycosyltransferase enzyme to retain its activity. Here, we have established a lipid-mediated *in vitro* assay to characterize the glycosylation of membrane proteins and provide valuable insights into the future use of SAR for these important proteins.

RESULTS

The purpose of this study was to develop a novel *in vitro* assay for glycosylating membrane peptides/proteins using *N*-glycosyltransferase (NGT) enzyme. As an initial step to produce *N*-glycosyltransferase, we expressed the protein from

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ACS Omega 2021, 6, 12133−12142

A. pleuropneumoniae (ApNGT) strain using *Escherichia coli* DH5α and expression was induced by the addition of 0.2% L-arabinose. NGT enzyme was expressed here as a His-tagged recombinant protein, which could be purified and detected by Ni^{2+}-NTA affinity chromatography because the string of histidine residues binds to immobilized nickel ions under specific buffer conditions. Our purification protocol theory consists of Ni^{2+}-NTA affinity chromatography and dialysis to yield a high-purity NGT enzyme. The calculated molecular mass of NGT was approximately 72 kDa, consistent with the results observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel analysis (Figure 1).

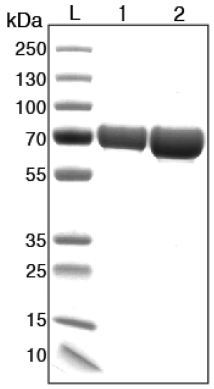

Figure 1. SDS-PAGE analysis of NGT purification and concentration. Lane 1 shows the elution fraction obtained from Ni-NTA affinity chromatography, and lane 2 contains the dialyzed and concentrated NGT. Lane L corresponds to the protein ladder, PageRuler Plus, that was used as the protein standard.

To assess whether ApNGT is capable of glycosylating peptides/membrane proteins having the NXS/T consensus sequence in detergent or lipid environments, we designed short peptides based on the glycosylation site of γ-sarcoglycan (-NVT-). We first designed a heptapeptide, TQNVTVA, and then a second highly hydrophobic WALP peptide, GGALW-$(LA)_6$LLAGATQNVTVA, with an NVT glycosylation sequence, WALP-NVT, to first test the *in vitro* glycosylation method. These peptides form a transmembrane helix and resemble an integral membrane protein. The NVT consensus sequence for glycosylation was engineered downstream of the WALP transmembrane helix. The third and most significant construct we hoped to glycosylate in this study was the membrane protein γ-sarcoglycan, which is a 32 kDa type II transmembrane protein (Table 1). This protein was found to be an ideal protein to study *in vitro* N-linked glycosylation for the following reasons: (1) γ-sarcoglycan has a single site for N-linked glycosylation in its large C-terminal extracellular domain, which is important for NMR and mass spectrometry studies, because having multiple glycosylation sites increases sample heterogeneity and complexity, (2) γ-sarcoglycan possesses a single transmembrane domain, which eases the solubility of relatively insoluble protein in mild detergents, and (3) γ-sarcoglycan is an extremely important glycoprotein expressed in skeletal and cardiac muscles. It stabilizes dystrophin−glycoprotein complex, which supports the linkage between the cytoskeleton and extracellular matrix. Mutations in any of the genes of sarcoglycans cause the destabilization of the complex, resulting in different forms of muscular dystrophy, which is a group of diseases characterized by progressive muscle weakness and degeneration. As sarcoglycans are N-glycosylated proteins, their function and role in muscular dystrophies are thought to involve N-glycosylation pathways. Therefore, it is important to study how glycan attachment affects γ-sarcoglycan structure and function. We were able to show previously that it was possible to express and purify full-length proteins.[29]

We aimed to perform these *in vitro* studies in lipid/detergent environments that ensured that membrane proteins could remain folded in a native confirmation while providing conditions that allow the glycosylating enzyme to retain its activity. Therefore, a wide range of detergents were tested to develop an assay for analysis of NGT activity toward membrane proteins with glycosylation sites. The tested detergents were 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), Fos-choline-16, Fos-choline-12, dodecyl maltoside (DDM), decyl maltoside (DM), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxyl-1-propanesulfonate (CHAPSO). Glycosylation assays were performed using these detergents as they are well known for their ability in solubilizing membrane proteins. Solubilization of proteins is dependent on the formation of micelles in the solution. Therefore, proteins were solubilized in detergents above their characteristic critical micelle concentration (CMC), the concentration above which monomers self-assemble into noncovalent micelles. When working with membrane proteins, it is important to use detergents present at concentrations above their CMC to solubilize the proteins.[30−35]

In the glycosylation assay, we solubilized the peptide/protein in detergents and uridine diphosphate glucose (UDP-Glc) and purified NGT enzymes were added. Previous works

Table 1. Amino Acid Sequences of the 7-Residue Peptide, the WALP-NVT Peptide, and Full-Length γ-Sarcoglycan[a]

name	sequence
heptapeptide	TQ**NVT**VN
WALP-NVT	GGALWLALALALALALWLAGATQ**NVT**VA
γ-sarcoglycan	MVREQYTTATEGISIERPENQYVYKIGIYGWRKRSLYLFVLLLLIILVVNLALTIWILKV SWFSPAGSGHLSVTKDGLRLEGESEFLFPLYAKEIHSRVDSSLLLQSTQV**NVT**NARNSEG EVTGRLKVGPKSVEVQNQQFQINSNDGKPLFTVDEKEVVVGTDKLRVTGPEGALFEHSVE TPLVRADPFQDLRLESPTRSLSSDAPRGVHIQAHAGKIEALSQSDILFHSSDGSLVLDAE TVSLPKLVQGTWGPSGSSQSLYEISVSPDGKLYLSVAGVSTTSQEHSHISL

[a]The glycosylation site, made up of the consensus sequence NVT, is bolded in each sequence. The transmembrane regions of WALP-NVT and γ-sarcoglycan are demarcated by a black solid bar below the sequence.

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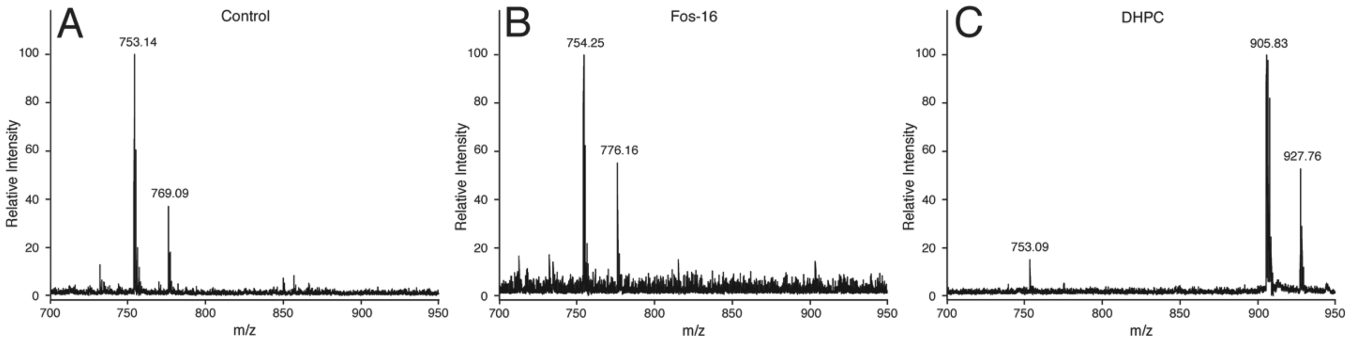

Figure 2. Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) analysis of *in vitro* glycosylation of the heptapeptide. (A) Control sample spectrum showing a large peak at 753.14 Da corresponding to the approximate size of the unglycosylated peptide. (B, C) Spectra of the heptapeptide postglycosylation in the presence of Fos-choline 16 and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) detergents, respectively. While the spectrum of the peptide glycosylated in Fos-16 does not demonstrate a significant increase in mass for the peptide, the spectrum of the peptide glycosylated in DHPC shows a clear increase in the mass of the peptide to 905.83 Da corresponding to the attachment of the sugar molecule.

have shown that for effective glycosyltransferase activity, acceptor peptide and sugar donor could be mixed in a ratio from 1:10 to 1:100. Therefore, for our work, peptide and UDP-Glc were mixed at a 1:25 mole ratio. Subsequently, the reaction mixture was incubated at 25 °C for 16 h with rocking. Analysis of reaction products was done by MALDI-MS and NMR. The addition of a single glucose moiety to the asparagine residue in a peptide or protein would increase the mass of peptide/protein by 162 Da. However, a 162 Da difference of unglycosylated and glycosylated peptides/proteins is too small to be clearly separated by SDS-PAGE. Therefore, we were not able to use SDS-PAGE to detect glycosyltransferase activity using peptides/proteins as substrates.

Since detergents can interfere with many analytical assays, detergent removal was necessary after initially using them to extract or purify protein or carry out the reactions.[36] Lipid/detergent removal methods were explored to avoid ion source fouling in MS analysis. The detergents having low-molecular-weight micelles (<10 kDa) and relatively high CMC values can be easily removed by dialysis or gel filtration chromatography. However, detergents that form high-molecular-weight micelles and have relatively low CMCs cannot be removed by dialysis because detergent micelles cannot pass through the cutoff filter during dialysis. So, we used β-cyclodextrin (β-CD), which can efficiently capture a variety of detergents by sequestering detergent monomers.[37] In the presence of β-CD, detergent micelles break down, bind inside the hydrophobic cavity as detergent monomers, and then the β-CD-detergent complexes can be removed by dialysis to isolate the proteins. So, the reaction mixtures containing Fos-choline-12, Fos-choline-16, DM, and DDM detergents were subjected to dialysis with β-CD to get rid of detergents from reaction products. After dialysis of β-CD, the protein samples were subjected to dialysis against water to remove β-CD from the dialysis tubing. Since CHAPS and CHAPSO form low-molecular-weight micelles, the reaction mixtures containing CHAPS and CHAPSO were subjected to dialysis in water prior to MS analysis.

In MS analysis, we observed that the glycosylated heptapeptide TQN(+Glc)VTVA, which was dissolved in DHPC, was found to have an increased molar mass. As shown in Figure 2A, MS analysis of unmodified TQNVTVA resulted in a major peak, matching the calculated molar mass of the peptide (753.14 Da). Following the reaction of the peptide dissolved in Fos-choline-16, with NGT and UDP-Glc, we observed a peak corresponding to unmodified TQNVTVA peptide (754.25 Da) (Figure 2B). As shown in Figure 2C, the reaction products in DHPC, resulted in major peaks at 753.09 Da and 905.83 Da corresponding to unmodified TQNVTVA peptide and TQN(+Glc)VTVA species respectively. Based on MS analysis, we observed efficient glycosylation of the TQNVTVA peptide in DHPC However, we observed no evidence of glycosylation in reaction mixtures in other detergents: Fos-choline-12, DM, DDM, CHAPS, and CHAPSO, so these spectra are not included here. Subsequently, we examined the effect of the addition of more NGT and UDP-Glc to the reaction mixtures. However, no change was observed in mass spectra, indicating that glucose was not attached to peptides. Thus, an approximate excess of the donor−substrate (∼25-fold) seems sufficient for an efficient glycosylation reaction (Figure 2).

To prove glycosylation of the 7-residue peptide directly and to characterize the site of the modification, we analyzed the glycosylation products using NMR spectroscopy. Short-range J-couplings (scalar couplings) were used to measure $^{1}H-^{1}H$ couplings of the side-chain amide groups for asparagine and glutamine. Here, the peptide was solubilized in deuterated DMSO and two-dimensional correlation spectroscopy (COSY) NMR experiment was carried out. Efficiency of the coherence transfer largely decreases with increasing line width, which is related to the molecular weight. The COSY experiment is therefore almost exclusively used for smaller (nonlabeled) peptides. Positions of cross-peaks in the COSY are characteristic spectra for the amino acids. As there is a proton−proton correlation in side-chain amides of asparagine and glutamine residues, we can observe characteristic cross-peaks in a region of 5−6 ppm. Figure 3 shows the expanded region of two overlaid COSY spectra obtained for unmodified (red) and Glc-modified peptide (blue). Since the 7-residue peptide sequence contains both asparagine and glutamine residues, side-chain $^{1}H-^{1}H$ correlations could be observed as cross-peaks in the unmodified peptide (red). The cross-peaks originated from glutamine side-chain $^{1}H-^{1}H$ correlation with and without glycosylation were consistent in both samples. However, the cross-peak belonging to the $^{1}H-^{1}H$ correlation of the asparagine side chain was not present in the glycosylated peptide (Blue). The glycosylation of the asparagine residue results in the replacement of one amide proton by a glucose

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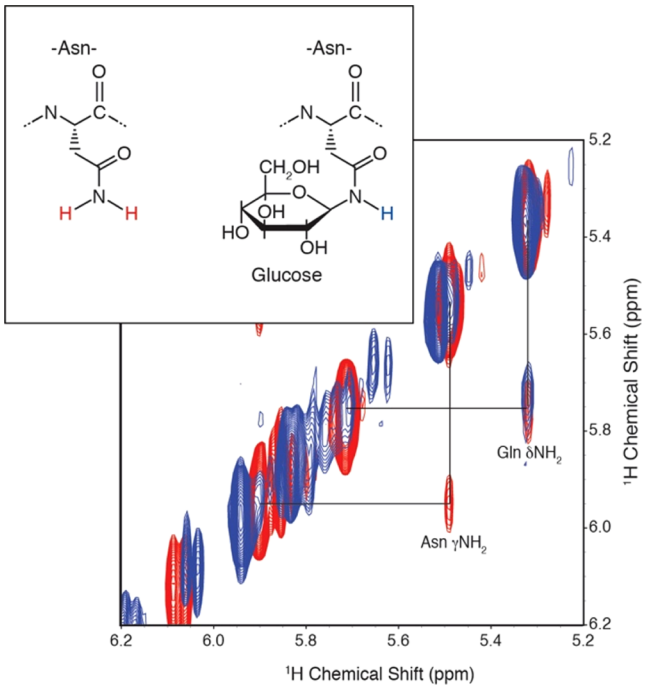

Figure 3. $^{1}H-^{1}H$ COSY spectrum of the heptapeptide. The cross-peaks in the spectrum allow for monitoring of glycosylation using the γNH_2 group of the asparagine residue. Since the glycan is attached at the side-chain amide group, the absence of the γNH cross-peak evident in the 2D spectrum indicates that glycosylation has occurred. The side chain of glutamine, which also has a side-chain amide group, can be used for comparison of the two samples. The overlayed spectra show the heptapeptide without (red) and with (blue) glycosylation.

molecule. Thus, the proton−proton correlation of the side chain is not present in the spectrum of the glycosylated peptide. This result provides confirmation that a glucose molecule is attached to the side-chain amide nitrogen of asparagine amino acid.

To investigate the glycosylation reaction of the WALP-NVT peptide, we performed a similar *in vitro* assay with different detergent solutions and, again, performed MS analysis. The unmodified WALP-NVT peptide showed a characteristic peak at 2692.01 Da, which matches with its calculated molar mass (Figure 4A). As shown in Figure 4B, two major peaks corresponding to WALP-N(+Glc)VT (2849.79 Da) and unmodified WALP-NVT (2689.33 Da) were observed after reaction with NGT and UDP-Glc in CHAPSO. Similar results were obtained for reaction mixtures in CHAPS for WALP-N(+Glc)VT (2853.76 Da) and unmodified WALP-NVT (2691.13 Da) (Figure 4C). These results demonstrate that hydrophobic peptides like WALP-NVT could be incorporated into detergents and soluble NGT can still mediate the sugar attachment *in vitro*. However, we could not observe any evidence of glycosylation for reaction mixtures in DHPC, Fos-choline-12, Fos-choline-16, DM, and DDM detergents by mass spectrometry. These spectra are not included since there was no indication that the glycosylation was successful. To investigate the possibility of enhanced glycosylation reaction, we repeated the assay by incubating the WALP-NVT peptide with higher amounts of NGT and UDP-Glc. Glycosylation was not affected with increased amounts of NGT and UDP-Glc (data not shown).

To extend our *in vitro* results and examine whether ApNGT is capable of glycosylating membrane proteins, a full-length γ-sarcoglycan protein was chosen for glycosylation. Here, the γ-sarcoglycan protein was expressed, purified, and subjected to the same glycosylation reaction in different detergents and lipid micelles. Initial results by MALDI-MS (not shown) indicated that glycosylation was successful. However, due to the larger molecular weight of γ-sarcoglycan protein, the use of MALDI-MS made it difficult to clearly visualize the small mass increase (∼162 Da) resulting from the addition of a single glucose molecule. By considering the possible cleavage sites of γ-sarcoglycan by protease enzymes, we hypothesized that the protein could be cleaved after the glycosylation reaction, particularly in DHPC micelles, and that a peptide fragment with a glucose modification could more easily be observed using a comparison of the control peptide fragment. Therefore, the next step was to cleave the glycosylated product by a suitable protease enzyme and identify possible glycosylated peptide fragments by peptide analysis of mass spectrometry. An SDS-PAGE gel of the control sample and the glycosylated γ-sarcoglycan protein sample was run. The protein bands were cut out from the gel for trypsinolysis and run through liquid chromatography-mass spectrometry (LC-MS) for identification.

After trypsinolysis, the parent peptide in question consisted of amino acids 98−115. This is the peptide after trypsinolysis that contains the glycosylation site NVT. This peptide did show glycosylation but did not show complete glycosylation of the parent peptide. The glycosylated peptides were found to

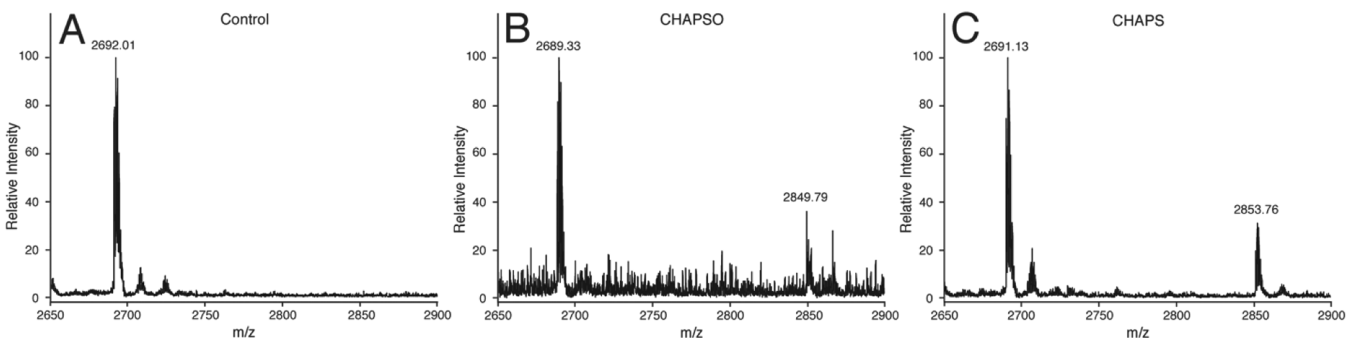

Figure 4. MALDI-MS analysis of the WALP-NVT peptide. (A) Control sample spectrum showing a large peak at 2692.01 Da corresponding to the approximate size of the unglycosylated peptide. (B, C) Spectra of WALP-NVT postglycosylation in the presence of CHAPSO and CHAPS detergents, respectively. The spectra of the peptide glycosylated in both detergents show a clear increase in the mass of the peptide indicating the attachment of the sugar molecule in both samples.

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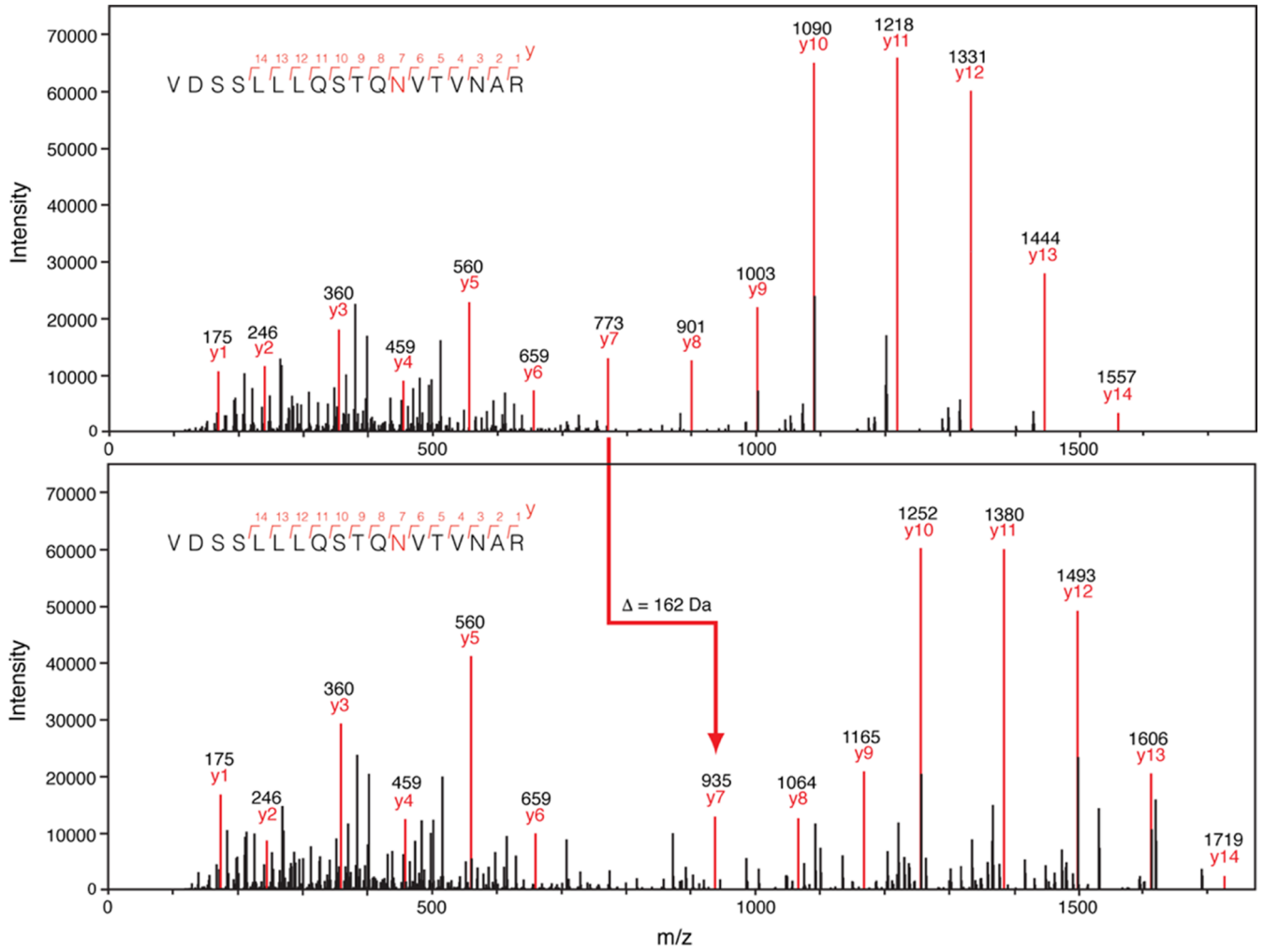

Figure 5. Quadrupole-Orbitrap MS/MS analysis of the tryptic products of γ-sarcoglycan. Top, the spectrum from the fragmentation of the unglycosylated protein. Bottom, the spectrum from the fragmentation of the glycosylated protein. y7 corresponds to the first fragment that contains the altered asparagine residue in which the glucose contributes to the mass increase.

have an observed m/z of 1054.0519 and a calculated m/z of 1054.0475. These numbers are shown to be within the 10 ppm of the instrument's specifications. The posterior error probability (PEP) or the probability of an incorrect peptide identification was 2.5×10^{-15}, showing a low chance of a misidentified peptide. The z, or charge of this peptide, was +2.

The control sample, which did not have NGT added, showed no glycosylation when looking at the parent peptide of amino acid residues 98−115. This same peptide without glycosylation showed an observed m/z 973.0216 and a calculated m/z of 973.0211 which was also within the 10 ppm specifications of the instrument. The PEP of the control peptide that was in question is 4×10^{-15}, which shows a low probability of misidentification. The z for this peptide was +2 as well. Also, high-accuracy/high-accuracy mass spectroscopy was used for testing these samples, which corresponds to high mass accuracy for first the parent peptide and second high mass accuracy for the MS-MS fragments. This shows, as further proof, that the peptide in question is correctly identified.

When comparing the aforementioned glycosylated protein to the control samples of the study, we can see a clear m/z movement at the y7 amino acid in the peptide spectral matches. The y7 fragment corresponds to the first truncated fragment that contains the asparagine in the glycosylation site. As shown in Figure 5, the mass difference between the glycosylated and control peptides at y7 is 162 Da, which corresponds to the expected mass difference in a glycosylated sample from the unmodified parent peptide. This shift can be seen in the m/z of every other amino acid after the asparagine (y7). This is shown by comparing every y fragment following y7 from the control and glycosylated samples from the peptide spectra. Further proof of glycosylation is the select reaction monitoring chromatograms from the LC-MS data. This data allows us to look at the elution time of the glycosylated peptide peak and compare it to the control at the same elution time. The control should be lacking the peak shown in the glycosylated sample. When looking at the select reaction monitoring data (Figure 6), the peak at the specific elution time of the glycosylated peptide had a relative intensity of 8.4 $\times 10^4$ and the control had a peak intensity of 3.08×10^3 at the same elution time. A small peak in the control is seen and is approximately 27-fold smaller than the intensity of the glycosylated sample. Therefore, the peak in the control spectrum can be attributed to possible contamination from the gel. The methods described here are crude, allowing us to determine qualitatively that we achieved glycosylation, but

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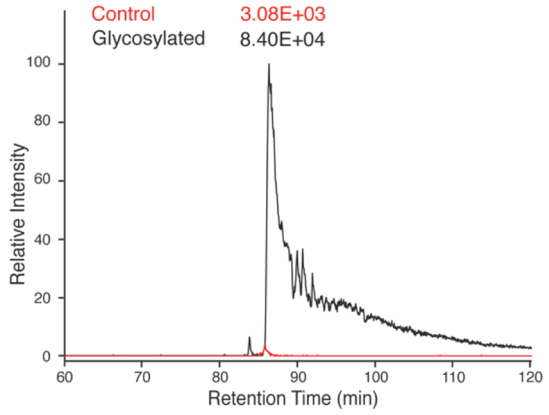

Figure 6. SRM chromatograms from quadrupole LC-MS data of control (red) and glycosylated (black) samples. The 27-fold greater intensity of the glycosylated samples at 87 min shows the presence of the glycosylated peptide, whereas the control does not. The small peak in the control can be attributed to possible gel contamination and is not believed to be a glycosylated peptide. This is also confirmed by the lack of a glycosylated peptide in the peptide spectral match (not shown here).

methods and further experiments are being developed to assess the percentage of glycosylation.

DISCUSSION

Studying and handling membrane proteins outside the native membrane environment are a significant challenge in protein biochemistry. Therefore, when performing *in vitro* studies of membrane proteins, extra attention is needed in supplying conditions that mimic the native membrane environment. In this *in vitro* glycosylation study, we used different detergents/lipids to solubilize the proteins to carry out the reaction while maintaining the membrane-like environment.

The expression and purification of *N*-glycosyltransferase from *A. pleuropneumoniae* enabled the catalysis of glycosylation of designed peptides having an NXS/T consensus sequence. The hydrophobic peptides and membrane proteins we used have an NVT sequence, and all were modified with a single glucose moiety in the presence of an NGT enzyme. According to our MS analysis data of glycosylation products, we observed characteristic peaks corresponding to the mass of unmodified peptides/proteins and peaks with a mass increase of 162 Da, which corresponds to the mass of an attached glucose moiety. This proves the successful glycosylation reaction by NGT in detergent environments.

When performing *in vitro* experiments, we sought to answer a basic question: do specific detergents at concentrations above their CMC allow the glycosyltransferase activity to be preserved so that glycosylation can be performed *in vitro*? Our results demonstrated that the ability of the enzyme to perform glycosylation is dependent on the detergent or lipid that is used in the reaction. Based on the experimental results, zwitterionic detergents worked better for the *in vitro* glycosylation of hydrophobic peptides and membrane proteins. Detergents with low CMC, DHPC and Fos-choline-16, worked for glycosylation of heptapeptides and detergents with high CMC, CHAPS and CHAPSO, worked for glycosylation of WALP-NVT peptides. In this study, we were able to determine that γ-sarcoglycan could be glycosylated in DPHC micelles. What is slightly surprising is that the results of glycosylation were not consistent among peptides and proteins. Based on the observed results, both the unmodified peptide/protein and glycosylated product were present in the reaction mixture and in most cases the percent of the unmodified peptide/protein was greater than the glycosylated product. Even after repeating the reaction with an increasing concentration of NGT and donor−substrate UDP-Glc, to obtain sufficiently high concentrations of glycosylated products, we did not observe a noticeable change in results.

In this work, we have shown that the soluble enzyme NGT modifies the asparagine side chains of peptides and membrane proteins with a single glucose moiety in detergents. Therefore, our work has initiated a novel *in vitro* assay to perform N-glycosylation of membrane proteins in detergent environments. Furthermore, we hope to expand the approach to produce more complex glycoproteins by introducing different glycan types to the attached glucose primer.

This work also highlights the fact that the glycosylation reaction is likely protein- and detergent/lipid-dependent. This means that more peptides and proteins need to be explored in a variety of hydrophobic environments, as there might not be a universal formula for the sample preparation of *in vitro* glycosylation of membrane proteins.

MATERIALS AND METHODS

***N*-Glycosyltransferase (NGT) Protein Expression and Purification.** Plasmids of NGT from ApNGT were received as a generous gift from the lab of Dr. Markus Aebi (ETH Zurich, Switzerland). Plasmids were transformed into BL21 *E. coli* using a standard heat-shock protocol. The expression of ApNGT (72 kDa) was performed according to the methodology described by Aebi and co-workers.[6] The expression cell pellet was resuspended in 30 mL of a resuspension buffer, containing 30 mM Tris (pH 8), 300 mM NaCl, and 20 mM imidazole. Cell lysis was performed by sonication (Fisher Scientific Sonic Dismembrator, www.fishersci.com) on ice for 4 min (2 s on, 10 s off), and the extract was centrifuged at 16 000*g* for 30 min at 4 °C (Thermo Scientific, SORVALL LINX 4000 Centrifuge). The protein was purified from the supernatant using Ni^{2+}-NTA resin (Thermo Scientific, www.thermoscientific.com) to purify the His-tagged protein by affinity chromatography. First, the column was washed with 15 mL of resuspension buffer. Then, the column was washed with 15 mL of wash buffer containing 30 mM Tris (pH 8), 300 mM NaCl, and 50 mM imidazole. The purified protein was eluted with an elution buffer containing 30 mM Tris (pH 8), 300 mM NaCl, and 300 mM imidazole. Pooled fractions of ApNGT were transferred to a 10 kDa dialysis bag (Spectrum Laboratories, Inc., spectrumlabs.com) and dialyzed in a dialysis buffer containing 25 mM Tris (pH 7.2) and 150 mM NaCl. After quantification of the protein by measuring its absorbance at 280 nm (Thermo Scientific, Evolution 60S UV−visible Spectrophotometer), the protein was concentrated to approximately 10 μM using an Amicon Ultra 10 kDa filter unit (Merck Millipore Ltd.). The protein purity was analyzed by SDS-PAGE and PageRuler Plus standard (Thermo Scientific).

Peptide Design. To evaluate the potential of ApNGT to *in vitro* glycosylate membrane proteins, two short model peptides were used. The first, a short 7-amino acid, soluble peptide with a single N-glycosylation site was designed. A second, hydrophobic peptide that would be incorporated into the detergent or lipid environment and have the same consensus N-glycosylation site was also designed. Both of these short peptide sequences were designed based on the N-glycosylation

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ACS Omega 2021, 6, 12133−12142

site of the γ-sarcoglycan protein and were terminally modified with N-terminal acetylation and C-terminal amidation. For the shorter 7-residue peptide, the -NVT- sequence was situated in the middle of the short sequence, TQNVTVA. This peptide was purchased from RS Synthesis (www.rssynthesis.com) and arrived in a dry powder form. The WALP-NVT peptide was designed with six leucine−alanine repeating units flanked by a tryptophan residue at the C-terminus to resemble the transmembrane helix. An NVT consensus sequence was engineered downstream of the WALP transmembrane helix. This peptide was purchased from Genscript (www.genscript.com) and arrived in a dry powder form.

Sample Preparation and Glycosylation of the 7-Residue Peptide. Detergents 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) (Anatrace), *n*-hexadecylphosphocholine (Fos-choline-16) (Anatrace), *n*-dodecylphosphocholine (Fos-choline-12) (Anatrace), *n*-dodecyl β-D-maltoside (DDM) (Anatrace), and *n*-decyl β-D-maltoside (DM) (Anatrace) were dissolved in a dialysis buffer. All experiments were executed at a concentration of 25 mM. The 7-residue peptide (0.25 mg) solubilized in a dialysis buffer with 20% dimethyl sulfoxide (DMSO) was added into each detergent solution and vortexed briefly to obtain a clear uniform solution. To initiate the glycosylation reaction, uridine diphosphate glucose (UDP-Glc) (5.2 mg) (Calbiochem, www.millipore.com) was added to the peptide solution at a 1:25 peptide-to-sugar molar ratio and incubated for 16 h at 25 °C in the presence of NGT (10 μM) in a 400 μL final volume of the dialysis buffer.

Sample Preparation and Glycosylation of the WALP-NVT Peptide. Detergent solutions of DHPC, Fos-choline-16, Fos-choline-12, DM, and DDM were dissolved in trifluoroethanol (TFE) (Acros Organics, www.acros.com) solution (500 μL) to a final concentration of 25 mM. Additionally, 3% (w/v %) of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Acros Organics, www.acros.com) and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxyl-1-propanesulfonate (CHAPSO) (Calbiochem, www.millipore.com) in a dialysis buffer were prepared separately. The 30-residue WALP-NVT peptide for glycosylation was prepared in two ways. For the first set of experiments, the peptide (1 mg) was dissolved in each 25 mM detergent solution and the organic solvent was then evaporated under a stream of nitrogen gas to obtain a dry peptide−lipid film and the sample was placed under a high vacuum overnight. In the second set, the WALP-NVT peptide (1 mg) was dissolved in TFE (500 μL) and the organic solvent was evaporated and the sample was placed under a high vacuum overnight. Then, solutions of CHAPS and CHAPSO (400 μL) were added separately to the dry film of peptide samples and vortexed to obtain a uniform solution.

The glycosylation reaction was initiated by adding the donor−substrate, UDP-Glc (5.7 mg), to each peptide solution in buffer (25 mM Tris pH 8, 150 mM NaCl) at a 1:25 molar ratio (peptide: UDP-Glc) with an NGT enzyme (10 μM). Glycosylation reactions were incubated for 16 h at 25 °C.

Protein Expression and Purification of γ-Sarcoglycan. The expression and purification of γ-sarcoglycan are described previously in the manuscript by Jamaleddine et al.[29] Briefly, γ-sarcoglycan was recombinantly expressed in BL21 *E. coli* in 4 L of LB media with carbenicillin and protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG). Lysis of the cells was then performed with resuspension buffers (50 mM Tris, 1 mM EDTA, 1 mM NaN_3, and 15% (v/v) glycerol, pH 8.0 and 50 mM Tris, 1 mM EDTA, 1 mM NaN3, 1% deoxycholic acid, and 1% IGEPAL CA-630, pH 8.0) to separate soluble proteins from the inclusion bodies containing the fusion protein. These inclusion bodies were then dialyzed against deionized H_2O, frozen, and dried on the lyophilizer. Following lysis, the isolated inclusion bodies containing the fusion protein were treated with cyanogen bromide in formic acid to chemically cleave the target protein from the fusion partner. The cleavage products were separated using a size exclusion column on a fast performance liquid chromatography (FPLC) system in a sodium dodecyl sulfate (SDS) running buffer (20 mM sodium phosphate, 4 mM SDS, 1 mM EDTA, 1 mM sodium azide, at pH 8.2). The purified protein in the SDS buffer was dialyzed against deionized H_2O, and the protein was precipitated out. This precipitated protein in water was then frozen and lyophilized. This final product of the purification is a dry powder, which is then used for glycosylation.

Glycosylation of γ-Sarcoglycan. Detergent stock solutions of DHPC (450 mM), Fos-choline-16 (100 mM), Fos-choline-12 (100 mM), CHAPS (3% w/v), and CHAPSO (3% w/v) were prepared in 500 μL of TFE. One milligram of the purified γ-sarcoglycan protein was first solubilized separately in 500 μL of TFE. The detergent stock solution was added to the protein solution, making the 25 mM final concentration of detergent in a final volume of 1 mL. The protein solutions were vortexed and kept in a bath sonicator to obtain a clear solution. The organic solvent was evaporated using a stream of nitrogen gas, and the sample was placed on the lyophilizer overnight.

After the protein−lipid film was hydrated with a buffer (25 mM Tris, pH 8, 150 mM NaCl), it was placed in the bath sonicator for solubilization. As a donor for the glycosylation reaction, UDP-Glc at a 1:25 molar ratio was added. In the presence of NGT (10 μM), glycosylation reactions were incubated for 16 h at 25 °C. Later runs, in hopes to optimize glycosylation, were run at 25 μM of NGT. Control samples for mass spectrometry testing were also prepared the same way as the glycosylation samples but NGT or UDP-Glc was left out of the reaction mixture, so the glycosylation of γ-sarcoglycan could not occur.

Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry Analysis. After the completion of all glycosylation reactions, the removal of detergents from glycosylation products was necessary because detergents are expected to interfere with mass spectrometry. The presence of detergents in protein/peptide samples severely suppresses the ionization of proteins/peptides in mass spectrometry.[38] Therefore, some common approaches for detergent removal were tested, such as methanol/chloroform protein extraction, trichloroacetic acid (TCA) precipitation, ammonium sulfate $(NH_4)_2SO_4$ precipitation, the use of Zip-tips, and molecular weight cutoff dialysis.[39]

MALDI time-of-flight spectrometry was performed on a Voyager DE Pro mass spectrometer (AB SCIEX). The dried powders of the 7-residue peptide and the WALP-NVT peptide were dissolved in TFE/acetonitrile (ACN)/trifluoroacetic acid (TFA) solution (60:40:0.1%) and (80:20:0.1%), respectively. A matrix stock solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid (Alfa Aesar, www.alfa.com) in ACN/H_2O/TFA (50, 50, 0.1%). For sample analysis, the peptide solutions were diluted 2-fold and 10-folds into the matrix solution. One microliter of peptide solutions was then

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aliquoted onto a target MALDI plate and allowed to dry at ambient temperature. Then, 1 μL of matrix solution was placed on top of each spot and allowed to dry again. The dried powder of the γ-sarcoglycan protein was dissolved in ACN/TFA (98:2%) solution and mixed 1:1 with a matrix solution of sinapinic acid (Asta Tech, www.astatechnic.com) (50% ACN and 0.1% TFA) for spotting onto the target plate. Samples were analyzed in the linear positive ion mode, with 30 laser shots collected at random across each sample spot and accumulated using the automated sample collection mode. Mass spectra were acquired in the mass range of m/z 500−3000 and 20 000−40 000 for peptides and sarcoglycans, respectively. All spectra were processed by a Data Analyzer smoothing and baseline correction.

Trypsinolysis LC-MS of Glycosylated γ-Sarcoglycan. After glycosylation, the dried protein powder of γ-sarcoglycan was then prepared for gel electrophoresis. Approximately 0.5 mg of the glycosylated protein sample was dissolved in 80 uL of LDS buffer (50 mM Tris−HCl, 50 mM Tris base, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 12.5 mM ethylenediaminetetraacetic acid (EDTA), 0.02% bromophenol blue). The protein was mechanically lysed with a 1 mL syringe, pulling the protein buffer solution up through the needle and back out 30 times. The sample was then boiled in a hot bath at 100 °C for 10 min.

The prepared glycosylated γ-sarcoglycan was loaded onto an SDS-PAGE gel at 20 uL. Four microliters of Page Rule prestained protein ladder was loaded with one empty well between the ladder and the sample to help avoid contamination. The samples were run on the gel at 150 V and 400 mA for 40 min. The gel was then soaked and microwaved in coomassie blue stain (50% methanol, 40% water, 10% acetic acid, 1 g Brilliant Blue G-250) for 45 s. The gel was left on a rocker for 1 hour before the coomassie blue stain was removed and destain (50% water, 40% methanol, 10% acetic acid) was added. The gel was microwaved again for 45 s in the destain solution and placed back on the rocker overnight. The gel was taken out of destain after 16 hours, placed in deionized H_2O, and stored until LC-MS is run on the excised γ-sarcoglycan band. The control samples of γ-sarcoglycan were prepared exactly the same for trypsinolysis as the glycosylated samples.

From these coomassie-stained gels, the γ-sarcoglycan protein bands were excised and digested with trypsin using standard methodologies (*e.g.*, Voruganti et al.).[40] Trypsinolytic peptides were injected onto a 75 μm × 50 cm nanocolumn (Acclaim PepMap, Thermo PN 164942) and separated using a water−acetonitrile gradient (3−30% acetonitrile in 120 min) containing 0.1% formic acid. Peptides were eluted through a stainless steel emitter and ionized within a NanoSpray Flex ion source (Thermo). Peptide ions were analyzed by a "high−high" "top-speed" data-dependent acquisition using a quadrupole-Orbitrap mass spectrometer (Thermo Fusion), wherein the parent peptide ions were analyzed in the Orbitrap sector at a nominal resolution of 120 000, the quadrupole sector was used to select peptide ions for HCD dissociation, and the fragment ions were analyzed within the Orbitrap sector at a nominal resolution of 30,000.

Peptides were identified by using the Byonic software application (Protein Metrics) to search RAW instrument files against a database of 4306 *E. coli* protein sequences downloaded from Uniprot and supplemented with 9 *N*-glycosyltransferase sequences representing recombinant proteins studied in this and related projects. Search settings were optimized using the Byonic Preview module, after which the searches were repeated, now including N-linked glycosylation (+162.0528) as an additional variable modification.

For select reaction monitoring, the quadruple was programmed for targeted MS/MS scans of ion m/z's 527.53, 703.03, and 1054.05, representing +4, +3, and +2 ions, respectively, of the parent peptide containing the glycosylation site consisting of amino acids 98−115 of γ-sarcoglycan. Each selected target ion was fragmented by HCD, followed by the wide-band scanning of fragment ions using the Orbitrap sector at a nominal resolution of 60 000. Ion-specific chromatograms were extracted using the parent ion scan filter, in conjunction with secondary filtering using the specific fragment m/z values indicated in figure legends.

NMR Spectroscopy. The control and glycosylated 7-residue peptides were lyophilized and dissolved in deuterated DMSO at 0.5 mM. The samples were mixed by vortexing, and undissolved proteins were removed by centrifugation 14 000*g* for 5 min. The supernatants containing the protein were transferred to 5 mm × 180 mm NMR tubes (Norrell) for experiments. Both samples were measured at room temperature on a 400 MHz AVANCE spectrometer equipped with a $^{1}H/^{15}N$ BBFO SmartProbe (Bruker, www.bruker.com). The DMSO peaks in the spectra were referenced to 2.50 ppm, and the pulse program cosyqf90 (www.bruker-biospin.com) was used and the proton pulse was calibrated for each sample. The standard two-dimensional $^{1}H-^{1}H$ COSY (homonuclear correlation spectroscopy) spectra were recorded using 2048 t_2 points and 256 t_1 points to measure $^{1}H-^{1}H$ cross correlations of the side chains of the asparagine and glutamine side-chain amide groups. Spectra were processed with NMRPipe[41] and analyzed by Sparky.[42]

■ ASSOCIATED CONTENT

Accession Codes

Accession Number for γ-sarcoglycan: NP_000222

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Notes

The authors declare no competing financial interest.

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