ISSN: 0976-0172
Journal of Bioscience AndTechnology
www.jbstonline.com

Creation of an HDX-MS-Enabled PNGase Rc Column for the Online Deglycosylation of Complex Glycoproteins

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Article Info

Received: 03-10-2019 *Revised: 26-11-2019* *Accepted: 27-12-2019*

ABSTRACT

Glycosylation of proteins is a prevalent post-translational modification (PTM), and it is present in several biopharmaceuticals, extracellular proteins, and cell surface receptors. Problems in identifying the HDX of protein segments containing glycans have, however, restricted HDX-MS investigation of these crucial glycoproteins until till now. For better glycoprotein analysis, we have created a column with immobilised PNGase Rc (from Rudaea cellulosilytica) that can be easily integrated into a standard HDX-MS system. By using HDX-MS in conjunction with the PNGase Rc column, we demonstrate that N-linked glycans can be efficiently removed online and the HDX of glycosylated areas in various complex glycoproteins can be determined. Furthermore, a thorough HDX-MS mapping of the binding epitope of a monoclonal antibody to c-Met, a complicated glycoprotein therapeutic target, is executed using the PNGase Rc column. The column maintained its high activity even when quench-buffer additives like urea and TCEP were present, and it continued to function consistently for 114 days of heavy operation. In summary, our research demonstrates that HDX-MS coupled with the integrated PNGase Rc column may provide online deglycosylation under severe quench conditions, allowing for thorough examination of intricate glycoproteins.

INTRODUCTION:

The majority of proteins modified after translation that are exposed to the extracellular environment undergo glycosylation of aspartate residues, also known as N-glycosylation.In addition to making proteins more solubile and stabilising their structure, 1,2 N-glycosylation also provides binding partner recognition mechanisms, which enable proteins to operate in environments outside than cells.3 Additionally, N-glycosylation is present on several extracellular proteins that hold promise as therapeutic targets or biopharmaceutical modalities.one, two This highlights the increasing importance of understanding proteins in their naturally glycosylated form for structural and functional research.4 Because of the enhanced kinetics and structural heterogeneity provided by N-glycosylations, conventional protein structural investigation techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) are not always suitable for natively glycosylated proteins.

To explore the interactions and conformation of dynamic or conformationally diverse protein systems in solution, hydrogen−deuterium exchange mass spectrometry (HDX-MS) may be a strong alternative approach.5 In high-resolution X-ray fluorescence mass spectrometry (HDX-MS), a protein is exposed to a deuterated solvent, and the MS can detect the hydrogen bond breaking between the backbone amide and the heavier deuterium atoms in the solvent. Protein higher-order structure is defined by its involvement in hydrogen-bond networks

HDX-MS is a sensitive tool for studying protein dynamics and structure due to the rate of backbone amide hydrogen exchange. Online proteolysis allows for the resolution of protein HDX down to the peptide level. Many protein states, such as glycoproteins, may be difficult to examine using traditional techniques; nevertheless, HDX-MS is useful for this because of its sensitivity and inherent capacity to tolerate sample complexity.5. HDX-MS is often used to analyse protein conformational changes that occur during function and regulation, including membrane proteins, interactions between proteins and ligands or drugs, the mechanism of action of biopharmaceuticals, and the mapping of therapeutic monoclonal antibodies' epitopes.5 minus 8

ISSN: 0976-0172
Journal of Bioscience AndTechnology
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cause MS analysis to detect a lower signal, leading to gaps in the sequence coverage in areas with N-linked glycosites. The retention of deuterium by acetamido groups within glycan structures may also affect glycan HDX interpretation and lead to inaccurate quantification of deuterium retention.9,11 One way to get around these problems is to use fragmentation in MS/MS analysis.12, 13 It is particularly challenging to use collision-induced dissociation (CID) MS/MS for this objective, however. This happens because of H/D scrambling that happens during CID and the preferred cleavage of glycosidic linkages in glycopeptides12, 13.14 Electron transfer dissociation (ETD),15 one of the non-scrambling fragmentation technologies, has shown selective peptide backbone breakage while maintaining the glycosidic link intact. Nevertheless, these choices are only available on MS systems that provide this capacity, and ETD has far worse fragmentation efficiency compared to CID.12, 13 Additionally, acid-stable endoglycosidases have been investigated for N-Glycan removal during HDX-MS. An off-line method of facilitating deglycosylation during quench conditions during HDX-MS of glycoproteins was first used by PNGase A, the first enzyme to be utilised for this purpose.9 Investigations into alternative acidic PNGases, such as PNGase H+ (derived from Terriglobus roseus), were initiated because to PNGase A's unusually high optimal pH of 5 and its poor tolerance to commonly used quench buffer additions such as TCEP and urea.10 In the same way as PNGase A can release a wide range of N-glycans, PNGase H+ can deglycosylate glycoproteins as well, giving it an edge over PNGase A.9 In contrast to PNGase A, it has a high tolerance for reducing agents like TCEP and an ideal pH of 2.6. Having said that, the expression yields and purity of PNGase H+ were poor. Some PNGase H+ variants exhibited strong deglycosylation activity thereafter. From Dyella japonica came PNGase Dj, an intriguing variation of PNGase H+. We demonstrated the use of PNGase Dj in HDX-MS during analysis of complex glycoproteins, including epitope mapping applications, and immobilised it on a microchip to make the use of such rare enzymes more accessible.16 Expanding upon this study, we have lately demonstrated, in partnership with Josef Voglmeir's lab at Nanjing Agriculture University, that PNGase Rc, an H+ variant of PNGase derived from Rudaea cellulosilytica, outperforms PNGase Dj in terms of intrinsic activity and expression yields. This is most likely because PNGase Rc contains only two disulfide bonds, as opposed to the three found in PNGase Dj.17 Glycans from plants and invertebrates, as well as core-fucosylated glycan structures that are not cleaved by PNGase F, are among the many N-glycans that PNGase Rc can cleave, much as PNGase H+.17, 18 Reasons like these make PNGase Rc a promising enzyme for glycoprotein HDX-MS techniques.17, 19

To further enhance the analysis of glycoproteins, we have created a column with immobilised PNGase Rc that can be integrated into an online HDX-MS process. When opposed to using PNGase enzymes in solution, immobilising PNGase Rc has several advantages, such as a greater enzyme to substrate ratio and the ability to reuse the enzyme. Furthermore, a significant benefit of HDX-MS is that it does not need any preincubation time. This leads to a quick online process, which improves throughput and reduces back-exchange.16 Determining the HDX of glycosylated areas and efficiently removing N-linked glycans online are both made possible by the PNGase Rc column.

on a number of glycoproteins, some of which are very complicated and difficult to map epitopes on. Repeated usage over more than 100 days does not diminish the column's strong deglycosylation activity, and it continues to work even when standard HDX-MS quench-buffer additives like urea and TCEP are present.

Section for Experimentation

Expression and Purification of PNGase Rc. Take a look at the SI and the data mentioned before.17, 19

Process for Making the PNGase Rc Column and Immobilising PNGase Rc). Refer to SI.

High-Definition X-Ray Mass Spectrometry on Glycoproteins with the PNGase Rc Column. A Synapt G2Si HDMS connected to an HDX- MS UPLC system (Waters Corporation, U.K.) was used to conduct research on haemoglobin and transferrin.

The process of starting the HDX of Haptoglobin (Hp 1−1, Sigma-Aldrich) began by diluting the protein to a concentration of 1 μM in labelling buffer (20 mM Tris pH(read) 7.0, 90% D2O). At different intervals, 50 μL of the HDX reaction was mixed with a 1:1 (v/v) amount of quench buffer, which consisted of 250 mM TCEP, 4 M urea, 0.5 M glycine, and a pH of 2.3, to stop the reaction. To facilitate protein unfolding and reduction, quenched samples were placed on ice for three minutes before being frozen on dry ice. During analysis, HDX samples were rapidly thawed and injected into the cooled (0 °C) HDX-MS UPLC system and subjected to online proteolysis (20 °C) in an in- house built column packed with agarose-immobilized pepsin, followed by online deglycosylation (20 °C) using a column placed immediately downstream packed with immobilised PNGase Rc (2.1 × 20 mm2, Wuxi GALAK Chromatography Technology Co, China), and finally desalting on a trap column (Waters Acquity BEH C18, 1.7 μm, 2.1 × 5 mm2 length) for 3 min with

ISSN: 0976-0172
Journal of Bioscience AndTechnology
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aqueous phase (0.23% FA) at 200 µL/min. A 9-minute gradient from 2 to 40% organic phase (0.23% FA in acetonitrile) was used at 0 °C to carry out separation on an analytical column (Waters Acquity BEH C18, 1.7 µm, 1 × 100 mm2). Using Protein Global Server 3.0 (Waters) and DynamX v3.0 (Waters), peptides were identified using data-dependent acquisition (DDA) and data-independent acquisition (DIA) modes, i.e., MSe MS/MS. Utilising DynamX, the deuterium uptake of the corresponding peptides was determined. There were three separate runs of the HDX experiment.

The aforementioned online digestion of Hp 1−1 and subsequent manual sample collection upon trapping were used to create maximum-labeled control samples of Hp 1−1. Hp 1−1 peptic peptides were subjected to the same labelling buffer for 5 hours as stated before in order to obtain maximum labelling. They were then applied to the same HDX-UPLC system that was described earlier, except that it was run without the pepsin column and in both the absence and presence of the PNGase Rc column. In order to determine the back-exchange (BE) values, the maximum deuterated control sample was compared to an undeuterated control. To dilute solid apo-human transferrin (Sigma-Aldrich) to a concentration of 10 µM in equilibration buffer (PBS, pH 7.4), the HDX of transferrin was carried out. The process of labelling began by adding 5 µL of each protein aliquot to 50 µL of labelling buffer, which is PBS produced in D2O with a pH of 7.0, creating a 90% D2O environment. Various time points were labelled. A solution of ice-cold quench buffer (50 mM KH2PO4, 4 M) was added in a 1:1 ratio to stop the labelling process. to a pH of 2.5, along with 200 mM TCEP and urea. The Waters Enzymate BEH pepsin column was used to digest the samples online at 20 °C. After that, they were deglycosylated online at 20 °C using a column that was positioned immediately downstream and packed with immobilised PNGase Rc (2.1 × 20 mm2, Wuxi GALAK Chromatography Technology Co, China). The peptides were held on a Waters BEH C18 VanGuard precolumn for three minutes in buffer A, which contains 0.1% formic acid and has a pH of 2.5, at a flow rate of 100 µL/min.

Using a linear gradient of buffer B (0.1% formic acid in acetonitrile ~ pH 2.5) and a flow rate of 40 µL/min, peptides were separated on a Waters BEH C-18 analytical column. To minimise back exchange, all separation and trapping were carried out at around 0 °C. Whether a state had or did not have a PNGase column, all data were acquired at the identical flow rates.

The MSE procedure was used to gather MS data. We took three separate readings at each time point during the experiment. The mass spectrometry data were acquired with lock mass correction using LeuEnk after a separate NaI calibration. Isotope uptake of individual peptides was ascertained using DynamX v3.0, whereas peptide assignments were made with the ProteinLynx Global Server (PLGS, Waters Corporation, U.K.) programme.

The HDX of VISTA was carried out according to the manufacturer's instructions by reconstituting the protein (Human B7−H5, Acro BioSystems) at a concentration of 5×. The pre-experiment deglycosylation by PNGase Rc was prevented by the 50% trehalose in the reconstituted solution. Thus, VISTA was mixed with PBS buffer by means of Zeba Spin Desalting Columns (7K MWCO Fisher Scientific, U.S.A.). Using a NanoDrop 2000 spectra photometer from Fisher Scientific in the United States, the final concentration was found to be 1.65 mg/mL. Incubation with 54 µL of PBS HDX buffer (1×, D2O pH 7.4) at 25 °C for 0, 25, 1, 10, 60, and 600 minutes deuterated six microliters of VISTA (90 µM in PBS). In order to decrease and quench 10 µL aliquots, a 1:1 (v/v) ice-cold quenching solution was added. This solution included 0.4 M TCEP and 4 M urea in 100 mM ammonium formate with a pH of 2.4. The final pH was 2.6, and the concentration was 0.2 M TCEP and 2 M urea. The samples were promptly placed in liquid nitrogen and kept at a temperature of -80 °C until analysis. Triplicates of each experiment were conducted independently.

This procedure begins with a Nepenthesin-2 column (AffiPro) and is followed by online glycosylation at 20 °C. A column is then inserted downstream, packed with immobilised PNGase Rc, then trapped and desalted on a Waters BEH C18 VanGuard precolumn. The flow rate is 200 µL/min for 3 minutes in buffer A, which contains 0.23% formic acid and has a pH of around 2.5. At a flow rate of 40 µL/min, peptides were isolated on an analytical column made of Waters BEH C-18 using a linear gradient of buffer B, which consisted of 0.23% formic acid in acetonitrile with a pH of about 2.5. To minimise back exchange, all separation and trapping were carried out at around 0 °C. Whether a state was utilising or not using the PNGase Rc enzymatic column, all data were acquired at the same flow rates.

utilising the following criteria and PEAKS Studio 11 (Bioinfomatic Solutions, inc.), peptide identification was carried out in three runs utilising DDA fragmentation with HCD, ETD, and EThcD: The MS/MS tolerance is 0.5 Da, whereas the peptide tolerance is 5 ppm. In order to find the deuterium uptake of the different peptides, Sierra Analytics's HDExaminer 3.0 was used.

In accordance with the suggestions made by the community, the study's HDX data, as well as an HDX summary table and HDX data tables (Table S1), are published in the Supplemental Information (SI).8.

ISSN: 0976-0172
Journal of Bioscience AndTechnology
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Trastuzumab (TZ) Digestive System. Refer to the SI and the data that was previously provided.sixteenth, an LC-MS assay to track the PNGase Rc column's online glycolysis activity. We used materials from a tryptic digest of Trastuzumab (TZ) to assess PNGase Rc activity, much as previously reported.16 A Waters cooled HDX-UPLC system (Milford, MA) was used to connect with the PNGase Rc column. Before desalting and separation by reversed-phase chromatography and MS analysis, the LC-MS setup and columns utilised in the HDX-MS experiments of Hp 1−1 were maintained. 50pmol of TZ tryptic digest was injected and passed through the column at a flow rate of 200 μL/min of LC mobile phase containing 0.23% formic acid (FA) and a pH of 2.5. Following earlier instructions,16 the column's online deglycosylation activity was assessed by comparing the signal intensities of the glycosylated and deglycosylated tryptic peptide EEQYNSTYR to those of the ATII (angiotensin II) peptide internal standard. The following equation was used to compute the deglycosylation efficiency:

An effort at complete deuteration labelling was made by subjecting the solution to deuteration for 50 hours and then incubating to a pH of 2.5, along with 200 mM TCEP and urea. The Waters Enzymate BEH pepsin column was used to digest the samples online at 20 °C. After that, they were deglycosylated online at 20 °C using a column that was positioned immediately downstream and packed with immobilised PNGase Rc (2.1 × 20 mm2, Wuxi GALAK Chromatography Technology Co, China). The peptides were held on a Waters BEH C18 VanGuard precolumn for three minutes in buffer A, which contains 0.1% formic acid and has a pH of 2.5, at a flow rate of 100 μL/min. Using a linear gradient of buffer B (0.1% formic acid in acetonitrile ~ pH 2.5) and a flow rate of 40 μL/min, peptides were separated on a Waters BEH C-18 analytical column. To minimise back exchange, all separation and trapping were carried out at around 0 °C. Whether a state had or did not have a PNGase column, all data were acquired at the identical flow rates.

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ISSN: 0976-0172
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An effort at complete deuteration labelling was made by subjecting the solution to deuteration for 50 hours and then incubating In percentage terms, the glycosylation efficiency may be expressed as ((I0 I)/I0) multiplied by 100%.

VISTA when 2 M urea-d4 in water is present. A quenching solution consisting of 0.4 M TCEP dissolved in 100 mM ammonium formate buffer, pH 2.6, was added to the samples after they had been incubated at 20 °C. The ratio of v/v was 1:1. Unfortunately, under these settings, full deuteration was not possible.

Characterization of c-Met Epitopes by High-Performance X-Ray MS. The Orbitrap Fusion Lumos from Thermo Fisher Scientific in the US and the Acquity UPLC system with HDX from Waters Corporation in the UK were used to conduct research on C-Met (Human, Proteros Biostructures GmbH, Germany). A LEAP HDX autosampler from Trajan was used to prepare the samples.

To conduct the HDX of both bound and unbound c-Met, a dilution of c-Met into a labelling buffer (20 mM Histidine, 140 μM NaCl, 90% D2O, pH(read) 6.0) was used. The resulting concentration of 0.35 μM was supplied by Roche Diagnostics GmbH, Germany. At different times, the 60 μL labelling process was stopped by adding ice-cold quench buffer (170 mM KH2PO4, 250 mM TCEP, 4 M urea, pH 2.8) in a 1:1 ratio. We performed online proteolysis at 20 °C using a cooled (0 °C) HDX-MS UPLC system after injecting HDX samples into the system. in where I is the normalised intensity of the glycopeptide obtained with the PNGase Rc column and I0 is the intensity obtained without the column. The Findings and Explanation Immobilisation of PNGase Rc. High yield and purity of PNGase Rc were achieved by recombinant expression in E. coli, using the previously published protocol.17, 19 The PNGase Rc Lys residues were used to immobilise POROS AL beads. Examining the PNGase Rc structure that was created by AlphaFold showed that all of the Lys residues were situated outside of the active site that had been expected (from examining the active sites of PNGase F and A, Figure S1).17 We evaluated the immobilisation effectiveness (ψ) by measuring the concentration of PNGase Rc in the supernatant after bead coupling. Figure S2 shows the HPLC analysis of standard dilutions of PNGase Rc utilising a C4 column. The concentration was then calculated using a calibration curve that used the UV 280 nm peak region. With an enzyme-to-bead (E:B) ratio of 1:10, the immobilisation efficiency was around 39%, but with an E:B ratio of 1:20%, it was 95%. Thus, with an E:B ratio of 1:20, it produces.

ISSN: 0976-0172
Journal of Bioscience AndTechnology
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Figure 1. (A) Structure of the Hp 1−1 heterodimer (AlphaFold) containing both the alpha and beta chain. Disulfide bonds are shown in yellow (intra) and red (inter) sticks, while glycans are shown in orange sticks and labeled. (B) Deglycosylation of Hp 1−1 (left) and Trastuzumab (right) by PNGase Rc analyzed by SDS-PAGE. Lanes 1 and 8 correspond to samples without PNGase Rc at pH 3.5 and 7.5, respectively. Lanes 2 and 7 correspond to samples following in-solution deglycosylation by free PNGase Rc and free PNGase F, respectively. Lanes 3 and 4 correspond to samples from in-solution deglycosylation using PNGase Rc coupled beads made with and without the use of sodium sulfate, respectively. Lanes 5 and 6 correspond to samples collected following online deglycosylation under HDX-MS compatible conditions (pH 2.3) using the PNGase Rc column at a flow rate of 100 μL/min. Lane M corresponds to the LMW protein standard. (C) Extracted ion chromatograms of the oxonium ion *m*/ *z* 366.13 (Hex-Hex-Nac glycan fragment) from LC-MS/MS analysis of a sample of Hp 1−1 (in 0.5 M glycine, 2 M urea, 100 mM TCEP, pH 2.3) following online proteolysis with or without downstream online deglycosylation using the PNGase Rc column at HDX-MS compatible conditions.quantitative coupling of the enzyme to the beads but most likely not the whole accessible surface is modified with enzyme. The activity of immobilized PNGase Rc was assessed as described previously using an LC-MS assay monitoring the deglycosylation of the intact IgG1 antibody Trastuzumab (TZ) that has a N297 glycosite.[9,16,19] In initial testing, immobiliza- tion with 1:10 E:B resulted in beads with higher deglycosylation efficiency than beads produced by immobiliza- tion with 1:20 E:B (Figure S4). However, upon further optimization (SI), an immobilization procedure was developed that required less enzyme (1:25 E:B) while producing beads with high deglycosylation efficiency (Figure 1) and the resulting PNGase Rc-functionalized beads were packed into HPLC/UPLC compatible columns (2.1 × 20 mm^2).

Haptoglobin 1−1 (Hp 1−1) was chosen as another model substrate to assess the deglycosylation activity of immobilized PNGase Rc. Hp 1−1 is one of the three phenotypes (Hp 1−1, Hp 2−1, Hp 2−2) of the human glycoprotein haptoglobin. Hp 1−1 is a heterotetramer composed of two heterodimers, each with a light α-chain and a heavy β-chain (Figure 1A). The two αβ heterodimers are held together by disulfide-bonds.[20] Four glycans are attached to the β-chain and are located at residues N23, N46, N50, and N80. PNGase Rc immobilized on beads was capable of deglycosylation of all N-linked glycans of Hp 1−1 at pH 3.5 and 2.3 as monitored by SDS-PAGE (Figure 1B) and LC-MS/ MS analysis (Figure 1C), respectively.[21] Upon deglycosylation, the band corresponding to glycosylated Hp 1−1 beta chain (∼45 kDa) was converted into bands corresponding toremoval of 1, 2, 3, and all 4 N-glycans (∼30 kDa for fully deglycosylated form). In-solution deglycosylation of Hp 1−1 by PNGase Rc at pH 3.5 was more effective compared to PNGase F at pH 7.4 under otherwise similar conditions. Moreover, efficient deglycosylation of the heavy chain of Trastuzumab could also be achieved by immobilized PNGase Rc (Figure 1B). Furthermore, LC-MS/MS analysis of Hp 1−1 following online proteolysis and deglycosylation at HDX-MS compatible conditions using the PNGase Rc column showed the absence of glycan-specific oxonium ions indicating highly efficient deglycosylation of all Hp 1−1 glycopeptides.

HDX-MS of Glycoproteins of Varying Complexity. To evaluate the implementation of the PNGase Rc column into HDX-MS setups in different laboratories, PNGase Rc columns were sent to three separate research laboratories and were used to perform HDX-MS experiments on three different glycoproteins of significant complexity in terms of disulfide- bonds and N-linked glycans. Namely, Hp 1−1, V-domain Ig suppressor of T cell activation transmembrane protein (VISTA) and transferrin.

As mentioned previously, Hp 1−1 forms a disulfide-bonded heterotetramer containing two α-chains and two β-chains with both α-chains linked to each other and to one β-chain, through disulfide bridges located at residues βC105 and αC72 for the α−β chain connections, and at residues αC15 and αC15 for the α−α chain connection (Figure 1A). In addition to those interchain disulfide bonds, each β-chain contains two intra- chain disulfide bonds at residues C148−C179 and C190− C219, and each α-chain contains one intrachain disulfide bond at residues C34−C68. Four glycans of Hp 1−1 are attached to each β-chain (N23, N46, N50, and N80).

VISTA consists of 180 residues with 6 N-glycosites at residues N17, N59, N76, N96, N103, and N158. VISTA also

ISSN: 0976-0172
Journal of Bioscience AndTechnology
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contains 3 intra disulfide bonds located at residues C10−C146, C22−C114, and C51−C113. Transferrin contains 679 residues with 2 N-glycosites at residues N413 and N614 and 19 disulfide bonds located throughout its structure.

To achieve maximal sequence coverage, HDX-MS analysis of each protein was performed with different concentrations of TCEP and urea in the quench buffer to facilitate denaturation and reduction of disulfide bonds. In all HDX-MS experiments, the PNGase Rc column was placed immediately downstream of the proteolytic column (Figure 2).

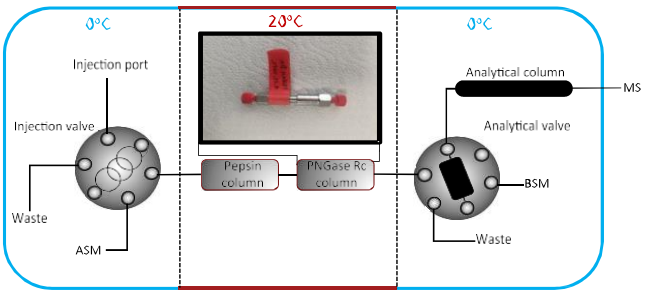

Figure 2. Integration of the PNGase Rc column within a conventional commercially available HDX-MS setup.

The α-chain attained a sequence coverage of 91.6% and the β-chain a sequence coverage of 84.5% for Hp 1−1, without online deglycosylation (Table 1, Figures S6). We were able to identify 66 peptides for the β-chain, but none of them covered the four N-linked glycosite areas (N23, N46, N50, and N80). There were two peptides found at the N80 N-linked glycosylation site that match to the nonglycosylated version. Figure 3 and Table 1 show that the α-chain had sequence coverage of 100% and the β-chain of 98.8% during HDX-MS using the PNGase Rc column. A total of 86 peptides with a redundancy of 5.33 were discovered for the β-chain. Figure 3 shows that the most noteworthy outcome was the complete coverage of all four glycosites, with high redundancy at glycosites N46,N50,andN80. The anticipated structure of Hp 1−1 by AlphaFold was mostly confirmed by its HDX. Figures S7 and S8 show that across the 10-hour time period, the areas surrounding N23 showed sluggish HDX, in contrast to the regions around N46, N50, and N80, which exhibited fast deuterium absorption. The location of N23 inside a β-strand is in agreement with the N-glycan sites within the β-chain structure. This area's solid hydrogen bonding makes it more stable and protected, which in turn slows down HDX. Figures S7 and S8 show that all of the other N-glycosites are situated in loop regions, which are known for their flexibility and the rapidity of their HDX. Figure 4, Figure S9, and Table 1 indicate that all glycosylated areas were covered by HDX-MS analysis using the PNGase Rc column of VISTA and transferrin. This deglycosylation was seen at all N-glycosites in the proteins. Figures S10 and S12 show that the N-glycosites in the more stable β-strands exchange more quickly than those in the more flexible loops, and this finding is consistent with the HDX of both VISTA and transferrin, which agrees well with their crystal structures. In general, the HDX-MS findings on these three glycoproteins that form disulfide bonds demonstrated that the addition of

Table 1. Overview of Glycoproteins Subjected to HDX-MS Analysis with the PNGase Rc Column

glycoprotein	sequence converage (%) without PNGase Rc column	no. of peptides (without PNGase Rc column)	sequence coverage (%) with PNGase Rc column	no. of peptides (with PNGase Rc column)	no. of N-linked glycosites	no. of deglycosylate d N-linked glycosites	quench conditions (after 1:1dilution)
c-Met	91	721	98	755	6	6	125 mM TCEP and 1.7 M urea pH 2.8
haptoglobin 1−1 (α chain)	92	9	100	10	0	0	125 mM TCEP and 2 M urea pH 2.3
haptoglobin 1−1 (β chain)	84.5	66	98	81	4	4	125 mM TCEP and 2 M urea pH 2.3
transferrin	65	87	71	92	2	2	100 mM TCEP and 2 M urea, pH 2.5
VISTA	73	50	97	110	6	6	200 mM TCEP and 2 M urea pH 2.6

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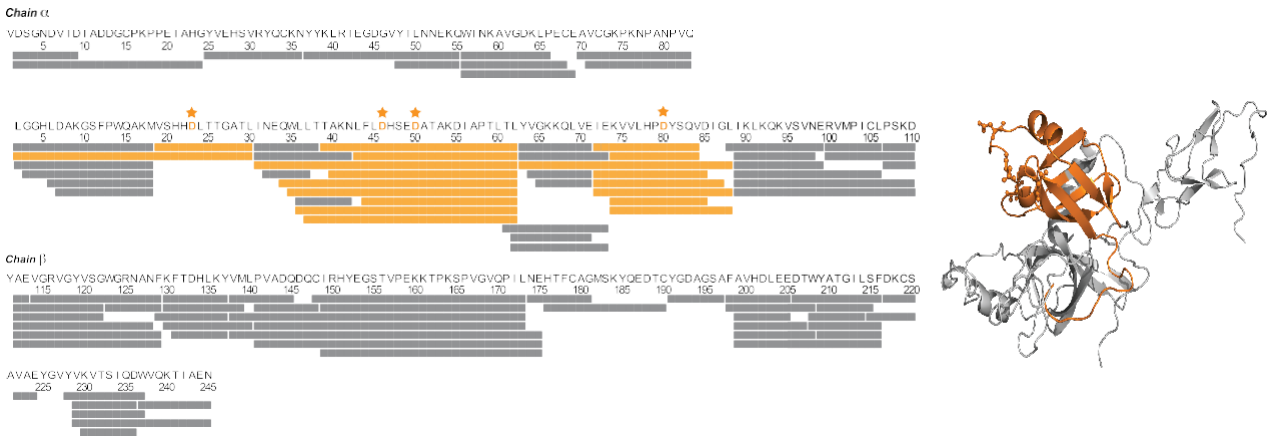

Figure 3. Sequence coverage map of Hp 1−1 obtained during HDX-MS with online deglycosylation by PNGase Rc. N-Linked glycosites are highlighted in orange and marked by stars. Deglycosylated peptides are colored in orange.

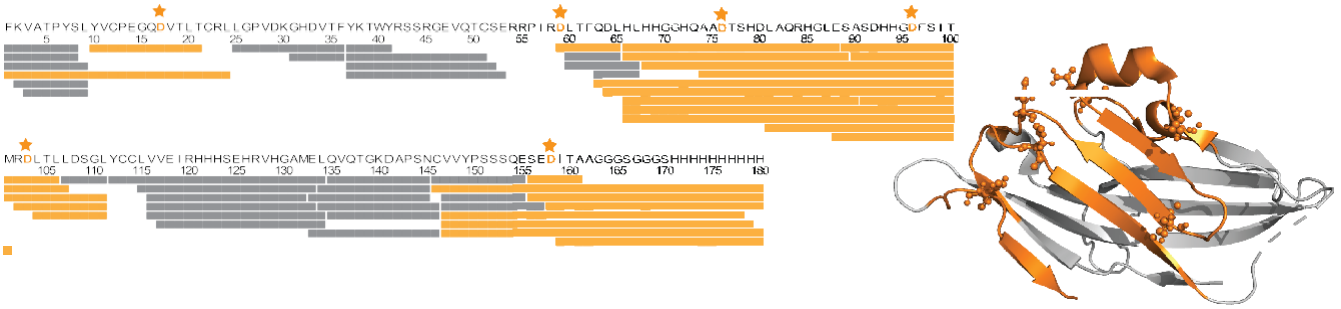

Figure 4. Sequence coverage map of VISTA during obtained during HDX-MS with online deglycosylation by PNGase Rc. N-linked glycosites are highlighted in orange and marked by stars. Deglycosylated peptides are colored in orange.Epithelial Transition factor), also named Hepatocyte Growth Factor Receptor (HGFR) is generated by the c-Met proto- oncogene and is regarded as a tumor assisted antigen and is an important signaling receptor for the tumor proliferating protein HGF (hepatocyte growth factor).[22] Inhibition of c-Met has been indicated as a promising strategy to treat certain cancer forms.[23]

The epitope mapping experiment was performed on the extracellular domain of c-Met (525 amino acids) that comprises an α- and a β-chain, with the α chain containing 4 N-glycans and 4 intrachain disulfide bonds and the β-chain containing 2 N-glycans and 1 intrachain disulfide bond. The two chains are connected by a disulfide bond between the C274 (chain α) and C36 (chain β).

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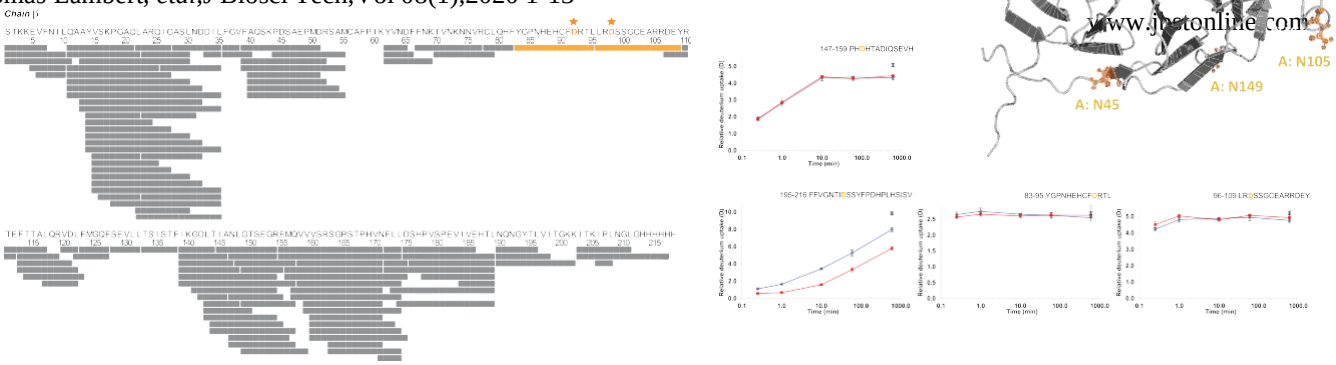

Figure 5. HDX-MS epitope mapping of c-Met with online deglycosylation. (A) Sequence coverage map of c-Met, with highlighted in red showed decreased HDX upon mAb binding. (B) HDX of all deglycosylated peptides spanning an N-linked glycosite. c-Met with mAb is shown in red curves, c-Met without mAb is shown in blue curves and the maximally labeled control sample is shown as a cross. Error bars represent the standard deviation from three technical replicate measurements. (C) Overlay of the HDX-MS data onto the crystal structure of c-Met (PDB: 1SHY) with the epitope region displaying reduced HDX upon mAb binding (res. 195 -216) shown in red and N-glycosites highlighted as orange sticks. deglycosylated peptides that contain an N-linked glycosite (denoted by orange star) highlighted in orange. Peptides uptake plots

The PNGase Rc-enabled HDX-MS epitope mapping experiment was done using a quench buffer containing TCEP and urea as this had previously been shown to yield the maximum sequence coverage of c-Met.[16] The HDX-MS experiment allowed identification and extraction of HDX information from 124 peptides covering 96.8% of c-Met. Importantly, this included peptides covering all 6 N-glycosites. Overall, the HDX of c-Met was in good agreement with the available crystal structure, with slow HDX in regions of stable structure and faster exchange in loops (Figure S11). For the six glycosylated regions, slow HDX was observed in regions 41− 50, 102−117, and 196−218 indicating the presence of significant secondary structure, with fast HDX in regions 83−95, 96−100, and 147−159 corresponding to dynamic loops. Upon binding the mAb, significant reductions in HDX was observed in several peptides spanning the region of the N202 glycosite. None of the other regions of c-Met, including the 5 other N-glycosylated regions showed significant changes in HDX (Figure 5B). Region 196−218 is thus clearly linked to binding of the mAb and it is very likely that the epitope is contained in this region. Notably, this region was previously inaccessible for HDX-MS analysis without the incorporation of the PNGase Rc column to allow efficient deglycosylation. A visual representation of the epitope when overlaid onto the crystal structure of c-Met (Figure 5C) shows that the region is highly solvent exposed and several residues are well-positioned for direct interaction with the mAb.

Reusability and Activity Assessment of the PNGase Rc Column. Reusability and activity of the PNGase Rc column was measured through the developed LC-MS assay using tryptic glycopeptides from the IgG1 Trastuzumab asubstrate.16 The PNGase Rc column's activity was examined at various temperatures by measuring its efficiency at both room temperature and 0 °C. Figure 6B shows that the deglycosylation effectiveness dropped from 99% at normal temperature to 72% at 0 °C. Nevertheless, even at 0 °C, a significant level of deglycosylation could be achieved, making it an ideal candidate for an HDX-MS experiment, should one be necessary. It should be noted that the HDX-MS studies for Hp 1−1, transferrin, and c-Met were conducted at room temperature with the column placed outside the cooling box, whereas the HDX-MS experiment for VISTA was carried out with online glycolysis at 0 °C. After then, for 114 days, we used LC-MS to track the concentrations of the glycosylated and deglycosylated forms of the glycopeptide to see if the PNGase Rc column could be reused. The column was often subjected to TCEP and urea during this time period due to its use in other HDX-MS research. With a deglycosylation efficiency of over 98%, the column performed well and remained stable throughout. Using various batches of substrate on days 68 and 110 might explain the fluctuations in glycosylated peptide intensity between days 34, 68, and 110. The overall deglycosylation efficiency remains high and basically unaltered even after 114 days

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of regular operation of the PNGase Rc column, as shown by both the intensities of the deglycosylated and glycosylated peptides (Figure 6C,D). Crucially, the column was routinely exposed to HDX-MS samples containing urea and TCEP during this time (Table 1).

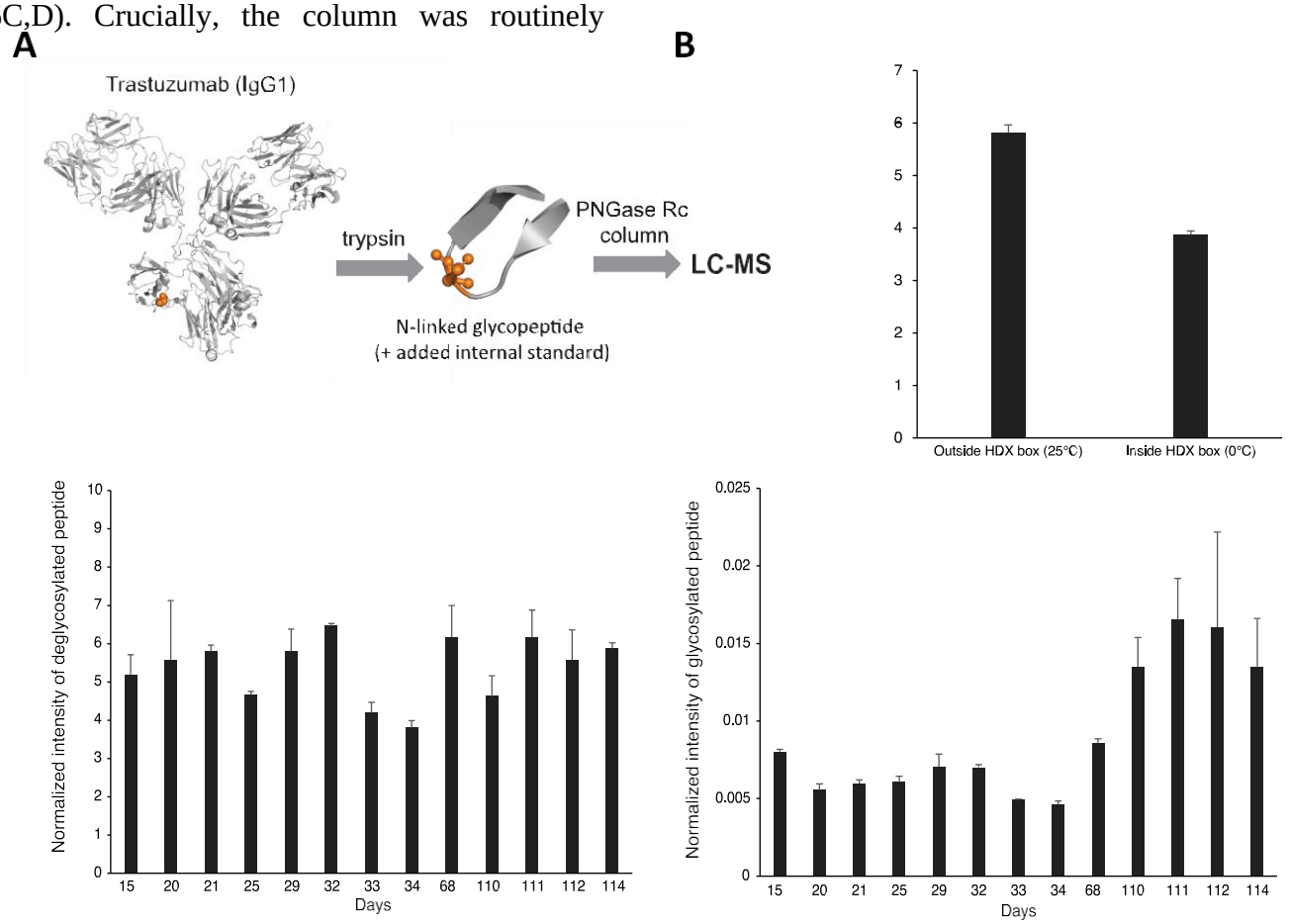

Figure 6. Monitoring the reusability and temperature dependence of the PNGase Rc column. (A) LC-MS assay used to monitor the activity of the PNGase Rc column. Intensities of the IgG1 N-linked glycopeptide EEQYNSTYR were normalized to intensities of the internal standard Angiotensin II. (B) Activity of the PNGase Rc column when placed inside (*0 °C*) and outside the HDX box (*20 °C*) at a flow rate of 200 *µ*L/min. (C,D) Reusability of the PNGase Rc column over 114 days (monitored by LC-MS assay). During this period, the PNGase Rc column was used regularly in various HDX-MS experiments exposing it to TCEP and urea (see Table 1). The column was used and equilibrated from day 0 to day 15.

The figure in question is 7. Reversibility of this decline in deglycosylation efficiency was a major finding. In addition, as shown earlier, the column demonstrated complete capability of performing thorough deglycosylation of the various glycoproteins in HDX-MS tests using quench buffers containing high concentrations of TCEP and urea (Table 1). Because of its excellent reusability and resistance to urea and TCEP, the newly designed PNGase Rc column should greatly benefit HDX-MS glycoprotein analysis. In a series of small-scale tests, we also examined how the PNGase Rc column performed when exposed to 1 M GndHCl or a mixture of 1 M GndHCl and 50 mM TCEP. A decrease in activity comparable to that caused by 400 mM TCEP was seen. The same holds true for the reversibility of this activity decrease (data not shown). We are aware that a study detailing the effective creation of a PNGase Dj column for use in HDX-MS was published simultaneously with our work (25,26).27 We have previously worked on immobilising PNGase Dj in a microprocessor; our work expands that method to a column format. Consequently, it seems that PNGase Rc and Dj may be immobilised on-column to facilitate glycoprotein

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analysis using HDX-MS. Figure 2 shows the present work using the PNGase Rc column downstream of the pepsin column; however, the column might instead be positioned upstream of the pepsin column to enable deglycosylation at the entire glycoprotein level instead of by glycopeptides. In our small-scale research, we compared deglycosylation before and after digestion, but we couldn't find any significant differences in terms of sequence coverage , number of peptides, or deglycosylation of N-glycosites. But it's worth noting that this might depend on proteins.

IN THE END

We demonstrate here that the designed PNGase Rc column allows for the online removal of N-linked glycans during glycoprotein HDX-MS with remarkable efficiency. In order to facilitate rapid online digestion and deglycosylation, the PNGase Rc column was easily integrated into each lab's standard HDX-MS configuration, positioning itself directly downstream from the pepsin column. VISTA (6 N-linked glycans, 3 S−S bonds), transferrin (2 N-linked glycans, 4 S−S bonds), Hp 1−1 (4 N-linked glycans, 7 S−S bonds), and c-Met (6 N-linked glycans, 5 S−S bonds) were the four complex glycoproteins that underwent HDX-MS experiments, which necessitated harsh quench conditions (up to 2 M urea and 200 mM TCEP). We were able to measure the HDX of all glycosylated areas that were previously unreachable without the PNGase Rc column, which dramatically boosted sequence coverage in all situations. One of the most difficult glycoprotein drug targets, c-Met, may have its epitope mapped using the PNGase Rc column. The use of a PNGase Rc column during HDX-MS led to a small increase (<2%) in the loss of deuterium label (back-exchange), as shown by the control studies. Even when popular quench-buffer additives like TCEP and urea are present, the column maintains a high level of activity. At doses ranging from 200 to 400 mM TCEP and 2.5 to 3M urea, there was a noticeable decrease in activity, however it is crucial to note that this decrease is completely reversible. The column's performance was steady for more than a hundred days and was consistent across many injections of TCEP and urea samples.

We predict that many so far difficult glycosylated proteins might be amenable to HDX-MS study of conformation and ligand interactions with the simple addition of the PNGase Rc column to standard HDX-MS setups. Many promising but as-yet-undiscovered therapeutic targets may be located among these proteins. The column's ability to provide rapid online deglycosylation of peptides or proteins before LC separation and MS makes it a potential component of various bottom-up proteomic procedures.

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Author Contributions

K.D.R., A.Z., M.K. designed the study. P.K. and D.D. produced PNGase Rc. M.G., P.P., and P.N. performed immobilization with efficiency and enzyme activity tests. P.N. performed SDS- PAGE analysis of deglycosylation of Hp 1−1. Luisa S performed HDX-MS of Hp 1−1. M.G. performed HDX-MS of VISTA. L.S. performed HDX-MS of transferrin. T.L. and

C.W. performed HDX-MS epitope mapping of c-Met. C.W., U.R., J.B., A.Z., and K.D.R. supervised the study. The draft of the manuscript was written by T.L. and K.D.R., which was revised through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Petr Pompach and Petr Novak are founders of

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ACKNOWLEDGMENTS

The research was supported by grants from the European Research Council (ERC Consolidator Grant no. 101003052, to K.D.R.) and the Technology Evaluation & Development programme for biologics at Roche. The HDX-MS investigation of VISTA was carried out using an Orbitrap Eclipse Tribrid Mass Spectrometer and an RSLC U3000 HPLC system, which were supported by the European Regional Development Fund (ERDF) and the State Ministry of Baden-Wuerttemberg for Economic Affairs, Labour, and Tourism (#3- 4332.62-NMI/69). Our sincere appreciation goes out to the Instruct-CZ Centre, CIISB's structural mass spectrometry facility, which has been generously supported by the MEYS CR (LM2023042) and the European Regional Development Fund-Project "UP CIISB" (No. CZ.02.1.01/0.0/0.0/ 18_046/0015974). Our sincere appreciation goes out to Jana Novakova of Affipro s.r.o., who kindly produced the PNGase Rc column.

REFERENCES

(1) The Characteristics and Localization of Human Protein Antibody Targets by Bakheet and Doig. The citation is from the 2009 issue of Bioinformatics, volume 25, issue 4, pages 451-457.
(Shental-Bechor and Levy, 2012). Glycoprotein Folding: A Biophysical Perspective on the Glycosylation Code. Publication: Current Opinion in Structural Biology, 2009, Volume 19, Issue 5, Pages 524-533.
The Biological Functions of Glycans (3) Varki, A. The Journal of Glycobiology, 2017, 27, 2.
The role of evolutionary forces in shaping the golgi glycolysis machinery: the universality of cell surface glycocans in all living cells (Varki, A., 2014). Article a005462 from Cold Spring Harbour Perspectives on Biology, 2011, volume 3, issue 6.
(5) The paper "Conformational Analysis of Complex Protein States by Hydrogen/Deuterium Exchange Mass Spectrometry (HDX-MS): Challenges and Emerging Solutions" was written by Trabjerg, Nazari, and Rand. 106, 125-138, 2018, TrAC - Trends in Analytical Chemistry.
(6) The use of hydrogen-deuterium exchange mass spectrometry to provide structural insight into multi-protein signalling assemblies was proposed by Harrison and Engen. Current Opinion in Structural Biology, 2016, 41, 187–193.
(7) The authors are Vasas and Burke. Examining the Dynamic Control of Peripheral Membrane Proteins using Hydrogen Deuterium Exchange-MS (HDX-MS)! The citation for the article is Biochemical Society Transactions, 2015, 43, 773−786.
(8) Masson, G. R.; Burke, J. E.; Ahn, N. G.; Anand, G. S.; Borchers, C.; Brier, S.; Bou-Assaf, G. M.; Engen, J. R.; Englander, S. W.; Faber, J.; Garlish, R.; Gryphon, P. R.; Gross, M. L.; Guttman, M.; Hamuro, Y.; Heck, A. J. R.; Houde, D.; Iacob, R. E.; Jorgensen, T. J. D.; Kaltashov, I. A.; Klinman, J. P.; Konermann, L.; Man, P.; Mayne, L.; Pascal, B. D.; Reichmann, D.; Skehel, M.; Snijder, J.; Strutzenberg, T. S.; Underbakke, E. S.; Wagner, C.; Wales, T. E.; Walters, B. T.; Weis, D. D.; Wilson, D. J.; Wintrode, P. L.; Zhang, Z.; Zheng, J.; Schriemer, D. C.; Rand, K. D. Recommendations for Performing, Interpreting and Reporting Hydrogen Deuterium Exchange Mass Spectrometry (HDX- MS) Experiments. Published in the journal Nature Methods in 2019, volume 16, issue 7, pages 595 to 602.
(9) The authors Jensen, Comamala, Trelle, Madsen, Jørgensen, and Rand conducted a study on N-linked glycoproteins and how PNGase a may remove N-linked glycosylations at acidic pH, which allowed for the analysis of these glycoproteins using hydrogen/deuterium exchange mass spectrometry. The article was published in 2016 in the journal Anal. Chem. and is numbered 88 (24), 12479−12488.
The authors of the tenth paper are Comamala, G., Madsen, J. B., Voglmeir, J., Du, Y.-M., Jensen, P. F., Østerlund, E. C., Trelle, M. B., Jørgensen, T. J. D., and Rand, K. D. H+Enables Hydrogen/Deuterium Exchange Mass Spectrometry Analysis of N-Linked Glycoproteins via Acidic Glycosidase PNGase Deglycosylation. The specific reference is "J. Am. Soc. Mass Spectrom. 2020, 31 (11), 2305−2312."
Lee, K. K.; Gottman, M.; Scian, M. Anal. Chem. 2011, 83 (19, 7492−7499) describes the use of mass spectrometry to trace hydrogen/deuterium exchange at glycoprotein glycan sites.
(12) The authors are Ford, K. L., Zeng, W., Heazlewood, J. L., and Bacic, A.
Protein N-Glycosylation Characterization via Tandem Mass Spectrometry with Complementary Fragmentation Methods. In: Front. Plant Sci. 2015, vol. 6, no. 18, pp. 147055.
Thirteen, Mechref, Y. Analysing Glycopeptides using CID/ETD Mass Spectrometry. Current Protocols in Protein Science, 2012, 68 (1). The electronic journal article has the DOI: 10.1002/ 0471140864.ps1211s68.
14. Rand, K. D., Zehl, M., and Jørgensen, T. J. D. Overcoming Gas-Phase Hydrogen/Deuterium Scrambling: Measuring the Hydrogen/Deuterium Exchange of Proteins at High Spatial Resolution by Mass Spectrometry. Journal of Applied Chemistry, 2014, 47 (10), 3018–3027.
(15)Protein Hydrogen Exchange Detected at Single-Residue Resolution using Electron Transfer Dissociation Mass Spectrometry by Rand, K. D., Zehl, M., Jensen, O. N., and Jørgensen, T. J. D. The article was published in 2009 in the journal Anal. Chem. and the number of pages ranged from 5577 to 5584.
Hydrogen/Deuterium Exchange Mass Spectrometry with Integrated Electrochemical Reduction and Micro-chip-Enabled Deglycosylation for Epitope Mapping of Heavily Glycosylated and Disulfide-Bonded Proteins (16) (Comamala, G.; Krogh, C. C.; Nielsen, V. S.; Kutter, J. P.; Voglmeir, J.; Rand, K. D.). The article is published in the journal Anal. Chem. in 2021 and can be found on pages 16330α-16340.
(17) PNGase H+ Variant from Rudaea Cellulosilytica with Improved Deglycosylation Efficiency for Rapid Analysis of Eukaryotic N-Glycans and Hydrogen Deuterium Exchange Mass Spectrometry Analysis of Glycoproteins, authored by Guo, R. R.,

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Zhang, T. C., Lambert, T. O. T., Wang, T., Voglmeir, J., Rand, K. D., and Liu, L. "Rapid Communications in Mass number one)A group of researchers including Wang, T., Gu, X. Q., Ma, H. Y., Du, Y. M., Huang, K., Voglmeir, J., and Liu, L. A New Highly Acidic Bacterial N-Glycanase, Discovered and Characterised, That Combines the Benefits of PNGase F and A. Science Reports 2014, volume 34, issue 6, pages 673 to 684. (19)This sentence lists the authors of the paper: Gramlich, Maier, Kaiser, Traenkle, Wagner, Voglmeir, Stoll, Rothbauer, and Zeck. Under difficult conditions, a new PNGase Rc enhances protein N-deglycosylation in bioanalytics and hydrogen-deuterium exchange coupled with mass spectrometry epitope mapping. Volume 94, Issue 9863, Anal. Chem. 2022. (20) The covalent structure of human haemoglobin: a serine protease homolog was described in a 1980 publication by Kurosky et al. in the Proceedings of the National Academy of Sciences, USA, volume 77, pages 3388–3392. (21)A team of researchers led by Guo, Comamala, Yang, Gramlich, Du, Y.M., Wang, Zeck, Rand, Liu, and Voglmeir discovered

Analysing Acidobacterial Genomes Rationally for Highly Active Recombinant PNGase H+ Variants. The article is published in the journal Front. Bioeng. Biotechnol. and has the DOI number 10.3389/fbioe.2020.00741.

(22) Matsuda, Y., Ohkuri, T., Oikawa, K., Ishibashi, K., Aoki, N., Kimura, S., Harabuchi, Y., Celis, E., and Kobayashi, H. C-To combat NK/T cell lymphomas, a new tumor-associated antigen called Met has been developed for use in T-cell immunotherapy. Publication: Oncoimmunology, Volume 4, Issue 2, Article Number: e976077, Journal: 2015. Spectrometry" 2022, 36 (21) Citation: 10.1002/rcm.9376. (

23. Mesteri, I.; Schoppmann, S. F.; Preusser, M.; Birner, P. Oesophageal adenocarcinoma, in contrast to squamous cell carcinoma, is associated with CMET overexpression, which in turn is associated with signal transducer and activator of transcription 3 activation and worse prognosis. Article published in the European Journal of Cancer in 2014, volume 50, issue 7, pages 1354-1360.

The Role of Disulfide Bonds in Endoplasmic Reticulum Protein Folding and Homeostasis (24), by Feige and Hendershot. Current Opinion in Cell Biology, 2011, 23, 167–175. (25). Lambert, T. O. T.; Gramlich, M.; Stutzke, L.; Smith, L.; Deng, D.; Kaiser, P. D.; Benesch, J.; Rothbauer, U.; Vankova, P.; Pompach, P.; Novak, P.; Koenig, M.; Wagner, C.; Zeck, A.; Rand, K. D. Allied Top, June 7, 2023, Proc. ASMS Conf. Mass Spectrom., WP 286, Development of a PNGase Rc Column for Efficient Online Deglycosylation of Complex Glycoproteins during HDX-MS. Texas (HOUSTON) May 4th, 2023 ASM S 2023.

(26)This list of authors includes: Lambert, T., Gramlich, M., Stutzke, L., Smith, L., Deng, D., Kaiser, P. D., Rothbauer, U., Benesch, J. L. P., Wagner, C., Koenig, M., Pompach, P., Novak, P., Zeck, A., and Rand, K. Making a PNGase Rc Column to Deglycosylate Complex Glycoproteins Online During HDX-MS. bioRxiv 2023, 2023.07.28.550801. hydrogen-deuterium exchange epitope mapping of glycosylated epitopes enabled by online immobilised glycosidase; O'Leary, T. R., Balasubramaniam, D., Hughes, K., Foster, D., Boyles, J., Coleman, K., and Gryphon, P. R. (2023, 95)