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Effects of *N*-butyldeoxynojirimycin and the Lec3.2.8.1 mutant phenotype on N-glycan processing in Chinese hamster ovary cells: Application to glycoprotein crystallization

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Abstract

Heterologous gene expression in either (1) the glycosylation-defective, mutant Chinese hamster ovary cell line, Lec3.2.8.1, or (2) the presence of the α-glucosidase inhibitor, *N*-butyldeoxynojirimycin facilitates the trimming of N-linked glycans of glycoproteins to single N-acetylglucosamine (GlcNAc) residues with endoglycosidase H (endo H). Both approaches are somewhat inefficient, however, with as little as 12% of the total protein being rendered fully endo H-sensitive under these conditions. It is shown here that the combined effects of these approaches on the restriction of oligosaccharide processing are essentially additive, thereby allowing the production of glycoproteins that are essentially completely endo H-sensitive. The preparation of a soluble chimeric form of CD58, the ligand of the human T-cell surface recognition molecule CD2, illustrates the usefulness of the combined approach when expression levels are low or the deglycosylated protein is unstable at low pH. The endo H-treated chimera produced crystals of space group $P3_121$ or $P3_221$, and unit cell dimensions $a = b = 116.4$ Å, $c = 51.4$ Å $\alpha = \beta = 90°$, $\gamma = 120°$, that diffract to a maximum resolution of 1.8 Å.

Keywords: CD58; cell–cell recognition; crystallization; deglycosylation; oligosaccharide processing

Recent years have seen a very rapid increase in the rate at which the crystal structures of biological macromolecules are solved, to the extent that it was recently estimated that the coordinates of new structures are being deposited at the Protein Data Bank (PDB) every 5 h (Wlodawer et al., 1998). For many, if not most crystallographic analyses, the production of well-diffracting crystals is now the key factor limiting progress toward high quality structure solutions. Many proteins of key interest are glycoproteins, and it has been recognized that the N-linked glycans present on these molecules can hinder their crystallization because they are flexible, heterogeneous, and unlikely to directly mediate crystal contacts; and because they obscure a substantial fraction of the potential crystal-contact forming protein surface (McPherson, 1982).

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A solution to this problem involves the expression of the protein of interest in mammalian cells in a way that prevents the processing of the glycans to complex forms, thereby rendering the product sensitive to endo H, which cleaves between the two GlcNAc residues at the base of N-glycans. With this approach, the protein is cotranslationally glycosylated in a normal fashion and is therefore likely to fold correctly in most instances, and the endo H-treatment of the protein leaves a single carbohydrate residue (GlcNAc) at each glycosylation sequon that may enhance overall protein solubility. Two methods based on this general approach have been established. In the first (Davis et al., 1993), glycoproteins are expressed in a mutant Chinese hamster ovary (CHO) cell line, Lec3.2.8.1, which is unable to effect N-glycan processing beyond the $Man_5GlcNAc_2$ stage (Stanley, 1981). The second method involves the use of the α-glucosidase inhibitor, *N*-butyldeoxynojirimycin (NB-DNJ), which prevents the initiation of processing and traps the N-glycans at the endo H-sensitive, $Glc_3Man_{7\text{-}9}GlcNAc_2$ stage (Davis et al., 1995).

These approaches have facilitated the crystallization and full structural analysis of soluble forms of the human and rat homologues of the T-cell surface recognition molecule CD2 (Jones et al., 1992; Bodian et al., 1994). However, experience with both approaches has indicated that the yields of fully endo H-sensitive material can be relatively modest, amounting in some instances to 12% or less of the total secreted protein. The combined use of these approaches, i.e., the expression of soluble recombinant glycoproteins in Lec3.2.8.1 lines cultured in the presence of NB-DNJ, is shown here to give, almost exclusively, fully endo H-sensitive product. The new protocol proved to be particularly useful for the crystallization of a chimeric form of CD58, the ligand of human CD2, which was expressed at relatively low levels and had limited solubility under the conditions of endo H-treatment.

Results

All of the glycoproteins used in this study, i.e., rat soluble CD2 (sCD2) (Jones et al., 1992), sCD58 (van der Merwe et al., 1994), human sCD2 (van der Merwe et al., 1994), human sCD80 (sCD80) (van der Merwe et al., 1997), and a chimeric form of CD58 (cCD58) consisting of domain 1 of CD58 and domain 2 of rat sCD2 (see Materials and methods below), are derivatives of cell surface molecules. These molecules are predicted to have similar structures composed of single V-set and C2-set immunoglobulin superfamily domains exhibiting various levels of glycosylation, as shown diagrammatically in Figure 1.

The expression of soluble glycoproteins in either Lec3.2.8.1 cells, or in CHO-K1 cells in the presence of 1.5 mM NB-DNJ, typically results in the generation of a mixture of glycoforms exhibiting variable sensitivity to endo H. For example, densitometric analysis of SDS-PAGE separated, endo H-treated preparations of rat sCD2 expressed in Lec3.2.8.1 cells, and of sCD58 expressed in CHO-K1 cells in the presence of NB-DNJ, reveals that only 30 and 12% of the total protein is rendered fully endo H-sensitive in each case, respectively (Fig. 2, lanes 6 and 9) (Table 1). While this represents a substantial increase in endo H-sensitivity over that of rat sCD2 expressed in wild-type CHO-K1 cells ($<$1%) (Fig. 2, lane 3) (Table 1), neither set of conditions gives complete inhibition of oligosaccharide processing beyond oligomannose forms. Since the target of NB-DNJ mediated inhibition of oligosaccharide processing, i.e., glucosidase I, and the steps in the glycosylation pathway defective in Lec3.2.8.1 cells, i.e., an undefined deficiency in sialic acid metabolism, defective CMP-sialic acid and UDP-galactose translocation, and disrupted N-acetylglucosamine transferase I (Stanley, 1981), are clearly distinct, it seemed likely that the combined effects of the two treatments might be complementary.

To test this possibility, the glycoforms generated under each set of conditions, and when the two sets of conditions are combined, were characterized. The released N-linked oligosaccharides of rat sCD2 expressed in wild-type CHO-K1 cells exhibit the expected pattern of heterogeneity associated with the synthesis of mostly complex-type glycans (Fig. 3A). The glucose unit values for the major peaks correspond to core fucosylated, mono- and disialylated biantennary glycans also found in human sCD2 and rat sCD48 expressed in these cells (Rudd et al., 1999). The pooled rat sCD2 oligosaccharides are largely insensitive to endo H digestion (Table 1).

Glycoprotein expression in Lec3.2.8.1 cells results in almost the complete absence of oligosaccharide processing beyond the $Man_5GlcNAc_2$ intermediate, as noted previously for both rat sCD2 and sCD4 (Davis et al., 1993) (Fig. 3B). However, it appears that mannosidase hydrolysis of this product in these cells produces significant amounts of $Man_3GlcNAc_2$ (Fig. 3B), a structure that is resistant to hydrolysis by endo H (Takahashi & Muramatsu, 1992). As a result, only 65% of the released oligomannose glycan pool and 36% of the total protein remain fully endo H-sensitive (Fig. 2, lane 6) (Table 1). Similarly, the incubation of CHO-K1 cells expressing sCD58 with NB-DNJ at relatively high concentrations (1.5 mM) is only partially effective in preventing complex-type glycan synthesis (Fig. 3C). The major peak, representing less than half the released glycans, corresponds to the endo H-sensitive $Glc_3Man_7GlcNAc_2$ glycoform previously shown to accumulate in inhibitor-treated CHO-K1 cells (Petrescu et al., 1997). Accordingly, only 45% of the released glycans and 12% of the protein remain fully endo H-sensitive (Fig. 2, lane 9) (Table 1). However, inhibition of α-glucosidase-mediated processing in the presence of threefold lower concentrations of NB-DNJ (0.5 mM), against the Lec3.2.8.1 genetic background, compensates for the deficiencies in NB-DNJ treatment and Lec3.2.8.1 expression alone. Under these conditions, both human sCD2 and sCD80 are substituted with equimolar amounts of $Glc_3Man_7GlcNAc_2$ and $Man_5GlcNAc_2$ (Fig. 3D,E). Between 92 and 94% of the total glycan pool, and virtually all of the protein, remains fully endo H-sensitive in each case (Fig. 2, lanes 12 and 15) (Table 1).

To take advantage of the enhanced endo H-sensitivity of glycoproteins expressed in Lec3.2.8.1 cells in the presence of low levels of NB-DNJ, a chimeric form of CD58 was expressed under these conditions. A large number of clones were screened and the best of these expressed cCD58 at levels of 2–3 mg/L (assuming

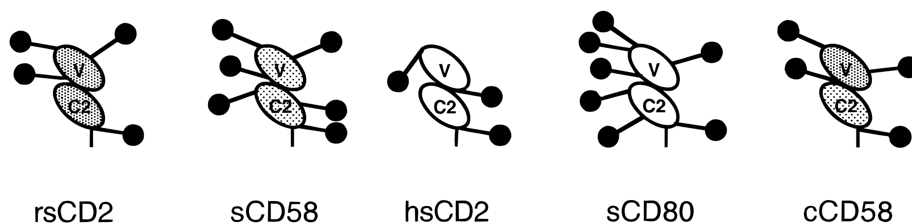

Fig. 1. Diagrammatic representations of the glycoproteins characterized in this study. IgSF domains are depicted with shaded or unshaded ovals and N-glycans represented by closed circles joined to the domains by thin lines. The IgSF domains are designated as V-set (V) or C2-set (C2) on the basis of sequence analysis (Williams & Barclay, 1988). Shown are soluble forms of rat CD2 (rsCD2), CD58 (sCD58), human CD2 (hsCD2), CD80 (sCD80), and the chimeric form of CD58 (cCD58) consisting of domain 1 of CD58 and domain 2 of rat sCD2.

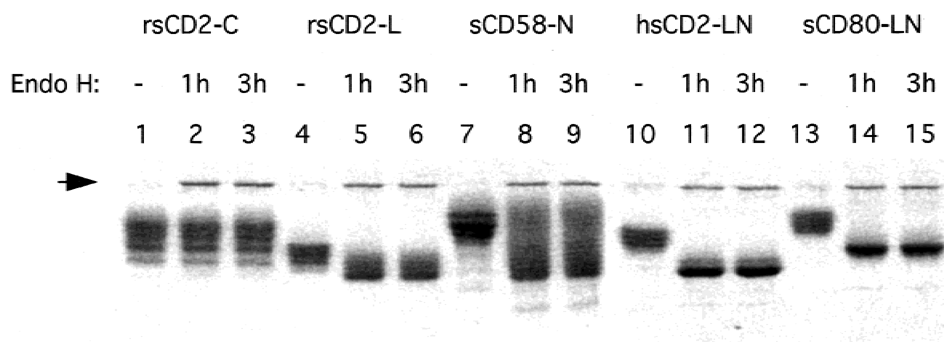

Fig. 2. Electrophoretic analysis of the endo H-treated glycoproteins. Protein at 0.5–1 mg/mL was digested with endo H at 8×10^4 U/mL for the indicated times prior to reduction, denaturation, and SDS-PAGE analysis on 15% gels. The arrow indicates the migration position of the endo H. The proteins analyzed are rsCD2-C (rsCD2 expressed in wild-type Chinese hamster ovary cells), rsCD2-L (rsCD2 expressed in Lec3.2.8.1 cells), sCD58-N (sCD58 expressed in wild-type Chinese hamster ovary cells in the presence of 1.5 mM NB-DNJ), hsCD2-LN (hsCD2 expressed in Lec3.2.8.1 cells in the presence of 0.5 mM NB-DNJ), and sCD80-LN (sCD80 expressed in Lec3.2.8.1 cells in the presence of 0.5 mM NB-DNJ).

$\epsilon = 1$ cm^2/mg). The endo H-sensitivity of cCD58 compensated for the relatively poor levels of expression of the glycoprotein (Fig. 4, lanes 1 and 2), and was comparable to that of human sCD2 and sCD80 expressed under identical conditions (Fig. 2). However, under the conditions of endo H digestion (pH 5.2), the deglycosylated protein proved to be relatively insoluble with >70% precipitating in the course of an 18 h incubation with endo H (Fig. 4, lanes 6 and 7). When the digestions were limited to 1 h duration and the pH of the protein solution then returned to pH 8, virtually all of the deglycosylated protein remained soluble (Fig. 4, lanes 4 and 5). It is also conceivable that increasing the pH of the incubation buffer may also have increased the yield of soluble protein. However, this was not attempted since it had been noted that the hydrolytic activity of endo H toward at least one glycoprotein, a soluble form of CD48, is fivefold lower at pH 6 than at pH 5.2 (S.J. Davis, unpubl. data).

In spite of the low levels of expression of the chimeric protein, and its insolubility under the conditions of deglycosylation, the endo H-sensitivity of the chimeric protein meant that sufficient deglycosylated protein could be obtained after 1 h incubation to permit crystallization trials. In contrast to the failure of native sCD58 to crystallize (E.A. Davies & S.J. Davis, unpubl. data), the chimeric form yielded crystals under a number of standard trial conditions, with the best growing in the presence of Tris-buffered ammonium sulfate. These crystals were of a trigonal morphology with typical dimensions of $0.15 \times 0.15 \times 0.4$ mm^3. In-house characterization indicated that the crystals were very well ordered, belonging to space group $P3_121$ (or an enantiomorph thereof) with room temperature unit cell dimensions of $a = b = 118.1$ Å, $c = 52.1$ Å. If it is assumed that the crystals contain one molecule in the crystallographic asymmetric unit, this gives a Matthews coefficient (V_m) of 4.38 Å^3/Da, which corresponds to a solvent content of approximately 72% (Matthews, 1968). Subsequent tests using synchrotron radiation indicated Bragg diffraction to 1.8 Å resolution. High resolution cryocrystallographic data collection at BM14 of the European Synchrotron Radiation Facility (ESRF) from two

Table 1. *Endo H sensitivity of the released oligosaccharides*

Protein	Cell line	NB-DNJ concentration in culture	% Endo H-sensitive oligosaccharides[a]	% Fully endo H-sensitive protein (predicted)[b]	% Fully endo H-sensitive protein (observed)[c]
rsCD2	CHO-K1	None	7	<1	<1
rsCD2	Lec3.2.8.1	None	65.6	19	36
sCD58	CHO-K1	1.5 mM	45.4	0.9	12
hsCD2	Lec3.2.8.1	0.5 mM	94.2	84	>95
sCD80	Lec3.2.8.1	0.5 mM	92.4	58	>95

[a]2AB-labeled oligosaccharides were analyzed by normal phase HPLC before and after digestion with endo H. Quantitation of the endo H-sensitivity of the oligosaccharides was done by comparing the areas of the peaks corresponding to each of the reaction products in the chromatogram (released 2-AB-labeled GlcNAc relative to nonhydrolysed 2-AB-labeled oligosaccharide).

[b]The proportion of the protein pool predicted to be fully endo H-sensitive was determined using the endo H-sensitivity of the released sugars (column 4) and the assumption that each site was processed independently (e.g., for sCD58, which has six sites, the predicted endo H-sensitivity of the protein, based on the endo H-sensitivity of the released sugars, is $(0.45)^6 = 0.009$).

[c]The actual proportion of the protein pool found to be fully endo H-sensitive was determined by densitometric analysis of the digestion products shown in Figure 2.

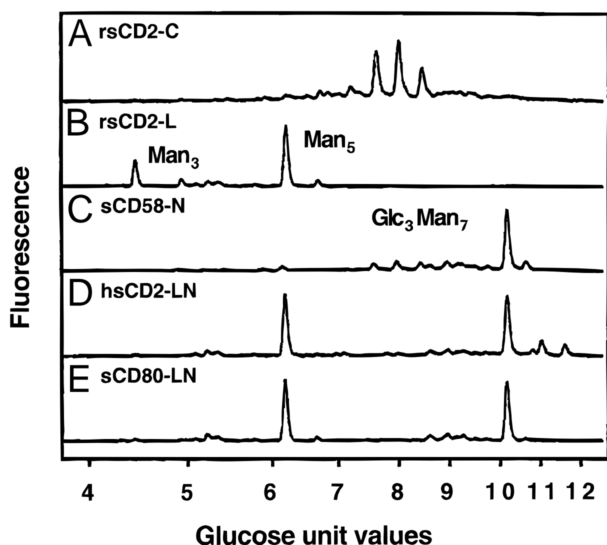

Fig. 3. HPLC analysis of the released N-glycans. The N-linked oligosaccharides released by hydrazinolysis of 0.5 mg of each protein were fluorescently labeled at their reducing end with 2-aminobenzamide and analyzed by normal phase HPLC. The nature of the glycans eluting in each peak was determined by comparison with the elution positions of standard oligosaccharides. The abbreviations used to identify each profile are those used in Figure 2.

crystals (cryocooled cell dimensions $a = b = 116.4$ Å, $c = 51.4$ Å) has yielded a 100% complete data set for the resolution range 30.0–1.8 Å (overall R_{merge} ($R_{\text{merge}(F)} = \sum|F - \langle F\rangle|/\sum F$) on intensities of 9.0% with 42.3% for the 1.86–1.8 Å resolution shell).

Discussion

The effects of the Lec3.2.8.1 phenotype and NB-DNJ on the endo H-sensitivity of the total oligosaccharide pool are essentially additive. That is, approximately two-thirds of the endo H-resistant glycans remaining after NB-DNJ inhibition are rendered endo H-sensitive by the defects in the Lec3.2.8.1 processing pathways, thus ensuring that the majority of glycans present within the total pool of secreted protein are rendered endo H-sensitive when the treatments are combined. The complementarity of these effects is consistent with the vectorial nature of oligosaccharide processing and the physical separation of the early and late stages of N-glycan processing in the endoplasmic reticulum and Golgi apparatus, respectively.

This analysis of the effects of the Lec3.2.8.1 phenotype and NB-DNJ required the examination of five different proteins, as no single protein has thus far been expressed under all conditions. It was assumed, however, that any protein- and site-specific effects would be dominated by the large effects on the oligosaccharide processing machinery of the Lec3.2.8.1 phenotype and NB-DNJ. This seems likely given that the profiles of the oligosaccharides released from both human sCD2 and sCD80 expressed under the combined conditions (Fig. 3D,E) are essentially identical, and in light of previous comparisons of the effects of the Lec3.2.8.1 phenotype on the glycosylation of rat sCD2 and sCD4 (Davis et al., 1993).

An unexpected result of the current analysis is the disparity between the observed endo H-sensitivity of the intact proteins and their predicted sensitivity based on the endo H-sensitivity of the released oligosaccharide pools. That is, for each glycoprotein preparation, the proportion exhibiting complete endo H-sensitivity was substantially higher than would have been predicted on the basis of the endo H-sensitivity of the oligosaccharide pools released from each protein by hydrazinolysis (Table 1). This disparity suggests that under conditions in which oligosaccharide processing is limited, either through the use of inhibitors or due to mutations in genes encoding the processing enzymes, processing at each site does not occur independently of that occurring at other sites. An explanation is that under these conditions the oligosaccharide processing enzymes are physically separated to some extent from the bulk protein-flow through the endoplasmic reticulum and Golgi apparatus. Accordingly, for a given molecule, processing is likely to go to completion or not to proceed at all, depending on whether the molecule reaches the processing enzymes. It is also possible that the discrepancy reflects glycan-dependent cooperative effects on the efficiency of cleavage by endo H, although this seems unlikely given that each of the glycoproteins was overdigested (Fig. 2).

The interaction of CD2 with its ligands has been highly informative in the context of the mechanisms of protein–protein recognition at the cell surface (Davis & van der Merwe, 1996; Davis et al., 1998). The crystal structures of rat and human sCD2 provided the first initial view of the complete extracellular region of a cell adhesion molecule, and the three- and two-dimensional affinities and kinetics of the interactions of CD2 with its murine and human ligands, CD48 and CD58, respectively, were the first to be rigorously characterized for any interacting pairs of cell surface molecules in solution and at the cell surface. Mutational analyses subsequently lead to the proposal that charged residues clustered in the ligand binding site of CD2 ensure that ligand recognition by CD2 is simultaneously weak and highly specific. The crystals of the chimeric, CD2-binding form of CD58, described and characterized here, will significantly enhance our understanding of the mechanisms of ligand recognition by CD2. The combined use of the Lec3.2.8.1 cell line and NB-DNJ represents an effective solu-

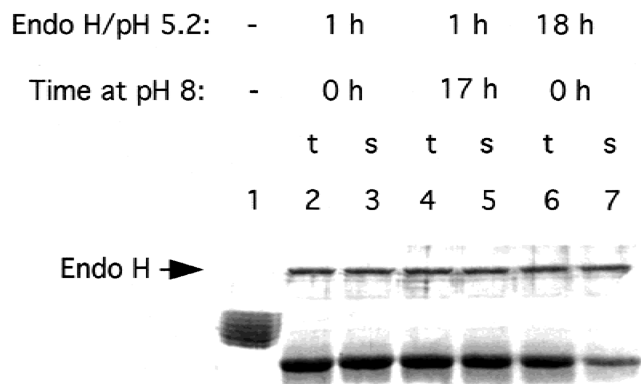

Fig. 4. Solubility of endo H treated cCD58. cCD58 was digested with endo H for 1 h as described in the legend to Figure 2. One-third of this material was then removed and the remainder split and either left at pH 5.2 for 17 h or transferred to buffer at pH 8.0 for 17 h. Prior to electrophoresis, aliquots of the three samples (t, total) were removed and the remainder centrifuged at 13,000 *g* for 5 min after which an equivalent aliquot of the supernatant (s) was removed. The “t” and “s” samples were then analyzed by SDS-PAGE analysis on a 15% acrylamide gel.

tion to the problem of efficiently deglycosylating heavily glycosylated, correctly folded glycoproteins prior to structural analysis.

Materials and methods

DNA constructs

For generating a chimeric form of CD58 (cCD58), the truncated cDNA encoding a soluble form of CD58 (van der Merwe et al., 1994) was subcloned into the vector pcDNA-3 (Invitrogen, Carlsbad, California). The codons for residues 94 [glu] and 95 [ser], which form part of the linker region between domains 1 and 2 of the mature polypeptide, were then changed to the Kpn I restriction site by in vitro mutagenesis using the Muta-Gene Phagemid Mutagenesis Kit version 2 (BioRad, Hercules, California). This allowed the sequence encoding domain 2 to be excised using the Kpn I site and a Bam H1 site downstream from the stop codon of the original construct and its replacement with the analogous rat sCD2 domain 2 sequence (Gray et al., 1993), engineered with a 5′ Kpn I site (at codons 99 [glu] and 100 [met]) using the polymerase chain reaction. The Kpn I site was then returned to the original CD58 cDNA codons by in vitro mutagenesis and the fidelity of the construct confirmed by dideoxy sequencing. The construct thus encoded a glycoprotein (cCD58) predicted to begin with the native CD58 NH_2-terminal sequence (FSQQ...), continuing to the CD58 domain 1-rat CD2 domain 2 junctional sequence (...LYVL′ EMVS...), and ending with the C-terminal sequence ...CPEK of domain 2 of rat CD2. The construct was subcloned into the pE14 vector of the glutamine synthetase-based gene expression system (Bebbington & Hentschell, 1987).

Protein expression and purification

The cCD58 construct was transfected into Lec3.2.8.1 cells using Pfx-8 lipids (Invitrogen). Clones resistant to 15 μM methionine sulfoximine were selected and one of these expressed the chimera at a level of 2–3 mg/L. For large-scale production of protein, the clone was grown in cell factories (Nunc, Roskilde, Denmark) in the presence of 0.5 mM *N*-butyldeoxynojirimycin (NB-DNJ, gift of Professor R.A. Dwek of the Glycobiology Institute, Oxford, United Kingdom). The protein secreted into the tissue culture supernatant was loaded onto an anti-CD58 antibody Bric-5 (Anstee et al., 1991) affinity column, which was then eluted with 0.1 M glycine, pH 2.5. The eluting fractions were immediately neutralized with one-tenth volume of 1 M Tris pH 8. Protein containing fractions were then pooled.

Oligosaccharide analysis

Samples of affinity purified protein (0.5 mg) were subjected to hydrazinolysis (Davis et al., 1993) and the released N-linked oligosaccharides fluorescently labeled at their reducing end with 2-aminobenzamide (2-AB) (Oxford Glycosystems, Abingdon, Oxon, United Kingdom). Labeled oligosaccharides were analyzed by normal phase high-performance liquid chromatography (HPLC) as described (Guile et al., 1996) both before and after digestion overnight with 5,000 U endo H (New England Biolabs, Beverly, Massachusetts) in the supplied incubation buffer. Quantitation of endo H-sensitivity was done by comparing peak areas for each of the reaction products in the HPLC chromatogram (released 2-AB-labeled GlcNAc relative to nonhydrolysed 2-AB-labeled oligosaccharide).

Protein deglycosylation and crystallization

The pH of the neutralized protein-containing solution was reduced to pH 5.2 by addition of one-thirtieth volume of 3 M sodium acetate, pH 5.2. The requisite amounts of endo H were then added to the mixtures and incubation carried out at 37 °C. The reaction was stopped by the addition of an equal volume of 1 M Tris, pH 8. The endo H-treated protein was then purified by gel-filtration chromatography (Sephadex G-100, Pharmacia, Uppsala, Sweden) and screened for crystallization using the Crystal Screen and Crystal Screen 2 crystallization kits (Hampton Research, Laguna Niguel, California). Crystallization trials were carried out at 20 °C by sitting drop vapor diffusion in MicroBridges® (Hampton Research). Typically, 1–4 μL drops of the protein, concentrated in 10 mM Tris, 150 mM NaCl, 0.05% NaN_3, pH 8 to the extent that the absorbance at 280 nm was 15, were mixed with an equal volume of the precipitant solution. Single crystals or microcrystalline precipitates appeared in 10 of 98 conditions tested. Crystallization conditions were optimized by dilution of the precipitant in water. The largest crystals grew in the presence of 1.1–1.5 M ammonium sulfate, 50 mM Tris pH 8.33.

X-ray diffraction data collection

Initial data were collected in house at 20 °C using an 18 cm MarResearch (Norderstedt, Germany) imaging plate detector mounted on a Rigaku RU200 rotating anode X-ray generator. Two high resolution data sets were collected on BM14 of the ESRF at 100 K using a 34.5 cm MarResearch imaging plate detector. For cryocrystallography the crystals were transferred into a cryoprotectant solution (consisting of the mother liquor supplemented with 15% glycerol) for 30 min prior to flash cooling using an Oxford Cryosystems Cryostream. All data were auto-indexed, integrated, and corrected for Lorentz and polarization effects with the program DENZO, and scaled and merged using the program SCALEPACK (Otwinowski & Minor, 1997).

Acknowledgments

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