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Production, crystallization and X-ray characterization of chemically glycosylated hen egg-white lysozyme

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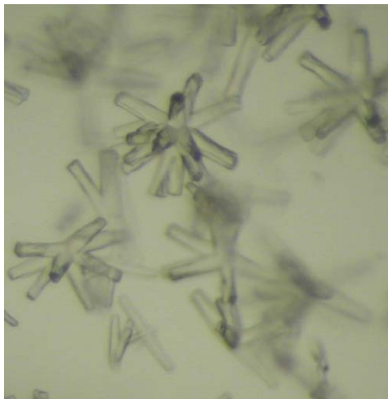


The crystallization of glycoproteins is one of the challenges to be confronted by the crystallographic community in the frame of what is known as glycobiology. The state of the art for the crystallization of glycoproteins is not promising and removal of the carbohydrate chains is generally suggested since they are flexible and a source of heterogeneity. In this paper, the feasibility of introducing glucose into the model protein hen egg-white lysozyme *via* a post-purification glycosylation reaction that may turn any protein into a model glycoprotein whose carbohydrate fraction can be manipulated is demonstrated.

1. Introduction

Although not yet fully understood by genome and proteome scientists, now that a considerable amount of structural information on proteins is available, our view of oligosaccharides is changing and glycomics is coming onto the scene as a continuation of proteomics (Hirabayashi & Kasai, 2000). Glycans represent a third class of bioinformative macromolecules whose potential has been highlighted in a recent article that identifies glycomics as one of ten emerging technologies that will change the world (Roush *et al.*, 2003). The US National Institutes of Health has allocated $34 million over 5 y to the Consortium for Functional Glycomics and the Pharma market is focusing on this research area, with several cancer vaccines undergoing clinical tests (Maeder, 2002).

One of the lessons learnt from proteomics is that despite important advances in protein production, X-ray diffraction, phasing and refinement, success critically depends on the crystallization stage, where the lack of knowledge is usually compensated for by massive screening (*i.e.* brute force; Hui & Edwards, 2003; Rupp, 2003; Luft *et al.*, 2003; De Lucas *et al.*, 2003) or by the manipulation of the protein to change its intrinsic characteristics by point mutation, truncations, deletions and fusion to other proteins (Dale *et al.*, 2003).

The state of the art for the crystallization of glycoproteins is even worse. Little systematic work on the crystallization of glycoproteins has been carried out (Stura *et al.*, 1992) and it represents a challenge since (i) natively glycosylated proteins cannot be produced in standard overexpression systems because the glycosylation pattern is not directly dependent on the genome but on the species and tissues, (ii) purification yields heterogeneous samples consisting of a mixture of molecules with partial deglycosylation of the carbohydrate fraction and (iii) carbohydrates can hinder the crystallization because they are flexible and can obscure a substantial fraction of the potential crystal-contact-forming protein surface. This negative view of carbohydrates has led to suggestions of their removal from the protein, despite the fact that the carbohydrate fraction is an important piece of information (McPherson, 1999).

We have approached the study of the crystallization of glycoproteins *via* a post-purification glycosylation reaction that is compatible with biological samples. In this paper, we report the glycosylation of the model protein lysozyme with glucose and its crystallization.

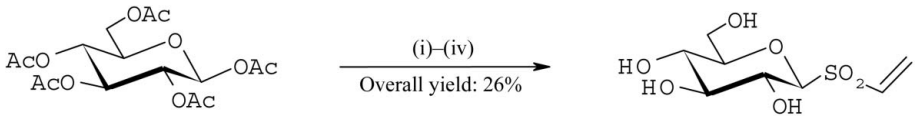


Figure 1
Synthesis of glucopyranosyl vinylsulfone from 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose. Reagents and conditions are as follows. (i) Thiourea, BF_3Et_2O, acetonitrile, reflux for 3 h then $BrCH_2CH_2Cl$, Et_3N, room temperature, 2 h. (ii) Et_3N, anhydrous MeOH, 313 K, 8 h. (iii) H_2O_2, AcOH, room temperature, 2 d. (iv) K_2CO_3, acetone, reflux, 8 h.

2. Materials and methods

Synthesis of glucopyranosyl vinylsulfone was carried out in four steps from 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose with an overall yield of 26% (Fig. 1). This product is an amorphous solid stable in the freezer for more than nine months (see supplementary material[1]).

2.1. Glycosylation of commercial hen egg-white lysozyme

The reaction was carried out by mixing and stirring 500 µl 50 mg ml^{-1} hen egg-white (HEW) lysozyme (Sigma) in 90 m*M* phosphate buffer pH 7.7 and 10%(*v*/*v*) 2-propanol (Sigma) with 10.5 mg glucopyranosyl vinylsulfone for 72 h at room temperature. To remove the excess glucopyranosyl vinylsulfone and stop the reaction, the mixture was transferred to a 3500 kDa cutoff membrane (Spectrum) and dialyzed against a total volume of 1500 ml of 20 m*M* acetate buffer pH 4.5 in three steps. The reaction was monitored every 24 h by native PAGE run with a BioRad apparatus with reversed polarity and stained with Coomassie Brilliant Blue (Hames, 1990).

2.2. Crystallization

Crystallization conditions were screened by the hanging-drop vapour-diffusion method at 293 K. Typically, drops contained 1.5 µl 25 mg ml^{-1} glycosylated lysozyme in 20 m*M* acetate pH 4.5 and 1.5 µl reservoir solution consisting of 100 m*M* Bis-Tris buffer pH 6.9 and 0.25–1.5 *M* $MgSO_4$ and were equilibrated against 500 µl reservoir solution. After three weeks of equilibration, 100 µl 2 *M* NaCl were added to the reservoir and 5 d later clusters of needle crystals appeared in conditions that had initially contained 0.75–1.25 *M* $MgSO_4$.

2.3. X-ray diffraction

Needle crystals from 1.04 *M* $MgSO_4$ (initially 1.25 *M*) were large enough to cut out and isolate fragments for use as single crystals in diffraction. Cryoprotection was carried out by soaking the crystals in increasing concentrations of glycerol up to 20%(*v*/*v*) in 50 m*M* Bis-Tris buffer pH 6.9, 1.04 *M* $MgSO_4$ and 0.3 *M* NaCl. Data collection was carried out using a MAR CCD at ESRF beamline BM16 (λ = 0.98 Å), placed at a distance of 105 mm. The oscillation angle was 0.3° and the exposure time was 10 s per frame. The data set consisted of 441 images that were processed with the *XDS* software package (Kabsch, 1988, 1993). Neither overloads nor incomplete reflections (*i.e.* those whose intensity estimate at the profile fitting was less than 75% of observed intensity) were included in the processing. A forward-directed search strategy (Kabsch, 1993) was followed; no unit cell or space group was imposed. The program was asked to determine the reduced cell, to compare it with all 44 possible lattices and to index the data in space group *P*1. The diffraction parameters and unit cell were refined in $P2_12_12$ and $P2_12_12_1$, which were the two space groups with the highest likelihood of being correct that were output by *XDS*. Finally, the data sets were reindexed with the refined unit cell in $P2_12_12$ and $P2_12_12_1$ and scaled independently with *XSCALE*.

2.4. Structure solution and refinement

Molecular replacement was carried out with *CNS* (Brünger *et al.*, 1998) using as a search model the atomic resolution structure at 0.94 Å reported by Sauter *et al.* (2001) (PDB code 1iee), omitting the solvent molecules. The rotation search was carried out as a direct rotation search (Grosse-Kunstleve & Adams, 2001) from 15 to 2.5 Å. For the translation searches the resolution range was 10–2 Å. At this stage, the space group was confirmed to be $P2_12_12_1$.

The structure was refined with *CNS* (Brünger *et al.*, 1998) using torsion-angle molecular dynamics and a cross-validated maximum-likelihood target function (Adams *et al.*, 1997), 10% of the data set being randomly selected to estimate the free *R* value (Brünger, 1992). Rebuilding of the model was carried out with *XtalView* on the basis of σ_A-weighted maps (McRee, 1993). No σ cutoff was applied and a solvent mask was automatically determined and updated by *CNS* to use the resolution range 10.0–1.6 Å. Inclusion of solvent molecules and ligands was based on peaks of at least 3σ and 1σ in the σ_A-weighted $F_o - F_c$ and $2F_o - F_c$ maps, respectively.

3. Results and discussion

The ideal system to study the effect of carbohydrates on the crystallization of glycoproteins would be one with an unlimited amount of a model glycoprotein whose carbohydrate fraction could be built up *à la carte*. Although feasible, the biological approach is not attractive, since it implies site-directed mutagenesis, production of the protein in

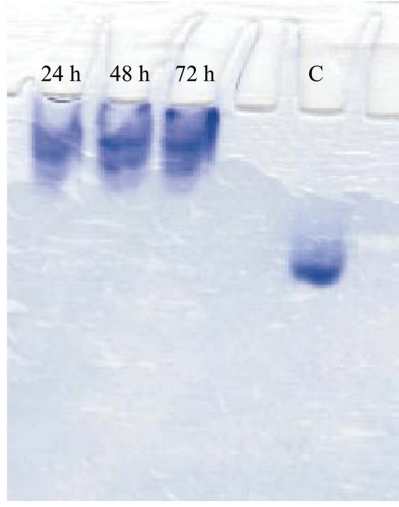


Figure 2
Native electrophoresis in 12% polyacrylamide gel. From left to right the samples are for the reaction after 24, 48 and 72 h, and for control HEW lysozyme.

[1] Supplementary material is available from Crystallography Journals Online (Reference: LL5017).

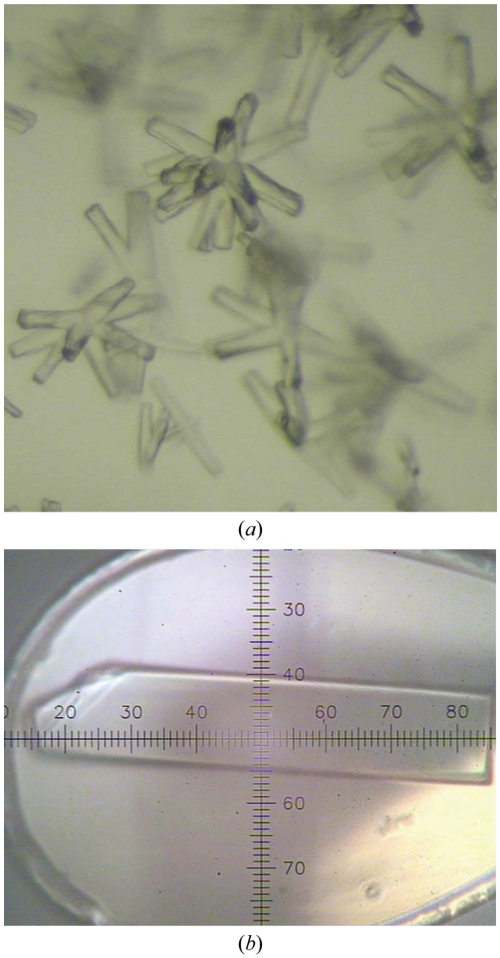
Table 1
Summary of the main features of the data set.

Values in parentheses correspond to the highest resolution shell.

Data collection	
X-ray source	BM16, ESRF
Wavelength (Å)	0.98
No. of crystals	1
Temperature of data collection (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 30.58, b = 32.97, c = 120.27
Data set	
Resolution range (Å)	15.00–1.6 (1.70–1.60)
Measured reflections	85461 (14050)
Unique reflections	16442 (2657)
Completeness [$I/\sigma(I) \geq 0$] (%)	97.9 (97.0)
Completeness [$I/\sigma(I) \geq 3$] (%)	91.6 (78.1)
$R_{\rm merge}$ (%)	3.8 (15.3)
Mean $I/\sigma(I)$	24.93 (10.71)

different overexpression systems to obtain different glycosylation patterns and purification of each specimen.

A more practical approach would be the glycosylation of the protein *in vitro* as a post-purification step to overcome the inconveniences of the biological approach. However, the main challenge for this chemical approach is to functionalize the carbohydrate to make it react specifically and precisely with the protein in a chemical environment compatible with the biological nature of the protein. One strategy for functionalization may be the introduction of a vinyl sulfone group that reacts with primary and secondary amines present in the protein as Lys, His and the amino-terminus.



(*a*)

(*b*)

Figure 3
Clusters of needle crystals (*a*) and the fragment used for diffraction (*b*) in a 0.3–0.4 mm loop.

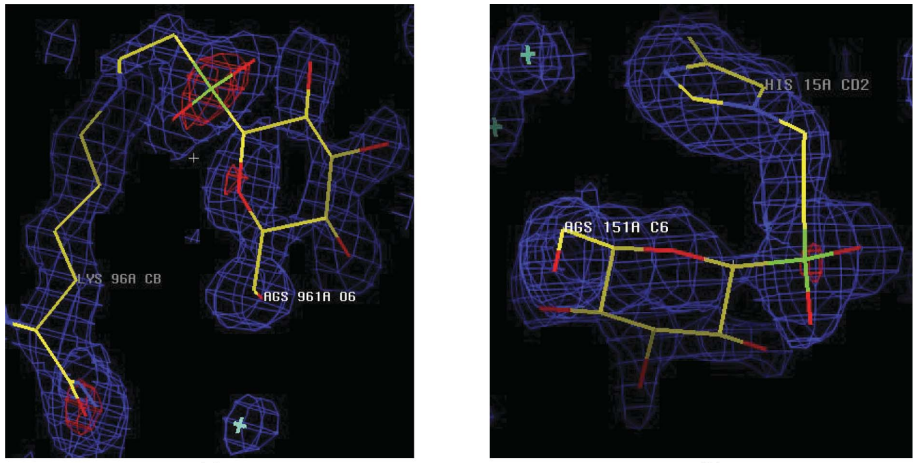
To test the feasibility of this approach, we have focused on the simplest case: glucose as the carbohydrate and HEW lysozyme as the protein. The functionalization of the glucose was achieved *via* a four-reaction protocol (Fig. 1) that will be described in detail elsewhere. Since one of the targets of the functionalized sugar is the amine group present in Lys, the modified lysozyme was expected to show a different charge density and hence a different migration pattern in native electrophoresis.

The glycosylation of HEW lysozyme was carried out in a mild environment compatible with most biological samples: room temperature, phosphate buffer pH 7.7 and gentle stirring. The reaction was monitored every 24 h to detect undesired byproducts and, as shown in Fig. 2, the glycosylation of 25 mg HEW lysozyme at a concentration of 50 mg ml^{-1} was completed within 24 h. The process was stopped after 72 h by dialysis against 20 m*M* acetate buffer pH 4.5 to shift the pH to acidic values that do not allow reaction and to remove the excess of free sugar and exchange the buffer system, since phosphate is prone to yield salt crystals with many common ions. In principle, any buffer system is suitable to carry out the reaction if (i) it yields alkaline pH and (ii) it does not bear primary and/or secondary amine groups.

The crystallization of the glycosylated protein was the prerequisite for the solution of the structure and to demonstrate the feasibility of our approach. Our attempts to crystallize the glycoslyated HEW lysozyme in acetate pH 4.5 and sodium chloride yielded clusters of thin needles and confirmed the prejudice among crystallographers, who have a negative view of carbohydrates based on the fact that they are flexible and located at the surface of the protein, disrupting potential crystal contacts that are important for the stability of the growing crystal. In addition to the tetragonal form, HEW lysozyme can crystallize in monoclinic, trigonal and orthorhombic space groups from sulfate salts (Forsythe *et al.*, 1999). According to Rypniewski *et al.* (1993), the methylation of lysine residues of HEW lysozyme prevent the protein from crystallizing in $P4_32_12$, but it is not incompatible with $P2_12_12_1$ and consequently we focused our effort on the search for crystallization conditions with $MgSO_4$, which was demonstrated to yield orthorhombic crystals.

The initial screening from 0.25 to 1.5 *M* $MgSO_4$ yielded a clear solution after three weeks, which is within the range of time expected for a system with salt as precipitant to reach equilibrium (Arakali *et al.*, 1995). Dehydration of the drops was forced by adding 100 µl 2 *M* NaCl to the reservoir and under these new conditions clusters of needle-shaped crystals grew in 0.65–1.04 *M* $MgSO_4$ (initially 0.75–1.25 *M*) within one week (Fig. 3). This morphology resembles that reported by Rypniewski *et al.* (1993) for the methylated HEW lysozyme, but the glycosylated lysozyme crystals were large enough to avoid the necessity for seeding. One fragment from the cluster of crystals was isolated and diffracted at BM16 (ESRF) to a resolution of 1.5 Å, the lattice being orthorhombic with a novel unit cell larger than those previously reported.

For structural purposes, a data set was collected at 1.6 Å (Table 1) and the space group was confirmed as $P2_12_12_1$ by molecular replacement using PDB code 1iee (Sauter *et al.*, 2001) as the search model. The refinement is still in progress ($R_{\rm free}$ = 0.230, R = 0.207), but the $2mF_{\rm o} - DF_{\rm c}$ electron-density map shows a clear peak at Lys96 and His15 that defines the sugar (Fig. 4). Additional electron density at covalent-bond distance from amino groups is found at Lys97 and the N-terminus of the molecule, which leads us to conclude that the experiment succeeded and that this reaction may be a powerful tool to allow a systematic approach to the crystallization of glycoproteins.



(*a*) (*b*)

Figure 4
$2mF_o - DF_c$ map contoured at 1.2σ (blue) and 4σ (red) showing the electron density that defines the sugar bonded to Lys96 (*a*) and His15 (*b*).

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