Increased Thermal Stability of Site-Selectively Glycosylated Dihydrofolate Reductase

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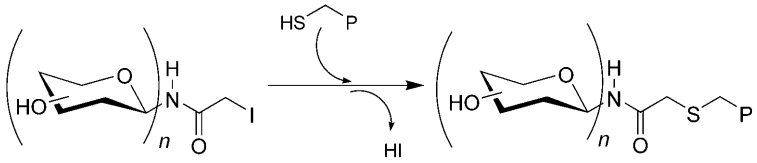

Scheme 1. Alkylation of site-selectively introduced cysteine mutants of DHFR;[12,16] $n = 0$–3.

Glycosylation is important for many molecular processes such as molecular recognition, degradation and stability. While it has long been established that the native conformation of many proteins can be stabilised against thermal denaturation through glycosylation,[1–3] the mechanism of such stabilisation is less clear. The removal of carbohydrates from naturally glycosylated proteins through the use of glycosidases[4] or by mutagenesis of glycosylation sites[5,6] can lead to decreased thermal stability of the protein. This is often accompanied by an increased tendency towards protein aggregation. Due to the heterogeneous nature of protein glycosylation both in vivo and in vitro, a comprehensive study of its effect on thermal stability has been elusive. However, studies of the stability of several unglycosylated proteins in the presence of high concentrations of saccharides such as glycerol, glucose, sucrose, galactose and α,α-trehalose have led to the conclusion that these glycans stabilise the folded protein due to preferential binding of the native state.[7–10] Such observations suggest that glycosylation of natural proteins could establish a microenvironment that resembles that of unglycosylated proteins in solutions of high carbohydrate content.

The scope of studies into specific in vitro glycosylation of proteins has until recently been limited. The use of natural enzymes allows the site-selective introduction of sugars but is limited to natural glycans and by often relatively poor yields. Chemical glycosylation reactions, however, which are more flexible with respect to the choice of glycan, suffer from limited site-selectivity. Such shortcomings have been overcome through a combination of the introduction of unique cysteine residues at required sites and a highly flexible but selective chemical derivatisation strategy (Scheme 1).[11–16] This methodology allowed us to study the effect of site-specific glycosylation on the thermal stability of the naturally nonglycosylated enzyme, dihydrofolate reductase (DHFR), systematically.

DHFR is a major target for antibacterial and anticancer drugs and has long served as a paradigm for the study of enzyme kinetics, protein dynamics and stability. DHFR has a compact folded form (Figure 1), which is comprised of two domains, the adenosine binding and loop domains. These domains are connected by a short polypeptide stretch centred around amino acid residues 86 and 87, known as the hinge region. Large-scale movements of external loops control access of the cofactor to the active site and propagation through the catalytic cycle.[17–19] Here we report dramatic increases in the thermal stability of *E. coli* DHFR as a result of site-selective glycosylation of individual surface residues.

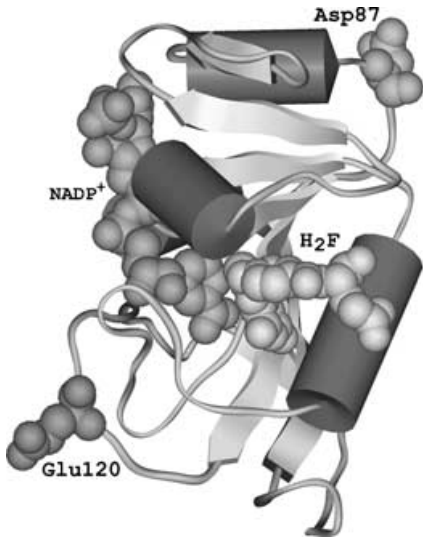

Figure 1. Diagram of the structure of *E. coli* DHFR indicating the positions of the two loop residues Asp87 and Glu120. The positions of dihydrofolate (H_2F) and cofactor ($NADP^+$) are shown in the space-filling models.[17]

Loop residues 87 and 120, located on opposite sides of the protein (Figure 1), were chosen for selective glycosylation. Glu120 is positioned on the water-exposed face of a surface loop next to Gly121, which appears to be crucial for the activity of the enzyme. Mutation of residue 121 significantly reduces the catalytic performance of DHFR through local conformational changes that affect the properties of the enzyme globally.[20–26] Asp87 is a solvent exposed residue within the hinge region that connects the adenosine binding and loop domains. The hinge region appears to lose its native structure relatively early in the temperature-induced unfolding process.[20,27] Site-selective glycosylation of positions 120 and 87 of DHFR was achieved through the replacement of Glu120 and Asp87 with cysteine by site-directed mutagenesis of the cysteine free double mutant DHFR-C85A/C152S (DM), which has folding, stability and kinetic properties very similar to those of the wild-type protein.[28] DM-E120C and DM-D87C were derivatised by

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treatment with the iodoacetamides of *N*-acetyl glucosamine, glucose, maltose, lactose and maltotriose (*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose) and purified as described previously.[16]

CD-spectroscopy indicated no significant differences between the structures of wild-type DHFR, DM-E120C and DM-D87C and their glycosylated forms (Figure 2). However, when

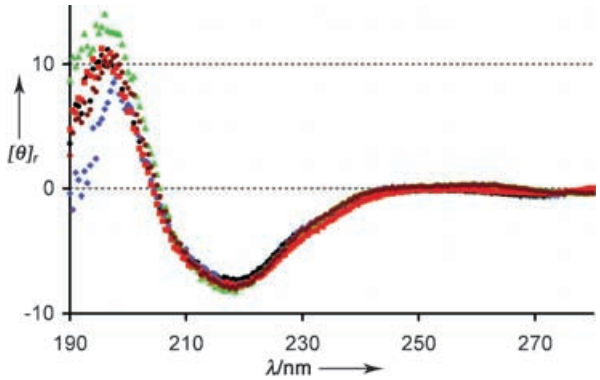

Figure 2. Representative CD-spectra for DHFR, DHFR mutants and their glycosylated forms. CD-spectra of DHFR (brown dots), DM (black dots), DM-D87C (red squares), DM-E120C (blue squares) and DM-D87C-lactose-acetamide (green triangles). All other glycan-carrying proteins described in the text exhibited CD-spectra that were essentially identical to the ones shown here. All protein concentrations were 10 μM and the spectra were recorded by using a JASCO-810 spectrometer at 25 °C with a path length of 1 mm in 10 mM KP_i, pH 7.0. Mean residue ellipticities $[\Theta]_r$ are reported in units of 10^3 deg cm^2 dmol^{-1}.

the thermal stabilities of the proteins were investigated by measuring their CD-spectra as a function of temperature, the presence of the glycans led to significant stabilisation of the protein folds (Table 1). Glycosylation of Cys120 led to a small but significant increase in the melting temperature of the protein. Interestingly, the extent of stabilisation observed for disaccharides was about twice that seen for monosaccharides.

Table 1. Melting temperatures [°C] of DM, DM-E120C and DM-D87C and their glycosylated forms. The thermal stabilities of nonglycosylated DM and DM-D87C in the presence of 0.5 M maltose are also indicated. The melting temperatures were determined from the denaturation curves obtained from the dependence of the mean residue ellipticity at 222 nm $[\Theta]_{r,222}$ on temperature. All protein concentrations were 10 μM and the spectra were recorded on a JASCO-810 spectrometer with a path length of 1 mm in 10 mM KP_i, pH 7.0.[20] The melting temperature for wild-type DHFR is 50.7 ± 0.2 °C.

Glycan	DM	DM-E120C	DM-D87C
none	50.8 ± 0.3	50.8 ± 0.4	40.9 ± 0.3
glucose		51.8 ± 0.2	47.1 ± 0.3
GlcNAc		52.5 ± 0.1	49.6 ± 1.1
lactose		54.1 ± 0.1	46.8 ± 2.1
maltose		54.2 ± 0.3	n.d.
maltotriose		n.d.	47.5[a]
acetamide		51.3[a]	39.6 ± 1.2
+0.5 M maltose	52.9 ± 0.4	n.d.	43.4 ± 0.4

[a] Single measurement.

Glycosylation of Cys120 with lactose or maltose stabilised the protein by approximately 3 °C.

The steady-state kinetic parameters measured for DM-E120C and its glycosylated forms were similar to those measured for DM and indeed the wild-type enzyme[29] (Table 2). This suggests that the structures were essentially unaltered. The surface FG loop, which contains Glu120, is connected through hydrogen bonds and van der Waals interactions to the M20 loop, which exists in two conformations depending on the state of the catalytic cycle.[17,30] Movements of the M20 loop, which display rates similar to that of the steady-state turnover, regulate ligand binding.[23] The similar reaction rates of DM-E120C and its glycosylated forms suggest that these conformational changes are not significantly affected by the presence of glycans in the FG loop. This could indicate a coordinated movement of the FG and M20 loops and is in agreement with results obtained by molecular mechanics simulations.[23,24,31]

Table 2. Steady state kinetic parameters of DHFR mutants and their glycosylated forms. Kinetic measurements were carried out at 25 °C as previously described.[29] The kinetic parameters for DHFR are $K_M^{NADPH} = 2.8 \pm 0.3$ μM and $k_{cat} = 8.8 \pm 0.2$ s^{-1}.

Glycan	DM		DM-E120C		DM-D87C	
	k_{cat} [s^{-1}]	K_M^{NADPH} [μM]	k_{cat} [s^{-1}]	K_M^{NADPH} [μM]	k_{cat} [s^{-1}]	K_M^{NADPH} [μM]
None	7.0 ± 0.4	2.7 ± 0.5	6.4 ± 1.4	2.3 ± 1.0	9.1 ± 0.1	4.9 ± 1.7
Glucose			7.8 ± 1.1	3.7 ± 1.4	7.3 ± 0.9	10.5 ± 1.5
GlcNAc			5.3 ± 1.7	3.9 ± 2.1	7.4 ± 0.1	6.3 ± 1.3
Lactose			7.1 ± 2.0	1.9 ± 1.7	6.1 ± 2.4	7.0 ± 1.8
Maltose			7.7 ± 1.9	6.8 ± 1.6	n.d.	n.d.

While replacing Glu120 with Cys had no effect on the stability of the protein, DM-D87C displayed significantly reduced stability compared to DM (Table 1). The melting temperature of DM-D87C was lowered by almost 10 °C and the thermally induced unfolding reaction displayed somewhat reduced cooperativity (Figure 3). However, the CD-spectrum of DM-D87C at 25 °C was comparable to that of DM (Figure 2); this suggests that no major conformational changes had occurred as a consequence of the amino acid change. Derivatisation of DM-D87C with glycosyl iodoacetamides dramatically increased the stability of the protein. Alkylation with *N*-acetyl glucosamine completely reversed the destabilising effect of Cys87, and the glycosylated protein displayed thermal stability that was identical to that of the parent protein (Table 1). The shape of the melting curve observed for glycosylated forms of DM-D87C was comparable to that of DM (Figure 3). As had been observed for glycosylated DM-E120C, the steady-state kinetic properties of the DM-D87C and its glycosylated forms at 25 °C, were similar to those observed for DM (Table 2). Together with the unaltered CD-spectra of the glycosylated proteins, the results suggested that the altered thermal stabilities of the proteins were not a consequence of global conformational changes, but due to the site-specific presence of glycans. Alkylation of DM-E120C and DM-D87C with nonglycosylated iodoacetamide alone did not lead to a significant increase of the

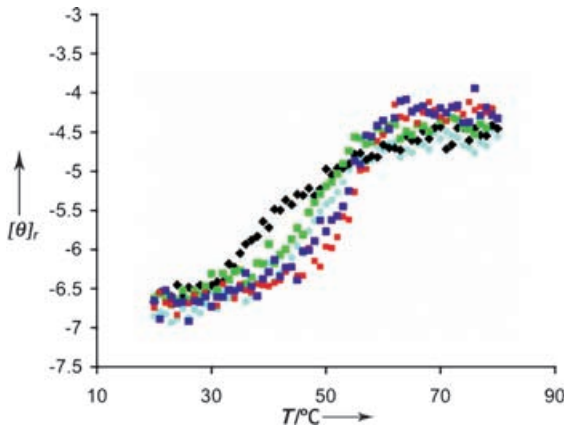

Figure 3. Representative melting curves for DHFR mutants and their glycosylated forms. The mean residue ellipticity at 222 nm was measured as a function of temperature for DM-D87C (black squares), DM-D87C carrying glucose (green squares) and *N*-acetylglucosamine (blue dots), DM-E120C carrying *N*-acetylglucosamine (purple squares) and lactose (red squares) by using a gradient of 20 °C h^{-1}. Conditions are as in Figure 2. Mean residue ellipticities at 222 nm $[\Theta]_{r,222}$ are reported in units of 10^3 deg cm^2 $dmol^{-1}$.

proteins' melting temperatures (Table 1). This indicated that the glycans were responsible for the increased stability of the glycoproteins. The unnatural linkage between the proteins and the glycans was clearly not the cause of their enhanced stability.

Asp87, which is located in the hinge region between the adenosine binding and loop domain of DHFR, contains one of the most solvent exposed side chains of the protein. Replacement of the polar carboxylate group of Asp87 with the hydrophobic side chain of cysteine might generate a local conformational change that affects the thermal stability of the protein. Glycosylation of residue 87 on the other hand reversed this effect most likely through increased solvation. However, the stability increase gained through glycosylation was significantly higher than that observed for the unglycosylated protein in the presence of high concentrations of maltose. The presence of 0.5 M maltose increased the melting temperature of all proteins examined by only approximately 3 °C (Table 1). The increased stability frequently observed with naturally glycosylated proteins, might therefore not simply be a nonspecific effect arising from changes in the solvation properties of the enzymes.[10] Site-specific effects can clearly also contribute to the overall thermal stabilisation of proteins, at least in the case of DHFR.

Our results show that site-selective glycosylation of DHFR can dramatically increase the resistance of the protein against thermal denaturation. The protein's melting temperature could be increased significantly through the site-selective attachment of simple glycans. It should be noted that the increase in thermal stability observed in our studies is of the same order of magnitude as the decreases observed upon deglycosylation of naturally glycosylated proteins, which were 3.4, 4.0 and 1.9 °C for yeast external invertase, bovin serum fetuin and glucoamylase, respectively.[4] Our data suggest that significant increases in the thermal stability of proteins can be achieved even with the small carbohydrates used in this study, rather than with the often much larger oligosaccharides found in naturally glycosylated proteins.

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Keywords: glycosylation · mutagenesis · oligosaccharides · selectivity · thermal stability

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