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Hydration of a glycoprotein: relative water affinity of peptide and glycan moieties

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Abstract Glycosylation, the most prevalent post-translational modification of proteins, affects a number of physical properties including the interactions with the surrounding aqueous solvent. Such glycan–water interactions have been discussed with respect to the increased solubility generally observed for glycoproteins, but experimental support of this correlation remains sparse. We have applied a two-channel calorimetric method to measure the free energy and enthalpy of hydration at 25°C for the glycoprotein phytase (Phy) and a deglycosylated form (dgPhy) of the same protein. Comparisons of results for Phy and dgPhy show that the polypeptide moiety has a higher affinity for water than the glycans. In fact, at moderate hydration levels (~0.3 g water/g macromolecule) the water uptake appears to be entirely governed by adsorption to the peptide groups. We conclude that strengthened interaction with the solvent is unlikely to be the mechanism underlying the increased solubility and lowered propensity of aggregation often reported to result from the glycosylation of proteins.

Introduction

Glycosylation is the most common post-translational modification of proteins, and one of its roles appears to be the suppression of aggregation and increase of solubility (Narhi et al. 2001; Price et al. 2003; Schulke and Schmid 1988a, b; Song et al. 2001; Tams et al. 1999; Tams and Welinder 1995). The mechanisms underlying these effects of glycans remain poorly understood, but it has been suggested that they may rely on the "hydrophilic nature" of the sugar residues (Creighton 1996). This implies that the sugar moieties interact more favorably with water than the peptide chain does and that these favorable interactions stabilize the dissolved monomeric state of the proteins over crystallized or aggregated states. In this work we address the question of the relative hydrophilicity of glycans and peptides through a novel calorimetric method, which simultaneously measures the free energy (the binding isotherm) and the enthalpy of hydration. Our model system is the hydrolytic enzyme phytase (Phy) E.C.3.1.3.26. from *Peniophora lycii*. This *N*-glycosylated enzyme consists of 47.5 kDa polypeptide and around 17.5 kDa glycans, mainly mannose residues, distributed on ten *N*-glycosylation sites (Lassen et al. 2001). Phy can be enzymatically deglycosylated with no change in the catalytic properties (and hence in the peptide structure). It follows that comparative investigations of Phy and its deglycosylated form (dgPhy) has a particular potential to elucidate the effects of glycans on hydration and other physico-chemical properties.

The conspicuous solubility in water of some carbohydrates may be taken as an indication of strong interactions between water and glycans. However, we have found some evidence based on thermodynamic and light scattering measurements that water may in fact interact more favorably with the peptide moieties than with the glycans (Bagger et al. 2003) (Nielsen et al., in preparation). If indeed so, the solubility promoting effect of glycans cannot rely on favorable solvent interactions but must depend on other factors e.g., steric inhibition of protein–protein contacts and the concomitant poor packing of aggregates or crystals. To elucidate this, we have investigated the effects of glycans on the water uptake by dried samples. This process may be considered the most fundamental measure of protein hydration (Rupley and Careri 1991) and thus essential to the understanding of glycan–water interactions.

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Materials and methods

Peniophora lycii Phy was expressed in *Aspergillus oryzae* and purified according to the previously published procedures (Lassen et al. 2001) and stored at −25°C in buffer solution (20 mM Tris-acetate, pH 6). The molecular mass of the glycosylated Phy (~65 kDa) and the purity of this enzyme (>95%) were determined by SDS-PAGE. The enzymatic deglycosylation of Phy producing dgPhy was performed by Endo-*β*-*N*-acetylglucosaminidase F_1 (Endo F_1) E.C.3.2.1.96 purchased from Calbiochem (Darmstadt, Germany). The deglycosylation procedure has previously been described in detail (Bagger et al. 2003). To ensure that the deglycosylation was quantitative, the reaction mixture was investigated by SDS-PAGE. Phy and dgPhy was extensively dialyzed (MWCO 12–14 kDa) against milliQ water at 4°C to avoid any salts (other than counter-ions) in the hydration experiments.

To produce dry enzyme samples Phy and dgPhy were lyophilized at 0.05 torr for 2 days. After drying the pressure was equalized with dry nitrogen gas and the cell containing the enzyme was immediately sealed with a close-fitting lid. To assure complete drying test samples of lyophilized enzyme was placed at 80°C to gravimetrically confirm that no further water evaporated.

The calorimetric measurements were conducted on a multi-channel thermal activity monitor (TAM, 2277) isothermal calorimeter (Thermometric A/B, Järfälla, Sweden). The experimental principles have been described in detail elsewhere (albeit for a different application) (Hansen et al. 2004). Briefly, two calorimetric channels are serially perfused with N_2(g) at a controlled flow rate and relative humidity. The exothermic heat flow (HF_A) associated with water adsorption to the enzyme in the first channel (A) quantifies the heat generation associated with water adsorption. The second channel (B) contains pure water and the endothermic heat flow (HF_B) associated with water evaporation in channel B reflects the amount of water adsorbed by the enzyme. To obtain the adsorption enthalpy, ΔH_{ads} (in kJ/mol H_2O) the results of the two channels are combined (Eqs. 1, 3). The temperature was 25°C and the flow rate was 75 ml/h throughout all trials. Channels A and B were equipped with 4 ml stainless steel cells connected to a perfusion unit of the Termometric 2250-series

To minimize the exposure of dry enzymes to (moist) air prior to the hydration experiment Phy and dgPhy were lyophilized in the calorimetric cell (cell A), which was directly connected to the perfusion unit. The enzyme was kept in the calorimeter under a flow of dry nitrogen gas until the hydration experiment was initiated. The total duration of an experiment was ~45 h. The first 2–6 h the relative humidity was set to 0% and kept there until the heat flow from channel A and B were stable for about 1 h. Subsequently, the RH was increased linearly from 0 to 98% over 20 h and kept at 98% for an additional ~20 h. This procedure was adopted from an earlier work (Liltorp 2003) which showed that two-channel calorimetry provided water binding isotherms and adsorption enthalpies for hen lysozyme similar to those reported by the other experimental approaches (Rupley and Careri 1991; Hnojewyj and Reyerson 1961; Smith et al. 2002).

Several reference experiments with an empty cell in channel A were conducted to measure the amount of water which adsorbs to the surface of the calorimetric cell during the RH ramp.

Results and discussion

Figure 1 exemplifies results from the calorimetric trials. Panel A shows the heat flow, HF_A, arising from the adsorption of water to 9.74 mg Phy when the RH is changed as illustrated by the solid line in the same graph (right hand ordinate). Panel A also shows the heat flow

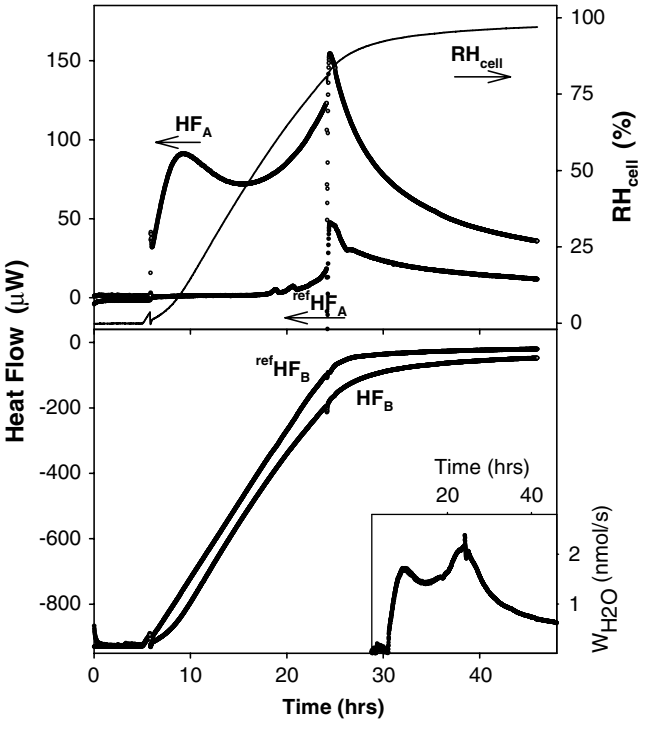

Fig. 1 A typical data set showing the calorimetric output for the hydration of 9.74 mg initially dry phytase (*Phy*) (and data from the accompanying reference experiment). The humidity of the purge gas (RH_{supply}) was zero for the initial 6 h of the experiment and it was subsequently increased linearly over 20 h to 98% RH. The uptake of water was followed for an additional 20 h during purging with 98% RH. **a** The exothermic heat flow for the protein sample (HF_A) and for the empty calorimetric cell ($^{ref}HF_A$). This panel also shows the ambient humidity in the calorimetric cell, RH_{cell}, calculated from Eq. 4. The amount of water adsorbed by the protein is illustrated by the data from channel B (**b**). The water removed from the purge gas through adsorption to the protein is quantified by the difference between HF_B and $^{ref}HF_B$. The water uptake, W_{H_2O} specified by Eq. 1 in nanomole per second is shown in the *inset*

$^{ref}HF_A$ generated by the adsorption of water to an empty calorimetric cell in a reference experiment with the same time-course of the RH. The amount of water adsorbed is illustrated in panel B by the (endothermic) heat flows associated with the saturation of the gas stream in the Phy trial (HF_B) and reference experiment ($^{ref}HF_B$). The amount of water taken up by the protein, W_{H_2O}, (in moles/s) is specified by the difference between the two curves in Fig. 1b and may be written

$$W_{H_2O} = -\frac{HF_B - {}^{ref}HF_B}{\Delta H_{evap}} \quad (1)$$

where ΔH_{evap} is the heat of evaporation of water at 25°C. The total amount of adsorbed water, h (in g H_2O/g enzyme), can be expressed as a function of the experimental time, t, according to Eq. 2.

$$h = \frac{M_{H_2O}\int_0^t W_{H_2O}\,dt}{m_P} \quad (2)$$

where M_{H_2O} and m_P are, respectively, the molar mass of water and the mass of protein in cell A. Once the water uptake has been quantified the molar enthalpy of water adsorption, ΔH_{ads} can be calculated as

$$\Delta H_{ads} = \frac{HF_A - {}^{ref}HF_A}{W_{H_2O}} + \Delta H_{evap}. \quad (3)$$

The raw data in Fig. 1a relates to the transition water(g) → water(ads). However, since ΔH_{evap} is added on the right-hand side of Eq. 3, ΔH_{ads} specifies the enthalpy of transferring one mole of water from the pure liquid to the surface adsorbed state.

The actual RH in the calorimetric cell in channel A, RH_{cell}, is slightly lower than the humidity of the incoming gas, RH_{supply}, due to the continuous uptake of water in the cell. However, RH_{cell}, which is the water activity governing the adsorption process, can be calculated from the calorimetric signal in cell B

$$RH_{cell}(\%) = \frac{HF_B - {}^{ref}HF_B(0)}{{}^{ref}HF_B(98) - {}^{ref}HF_B(0)}98\% \quad (4)$$

where $^{ref}HF_B(0)$ and $^{ref}HF_B(98)$ are the heat flows from cell B in the reference experiment when RH_{supply} is 0 and 98%, respectively.

The binding isotherm, i.e., the total amount of water uptake, h, plotted as a function of the water activity expressed by the relative humidity in cell A, RH_{cell}, is illustrated for Phy and dgPhy in Fig. 2. Each curve is calculated as the average of three runs and the error bars indicate the standard deviation. The figure shows the degree of hydration, h, both in units of gram water/gram enzyme (panel A) and mol water/molar enzyme (panel B).

The results in Fig. 2 clearly show that the glycans of Phy do not bring about an increased uptake of water.

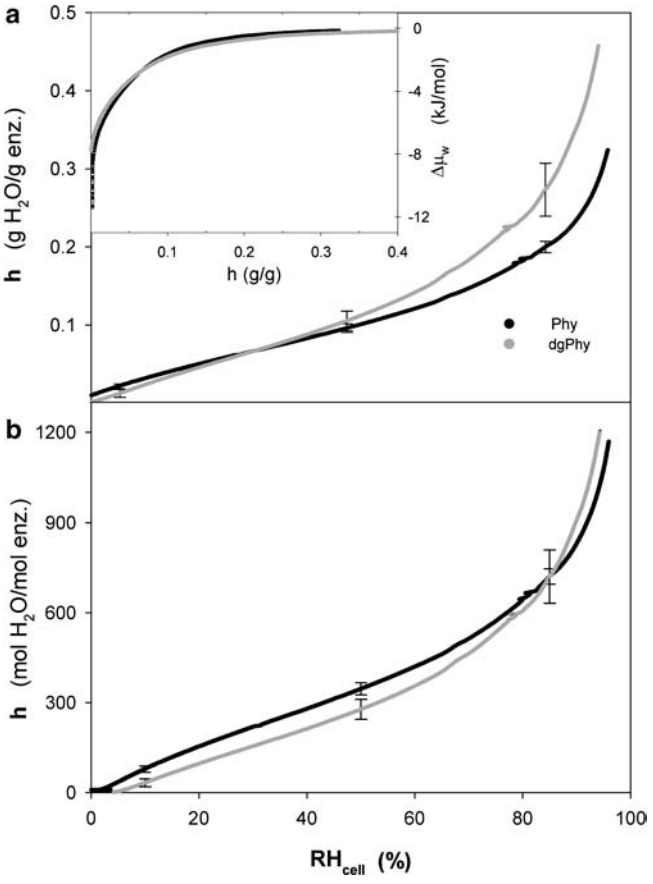

Fig. 2 Water adsorption data for the two glyco-forms of Phy and dgPhy. **a** The hydration isotherm illustrated as the hydration level, h (g H_2O/g enzyme) as a function of the relative humidity in cell A, RH_{cell}. Both isotherms represent the average of three separate trials in which the amount of enzyme varied from 2.91 to 15.70 mg Phy and 2.71 to 15.85 mg dgPhy. *Error bars* represent the standard deviation. In the *inset* of **a** the same data is plotted as the chemical potential of water, $\Delta\mu_w$ (see text) as a function of h. **b** The water uptake in units of mole water per mole of enzyme

Hence, no significant difference in the mass specific uptake (panel A), between Phy and dgPhy could be detected for $RH_{cell} < 50\%$, and at higher humidity, the deglycosylated form adsorbs more than the glycoprotein. For $RH_{cell} = 90\%$, for example, the polypeptide has an equilibrium water content of 0.34 g H_2O/g macromolecule while the glycosylated protein holds only 0.23 g H_2O/g macromolecule. If, for simplicity, the adsorption of water to the polypeptide and the glycan groups are considered independent, these numbers suggest that the glycans contributes negligibly to the total water adsorption at 90% RH. [Phy is ~73% (w/w) polypeptide corresponding to a contribution from the peptide of $0.73 \times 0.34 = 0.25$ g H_2O/g macromolecule to the water uptake of Phy. This value accounts for the full water adsorption of the glycoprotein and thus suggests an unimportant contribution of the glycans]. This is further illustrated in Fig. 2b, which shows the water uptake in mol/mol. It appears that at high humidities

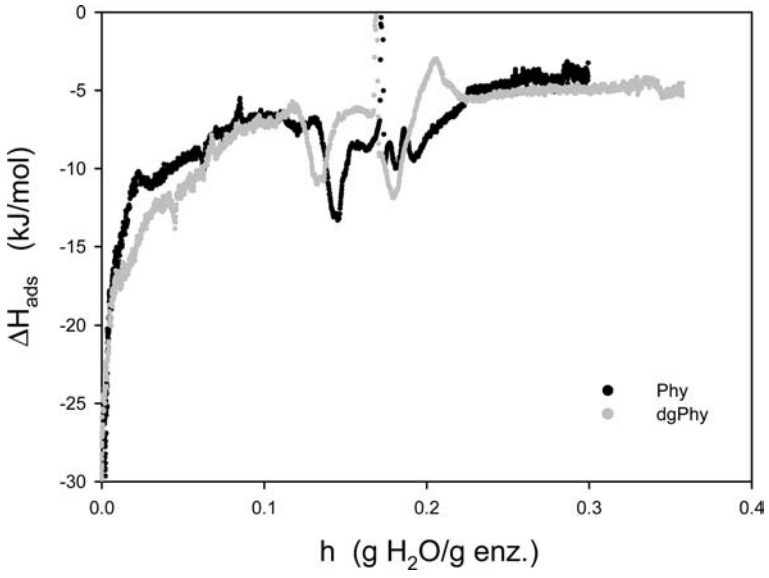

Fig. 3 Adsorption enthalpies for water, ΔH_{ads}, at 25°C as a function of the hydration level, h, in g water per gram macromolecule. ΔH_{ads} specifies the enthalpy associated with the transfer of water from the pure liquid to the surface adsorbed state. The *black curve* represents water adsorbing to Phy and the *gray curve* is the water adsorption by the deglycosylated phytase (*dgPhy*)

(RH > 80%) the difference between the two curves is smaller than the experimental uncertainty. Hence, the number of water molecules taken up by one enzyme molecule is not increased by the glycans in the high RH range. In the lower RH range where the amount of water is less than one molecule per amino acid residue, the molar uptake of the glycosylated protein is slightly larger.

The adsorption isotherms in Fig. 2 quantify the free energy of the absorbed water molecules (with respect to an appropriate standard state). To illustrate this more clearly and to facilitate comparisons with Fig. 3 (below), the data were re-plotted (in the inset) to show the chemical potential of water as a function of the water content. Assuming that the adsorption process is practically equilibrated[1] during the slow increase in RH_{cell} the chemical potential of adsorbed water may be written $\mu_w = \mu_w^* + RT \ln[RH/100\%]$. In other words the lowering in the chemical potential of adsorbed water with respect to saturated vapor, $\Delta\mu_w$ is $\Delta\mu_w = RT \ln[RH/100\%]$. This function is shown in the inset of Fig. 2a, and will be discussed below.

The weak hydration of glycans suggested by the results in Fig. 2 strongly speak against a hydrophilic mechanism for the enhanced solubility and reduced propensity for aggregation generally observed as a result of glycosylation (see Introduction).

The hydration of glycans is further elucidated by the adsorption enthalpy, ΔH_{ads}, which is plotted as a function of h for Phy and dgPhy in Fig. 3.

It appears from this figure that water binds rather strongly to the enzymes at the lowest hydration levels. Thus, the enthalpy of adsorbed water is initially 20–30 kJ/mol lower than for the pure liquid. The pronounced positive slope of ΔH_{ads} in this range, however, suggests that both forms of the enzyme only have few sites with this favorable binding enthalpy. A similar behavior of ΔH_{ads} at low hydration levels, corresponding to one-water molecule for every 2–3 amino acid, has previously been reported for other proteins (Smith et al. 2002). While this behavior may be important for the general understanding of protein hydration we suggest that these very few strongly bound water molecules are of minor relevance in attempts to single out the effects of glycans on proteins in solution. At higher water contents, ΔH_{ads} gradually increases from −10 to −4 kJ/mol. Comparisons of these results and the free energy data (inset of Fig. 2a) allows us to single out the enthalpic and entropic contributions to the net driving force of the hydration. At very low hydration we find, $\Delta\mu_w \sim -10$ kJ/mol and $\Delta H_{ads} \sim -25$ kJ/mol. In other words the entropic contribution $T\Delta S_w$ is about −15 kJ/mol. This substantial entropic penalty appears intuitive for the transfer of water from the pure liquid to the adsorbed state. At intermediate hydration levels, the entropic contribution becomes rather small, and is only about −4 kJ/mol at 0.2 g/g. A similar behavior has been observed for other proteins (Smith et al. 2002) and may rely on the "lubricating" effect of water on biomolecular movement (Barron et al. 1997). For the current discussion it is particularly important to notice that no clear differences between the two variants of Phy can be detected in Fig. 3. Also, the course of the ΔH_{ads} function found in Fig. 3, resembles that reported for lysozyme in a recent high-sensitivity calorimetric study (Smith et al. 2002). Hence, the results show no signs of particularly favorable hydration energy of glycan moieties. We consistently found an exaggerated experimental noise at $h \sim 0.13$–0.20 for both Phy and dgPhy (see Fig. 3). The origin of this could not be established, but it may reflect lyotropic (hydration dependent) rearrangements in the freeze-dried protein.

[1]The observation that the adsorption data did not depend on the amount of protein in the cell (3–16 mg) supports the assumption of equilibrium.

In conclusion we have applied a two-channel perfusion calorimetric method to the investigation of a glycoprotein and its carbohydrate-depleted variant. Comparison of these two-model systems shows that the peptide groups adsorb more water than the glycans. In other words, the free energy of interaction with water is more favorable for the peptide than the glycan moieties of Phy. Moreover, the adsorption energy, ΔH_{ads}, is similar for the two variants and close to that observed for other model proteins. While specific hydration effects at still higher hydration levels (i.e., in dilute solution) cannot be ruled out, these observations suggest that particularly favorable interactions of water and glycans cannot be the main reason for the high solubility and limited aggregation propensity observed for glycosylated proteins.

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