Preferential Binding of Two Compatible Solutes to the Glycan Moieties of *Peniophora lycii* Phytase[†]

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ABSTRACT: Regulation of hydration behavior, and the concomitant effects on solubility and other properties, has been suggested as a main function of protein glycosylation. In this work, we have studied the hydration of the heavily glycosylated *Peniophora lycii* phytase in solutions (0.15−1.1 *m*) of the two compatible solutes glycerol and sorbitol. Osmometric measurements showed that glycerol preferentially binds to phytase (i.e., glycerol−glycoprotein interactions are more favorable than water−glycoprotein interactions resulting in a preferential accumulation of glycerol near the protein interface), while sorbitol is preferentially excluded from the hydration sphere (water−glycoprotein interactions are the more favorable). To assess contributions from carbohydrate and peptide moieties, respectively, we compared phytase (Phy) and a modified, yet enzymatically active form (dgPhy) in which 90% of the glycans had been removed. This revealed that both polyols showed a pronounced and approximately equal degree of preferential binding to the carbohydrate moiety. This preferential binding of polyols to glycans is in contrast to the exclusion from peptide interfaces observed here (for dgPhy) and in numerous previous reports on nonglycosylated proteins. Despite the distinct differences between peptide and carbohydrate groups, glycosylation had no effect on the stabilizing action provided by glycerol and sorbitol. On the basis of this, it was concluded that the carbohydrate mantle of Phy is equally accessible in the native and thermally denatured states, respectively (most likely fully accessible in both), and thus that its interactions with compatible solutes have little or no effect on conformational equilibria of the glycoprotein. For solubility and aggregation equilibria, on the other hand, the results suggest a polyol-induced stabilization of monomeric forms.

The interaction of polyhydroxy alcohols (polyols) and proteins has attracted considerable research interest over decades. For example, polyols belong to the evolutionary conserved group of so-called compatible solutes, which are accumulated in a variety of cell types in response to high external osmotic pressures (*1*−*3*). While the primary role of this buildup is the colligative decrease in intracellular water activity and the concomitant regulation of cellular volume, insight into biomolecule−polyol interactions is necessary to elucidate in vivo effects of these compounds. For example, this type of information may elucidate the remarkable noneffect of polyols, even in molar concentrations, on metabolic processes (*2*). Furthermore, recent work has suggested that at least some compatible solutes are not merely inert effectors of cellular volume but provide a necessary protection of macromolecular structure in the low-water environment that prevails even after accumulation of the organic solutes (*4*−*6*). This aspect, which has led to terms such as "compensatory solutes" for the compounds (*5*, *7*, *8*) and "osmophobic effects" for their stabilizing mechanism (*9*), further stresses the relevance of molecular information on biomolecule−polyol interactions.

A main approach of elucidating polyol−protein interactions has its roots in the theory of preferential interactions (see, e.g., refs *10* and *11*). Experimental work along these lines has shown that polyols are in general moderately to strongly excluded from the protein−solvent interface (*12*−*14*). This implies that the local concentration of the polyol in the protein hydration sphere is lowered compared to the bulk. By use of the so-called Wyman relationships (*15*), changes in the extent of exclusion between different states can be directly related to the effect of the polyol on protein equilibria, such as thermal unfolding or aggregation. This connection has motivated the use of arguments based on preferential interaction theory in discussions of mechanisms underlying numerous processes within biochemistry, medicine, and biotechnology. Some examples include adaptative responses to hyperosmotic environments mentioned above (*4*, *5*, *16*, *17*), stresses associated with freezing (*18*), gelation processes (*19*) and the well-known in vitro stabilization provided by polyols (*14*, *20*, *21*).

In this work we have used dew point osmometry (*12*, *22*) to measure the preferential interactions of a heavily glycosylated phytase and two polyols, glycerol and sorbitol. The work was motivated by several factors. First, glycoproteins have not previously been directly investigated in this respect, and insight into the properties of glycosylated interfaces is clearly required in discussions of polyol effects in vivo. Second, polyol−glycoprotein interrelationships may be of

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particular interest since glycosylation covalently modifies physical properties such as solubility, thermal stability, and aggregation behavior (*23*−*32*). These same properties can also be regulated by polyols through noncovalent (solvent) effects (*33*), and the interplay of these two mechanisms appears to be of interest. Third, the fact that phytase (like several other glycosylated enzymes) can be readily deglycosylated without significant changes in the enzymatic activity (and hence presumably in polypeptide structure) may provide an approach to single out separate effects of peptide and carbohydrate and to assess driving forces underlying the measured preferential interactions. Recent investigations of the latter problem (*34*−*37*) have focused on the balance between soft and hard interactions (i.e., respectively "soft" intermolecular binding such as van der Waals forces or hydrogen bonding and "hard" steric repulsions). Most of these works have highlighted the importance of the hard contributions to polyol−protein interactions. Due to the pronounced differences in chemical structure and steric properties of the interface of phytase (Phy)[1] and deglycosylated phytase (dgPhy), respectively, a comparative analysis of the Phy/dgPhy system may provide an experimental approach to this discussion.

MATERIALS AND METHODS

Glycerol (99%) and D-sorbitol (>98%) were purchased from Sigma-Aldrich (St. Louis, MO) and used as supplied.

Peniophora lycii phytase was expressed in *Aspergillus oryzae* and purified according to previously published procedures (*38*). SDS−PAGE showed >95% purity and a molecular mass of about 65 kDa. The purified protein solution was stored at −25 °C. When needed, the samples were thawed and extensively dialyzed (MW cutoff 8 kDa) against milliQ water.

The systematic name of phytase (EC 3.1.3.26) is *myo*-inositol hexakisphosphate phosphohydrolase, and it catalyzes the hydrolysis of phosphomonoester bonds of phosphorylated *myo*-inositol, phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate). The products are lower forms of *myo*-inositol phosphates and inorganic phosphates (*38*). The enzyme is N-glycosylated at 10 sites with heterogeneous glycan chains dominated by structures of two *N*-acetylglucosamine units connecting the protein and a high mannose moiety (*38*). To produce deglycosylated phytase (dgPhy), 6.3 mL of the purified sample (23.7 mg/mL) was mixed with a 1.6 mL solution (0.093 mg/mL) of the enzyme Endo F_1 (a kind gift from Hoffmann-La Roche, Basel, Switzerland) and 32 mL of 50 mM sodium citrate and 20 mM EDTA, pH 6.0. The mixture was allowed to react for 24 h at room temperature. Endo F_1 (endo-β-*N*-acetylglycosaminidase; EC 2.2.1.96) hydrolyzes the glycosidic bond between the first and second *N*-acetylglycosamine groups linking the saccharides to the protein. Thus, the deglycosylated protein used in this work may retain ten *N*-acetylglycosamine groups (one on each glycosylation site). Following the hydrolysis with Endo F_1, the mixture was extensively dialyzed (MW cutoff 14 kDa) at 4 °C against milliQ water to remove salts and oligosaccharides. Endo F_1 (MW 32 kDa) remains in the phytase solution, but since the phytase/Endo F_1 molar ratio is about 500:1, the effect of this impurity is deemed negligible in the osmometric measurements, and no further purification was made.

The enzymatic activity of Phy and dgPhy, specified as the turnover rate at high substrate concentration, was tested by the method of Lassen et al. (*38*). Briefly, dialyzed and lyophilized phytase (Phy and dgPhy) was dissolved in 0.1 M sodium acetate and 0.01% (v/v) Tween-20, pH 5.5, to an enzyme concentration of 1.3×10^{-5} mM. The enzyme was further diluted 26-fold into a preincubated (37 °C) substrate solution (5 mM sodium phytate in 0.1 M sodium acetate and 0.01 vol % Tween-20, pH 5.5). After 30 min at 37 °C, the reaction was stopped by adding an equal volume of 10% trichloroacetic acid. Free inorganic phosphate was measured by the addition of an equal volume of molybdate reagent (7.3 g of $FeSO_4$, 1.0 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, and 3.2 mL of H_2SO_4 diluted in milliQ water to 100 mL). Absorbance was measured at 750 nm (Vmax microplate reader; Molecular Devices, Sunnyvale, CA), and the phosphate concentration was determined relative to prepared standard solutions. The assay was repeated in substrate solutions containing 2.2 M glycerol or sorbitol.

The water activity (osmolality) of two-component (water + polyol) and three-component (water + polyol + Phy or water + polyol + dgPhy) solutions was measured using a Vapro 5520 osmometer (Wescor Inc., Logan, UT). This instrument measures the thermodynamic activity of water in a solution by recording the difference between the temperature of a liquid and the dew point of its equilibrated gas phase. In this work, the osmolality of two- and three-component systems was measured as a function of the polyol concentration in the 0.15−1.1 *m* range. All experimental trials were initiated by calibrating the osmometer using standard solutions (100, 290, and 1000 mOsm, respectively) provided by Wescor. If standard measurements did not provide reproducible readings, the osmometer thermocouple was cleaned with an ammonium hydroxide solution (Wescor "cleaning solution") and deionized water. The calibration was frequently tested (at least once for every 10 readings) against the standard solutions. If the reading deviated more than 2 mOsm from the nominal value, the osmometer was recalibrated. All data reported here were recorded at 25 ± 1 °C and a laboratory relative humidity of 20−30%. [Courtenay et al. (*12*) have found that measurements on this instrument become less reproducible at an ambient humidity exceeding 42%.] The quality of the data is crucially sensitive to the exact control of the sample composition. Hence, the following gravimetric procedure for sample preparation was adopted. First, two-component batch solutions of glycerol and sorbitol, respectively, were prepared gravimetrically from milliQ water and newly delivered polyol. Subsequently, 1000 μL of the dialyzed samples of Phy and dgPhy, respectively, was transferred to tared plastic tubes, weighed, and lyophilized in the tubes for 48 h. Immediately after removal from the lyophilizer, the dry protein was dissolved in ≈200 μL of the two-component batch solution of polyol added directly to the plastic tube. The sample was sealed, weighed, and stored at 5 °C. For the osmometric measurements, 7 μL aliquots of the two-component batch solution and protein sample (three-component solution), respectively, were ana-

[1] Abbreviations: Phy, *Peniophora lycii* phytase (*myo*-inositol hexakisphosphate phosphohydrolase); dgPhy, deglycosylated *P. lycii* phytase; Endo F_1, endo-β-*N*-acetylglycosaminidase; DSC, differential scanning calorimetry.

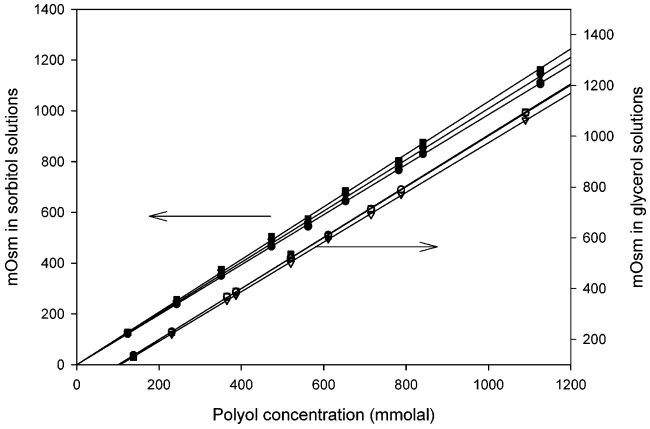

FIGURE 1: Osmolalities (water activities) in solutions of sorbitol (filled symbols, left-hand ordinate) and glycerol (open symbols, right-hand ordinate) as a function of the polyol concentration in millimolal. For each polyol, the osmolality is shown for binary aqueous solution (circles) and for solutions containing 0.88−0.99 m*m* phytase (squares) or 0.92−0.98 m*m* deglycosylated phytase (triangles). To facilitate reading of the two nearly superimposed families of curves, the two ordinates are displaced by 100 mOsm.

lyzed in the osmometer using the standard procedure recommended by the manufacturer. Three to six parallel measurements were made for each solution, and two experimental series with separate two-component batch solutions and Phy and dgPhy preparations were conducted.

The thermal stability of Phy and dgPhy was investigated by scanning calorimetry (MC 2-DSC; MicroCal, Northampton, MA). Dialyzed and lyophilized protein was added to the two-component batch solution to yield a total protein concentration of about 5 mg/mL. The calorimeter was loaded with the protein−polyol solution (cell) and a binary polyol solution of the same concentration (reference), and the heat capacity trace was recorded during heating from 30 to 90 °C at 45 °C/h. The thermal stability was measured at about 10 different polyol concentrations in the 0−1500 m*m* range.

RESULTS AND DATA TREATMENT

Representative raw data from the dew point osmometry are illustrated in Figure 1. On the basis of the reproducibility of the calibration measurements and the observation that lyophilized protein samples have a residual moist content up to 4% (w/w) (weight loss after lyophilization in samples dried to constant mass over P_2O_5 at 80 °C), the experimental precision in Figure 1 is estimated to be ±5 mOsm. It appears that (within the experimental resolution) the osmolality of all systems increases linearly with the polyol (glycerol or sorbitol) concentration. The figure reveals a small difference between the two- and three-component data, which depends systematically on the concentration of the polyol. This difference reflects the effects on water activity of protein−solvent interactions, and it may be quantified by the so-called isoosmotic preferential binding (or interaction) parameter, Γ_{μ_1} (*12*). Using the conventional notation of subscripts 1,2, or 3 for water, protein, and polyol, respectively, this parameter is defined as

$$\Gamma_{\mu_1} \equiv (\partial m_3/\partial m_2)_{T,P,\mu_1} \quad (1)$$

where m denotes molal concentrations, μ is chemical potential, and T and P have their usual meaning. Equation 1 shows that the isoosmotic binding parameter specifies the number of polyol molecules which have to be added or removed to reestablish the chemical potential of water upon addition of one protein molecule to an aqueous polyol solution. Its rigorous relation to solution thermodymamics, other preferential binding parameters, and conventional binding theory as well as its molecular interpretation have been discussed in detail in a number of works (*10*, *11*, *39*−*41*) and will not be reviewed here. We only note that the sign of Γ_{μ_1} can be interpreted as indicating whether the small solute is accumulated in (preferential accumulation or "binding", $\Gamma_{\mu_1} > 0$) or depleted from (preferential exclusion, $\Gamma_{\mu_1} < 0$) the zone near the protein interface.

For nonvolatile solutes, Γ_{μ_1} can be derived directly from dew point osmometry measurements (*12*)

$$\Gamma_{\mu_1} \approx (m_3 - m_3^{\Delta})/m_2 \quad (2)$$

where m_3^{Δ} is the polyol molality of the two-component solution, which has the same water activity (osmolality) as the three-component solution. Hence, the numerator of eq 2 is the horizontal distance between a set of curves (with/without protein) in Figure 1. This difference was determined using each data point for the two-component systems and the isoosmolal composition of the three-component solution calculated from the regression line shown in Figure 1.

Plots of Γ_{μ_1} vs polyol concentration (not shown) revealed linear correlation. The slopes of the regression lines (±95% confidence limit) were respectively $-22 \pm 4.1\ m^{-1}$ (Phy) and $-43 \pm 3.6\ m^{-1}$ (dgPhy) for sorbitol and $31 \pm 2.6\ m^{-1}$ (Phy) and $-5 \pm 2.4\ m^{-1}$ (dgPhy) for glycerol. These results reflect protein−solvent interactions, but it has been argued (*41*) that a different preferential binding parameter, Γ_{μ_3}, provides a more meaningful approach to discussions of thermodynamic effects and their molecular interpretation. This latter binding parameter is defined analogously to Γ_{μ_1} (eq 1), the only difference being that the chemical potential of polyol (μ_3) rather than that of water (μ_1) is kept constant in the partial derivative. Γ_{μ_3} is not readily accessible by experiment but can be estimated from Γ_{μ_1} as described by Courtenay et al. (*12*). Using eq 12 of this work, we have calculated Γ_{μ_3} for the present systems and plotted it against the polyol concentration, m_3, in Figure 2. The numeric difference between Γ_{μ_1} and Γ_{μ_3} is very small. Thus, the data for sorbitol (filled symbols) shows negative slopes, indicating that both Phy and dgPhy are preferentially hydrated in aqueous sorbitol. The slopes of the curves are -24 ± 6 and $-45 \pm 6\ m^{-1}$, respectively, suggesting that the preferential hydration (or preferential exclusion of sorbitol) is stronger by a factor of 2 for the deglycosylated protein. For glycerol (Figure 2, open symbols) the Phy data show a distinctive positive slope ($25 \pm 5\ m^{-1}$), while Γ_{μ_3} decreases slowly with m_3 for dgPhy ($\Gamma_{\mu_3}/m_3 = -6 \pm 5\ m^{-1}$). Hence, glycerol is preferentially bound to the glycosylated protein while the peptide moiety shows a slight preference for water. Comparison of all data in Figure 2 reveals the general trend that glycans interact more favorably with the polyols than with water. Interestingly, the (negative) change in slope observed as a result of the deglycosylation is approximately the same for the two investigated solutes. This signifies favorable interactions of similar strength between the two polyols and the glycan moieties.

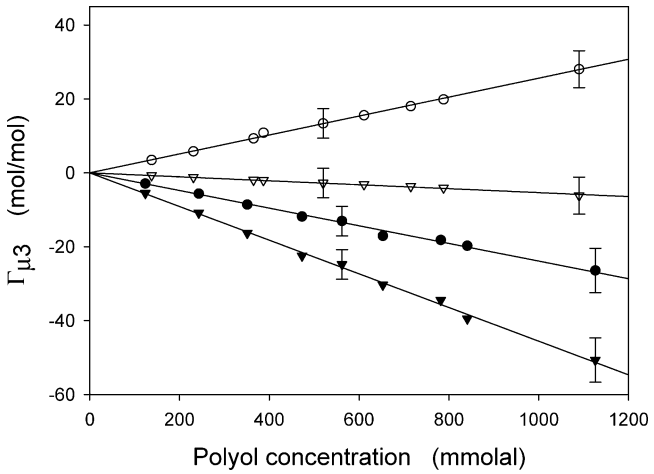

FIGURE 2: Preferential binding parameter, Γ_{μ_3}, specified by constant polyol activity (see text and ref *12* for details) plotted as a function of the polyol concentration. Filled and open symbols indicate sorbitol and glycerol, respectively. Data for the glycosylated protein (Phy) is given by circles while triangles refer to the deglycosylated variant (dgPhy). A noticeable feature of the figure is the substantial negative change in slope resulting from the deglycosylation. This change, which is similar for the two polyols, signifies favorable interactions of polyols and glycan moieties.

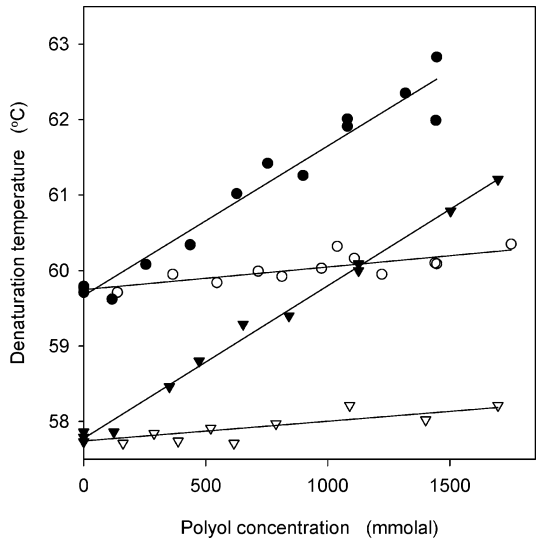

FIGURE 3: Denaturation temperature specified as the maximum of the DSC trace for phytase (circles) and deglycosylated phytase (triangles) as a function of the polyol concentration. Filled symbols represent sorbitol while open symbols are data for glycerol. The parallel course of the data for glycerol (slope 0.15 °C/*m*) and sorbitol (slope 1.8 °C/*m*), respectively, shows that the stabilizing effect of the two polyols is independent of glycosylation.

Results from the scanning calorimetric studies of the thermal denaturation of Phy and dgPhy are illustrated in Figure 3. Transition temperatures, T_m, defined as the maximum of the heat capacity traces (not shown), were found for both Phy and dgPhy as a function of the glycerol or sorbitol concentration and plotted against m_3 in the figure. In accordance with a previous report (*38*), T_m of the glycosylated protein is about 60 °C in aqueous buffer. It appears that deglycosylation introduces a slight (1.9 °C) decrease in T_m and that the thermal stability of both Phy and dgPhy is enhanced by the polyols. This latter effect is much stronger for sorbitol (the slope T_m/m_3 is 1.8 °C/*m*) than for glycerol ($T_m/m_3 = 0.15$ °C/*m*). For the current discussion, however, the most consequential result of Figure 3 is that the degree of stabilization brought about by sorbitol and glycerol, respectively, is practically identical for Phy and dgPhy (the lines in Figure 3 are pairwise parallel). Selected samples were taken through a second heating scan, and this suggested that the thermal denaturation of Phy and dgPhy was almost fully reversible.

The enzyme activity assays showed that deglycosylation brought about a small but systematic reduction in turnover rate. Thus, the activity of the deglycosylated samples fell within the 90−95% range of glycosylated protein from the same sample. The presence of 2.2 M glycerol or sorbitol in the reaction buffer did not change this behavior.

DISCUSSION

Carbohydrates are posttranslationally attached to most secreted eucaryotic proteins and appear to serve a number of purposes (*42*), which may be coarsely categorized in two groups. First, glycosylation has been connected to molecular recognition with respect to, e.g., targeting, immunological properties, protease susceptibility, and interactions with chaperone proteins (*42*−*45*). Second, the glycans modify physical (and hence functional) properties of enzymes. In particular, glycosylation has been related to solubility, but other properties such as oligomeric aggregation behavior and conformational stability have also been discussed intensively (*23*−*32*). The understanding of this latter group of effects relies on the elucidation of protein−solvent interactions, and the scope of the current work is to address this through measurements of preferential interactions in Phy−polyol and dgPhy−polyol systems, respectively.

The main result of the preferential interaction data in Figure 2 is that glycerol and sorbitol preferentially bind to the glycan groups of Phy. This effect is rather pronounced. Thus, the additional glycosylation of Phy (approximately 20 kDa oligosaccharides compared to approximately 2 kDa monosaccharides on dgPhy) increases the value of Γ_{μ_3}/m_3 (i.e., the slope in Figure 2) by 20−30 m^{-1} for both polyols. To our knowledge this is the first direct quantification of such interactions. The favorable interactions of glycans and polyols are in contrast to their preferential exclusion from polypeptide interfaces found here (data for dgPhy in Figure 2) and in several previous reports on nonglycosylated proteins (*12*−*14*). Comparison with these works shows that the preferential exclusion of glycerol from dgPhy found here is similar to what has been reported for (nonglycosylated) proteins. Thus, the slope in Figure 2 (-6 ± 5 m^{-1}) for glycerol/dgPhy (MW 48 kDa) compares well with results for bovine serum albumin (66 kDa), chymotrypsinogen (26 kDa), α-chymotrypsin (25 kDa), and RNase A (14 kDa), which fall in the −10 to −2 m^{-1} range according to their size (*12*). For sorbitol the preferential exclusion (-45 ± 6 m^{-1}) found here for dgPhy is stronger than what has previously been measured for RNase A (approximately −8 m^{-1}) (*14*). Even if the results are normalized with respect to the sizes of the proteins (i.e., surface areas assuming that this parameter is proportional to $MW^{2/3}$), the preferential exclusion of sorbitol from dgPhy is more than twice the value in RNase A solutions. The degree of preferential exclusion in dgPhy−sorbitol is comparable to that found for the BSA−betaine system, where it roughly corresponds to the complete exclusion of solute from a monolayer of water at the protein interface (*12*).

Preferential interactions can be related to the thermodynamic stability of protein conformations through the Wyman relationships (*15*). For a simple two-state (native ↔ dentured) equilibrium, for example, a solute will stabilize the conformation with the highest Γ_{μ_3} (i.e., least negative Γ_{μ_3} for preferentially excluded solutes). Since, in many cases, the preferential interactions are rather independent of the solution conditions and surface properties of the protein (*11*, *33*), preferential exclusion of a solute from the native state is often tantamount to its stabilization. In that case, the degree of exclusion in the denatured state is larger, and hence the stability of the native state is increased, simply as a result of the enlargement of the interface upon denaturation. This type of stabilization is believed to occur for a range of compatible (or compensatory) solutes, and it has been suggested that "a major characteristic of the compensatory solute is that it is preferentially excluded from the protein surface and its immediate hydration sphere" (*4*). While the current data (Figures 2 and 3) suggest that this picture applies to dgPhy, it may not be readily applicable to glycoproteins, since the polyols showed a considerable preferential binding to the carbohydrate mantle of Phy. The extent of this effect may be illustrated by comparing the influence of the glycans on the slope Γ_{μ_3}/m_3 (20−30 m^{-1} according to Figure 2) and the preferential binding to intact proteins of denaturants such as urea and guanidine hydrochloride. For urea the preferential binding to small globular proteins typically corresponds to Γ_{μ_3}/m_3 values in the 5−15 m^{-1} range (*11*, *22*, *46*) although negative values have been observed (*11*). The affinity of serum albumin for guanidine hydrochloride is slightly higher, with a slope (Γ_{μ_3}/m_3) of about 18 m^{-1} in dilute solution (*47*, *48*). This comparison might seem puzzling with respect to the cellular compatibility of these solutes, but as illustrated in Figure 3, the stabilization conferred by the polyols is in fact independent of the extent of glycosylation. These observations can be reconciled if the oligosaccharides are equally exposed to the solvent in the native and thermally denatured states. Such lack of additional exposure upon unfolding most likely implies that the glycans are fully exposed in the more condensed (native) state. If so, their preferential interactions will not change as a result of structural changes such as thermal unfolding, and hence polyol−glycan interactions will not perturb conformational equilibria of the protein molecule. It is interesting to note that if this mechanism turns out to be general for glycosylated proteins, it may set a maximum limit for the tolerated load of carbohydrates on a protein. At very high levels of glycosylation, some degree of (temporary) steric restrictions will limit the solvent accessibility of the carbohydrates in the native state. When these restrictions are reduced in a less compact, unfolded state, the increased accessibility would promote preferential binding of the polyol and consequently stabilize this state over the native glycoprotein. It follows that, rather than being compensatory solutes, polyols would be denaturants of such heavily glycosylated proteins. On the basis of these considerations, we suggest that the oligosaccharides of Phy are fully hydrated in both the native and thermally denatured state. As a result, the polyol-induced stabilization of Phy is suggested to arise from unfavorable polyol−polypeptide interactions (preferential hydration of the peptide moiety), while glycan−polyol interactions are of minor importance for the conformational stability of the protein.

As mentioned in the introduction, many reports have discussed glycosylation with respect to solubility (and changed oligomeric aggregation behavior). The current results suggest that, in an environment containing polyols, glycosylation will strongly promote solubility (and the stability of nonaggregated forms) and thus counteract the "salting-out" effect of polyols on peptides. This follows from the favorable glycan−polyol interactions which will favor forms with larger accessible carbohydrate surface. Analogous arguments have been put forward by Tams et al. (*49*), who found that the solubility of a glycosylated, fungal peroxidase was enhanced by ammonium sulfate and reduced by acetone. They further showed that this effect on the solubility scaled with the carbohydrate content in a series of N-glycosylation mutants. Tams et al. suggested that the results for ammonium sulfate reflected favorable interactions, with the glycans, and this conclusion has an interesting analogy to the present observations. Thus, both polyols and sulfate generally show preferential exclusion from peptide interfaces (*11*, *33*) but appear to preferentially bind to the carbohydrate moiety of glycoproteins. This distinct difference raises the question of what sources drive the interactions. For nonglycosylated proteins, the interaction with sulfate is rationalized through the strong structure-making (cosmotropic) properties of the ion. This (Hofmeister) effect of sulfate relates to its strong hydration and the concomitant affinity for bulk rather than interfacial positions (*50*). For polyols, analysis of the interaction through scaled particle theory suggests that steric restriction is a major contributor to the preferential exclusion (*34*, *36*). However, both of the above mechanisms are independent of the chemical structure of the interface, and hence they cannot account for the observed difference between peptide and carbohydrate surfaces. Therefore, we suggest that preferential binding of polyols to glycans as well as the "non-Hofmeister" behavior of sulfate relies on direct (i.e., soft) interactions of carbohydrates and the solutes. For the polyols, this probably involves hydrogen bonding to the glycans, although this explanation is far from unequivocal. Thus, thermodynamic properties of most aqueous polyols and carbohydrates (*51*−*53*) show that interactions with water are favored over mutual solute interactions. It follows that there is no a priori reason why glycans should attract polyols stronger than they do water, and further insight into this mechanism will probably require systematic studies of model systems.

The results for polyol−glycan interactions presented here may also be discussed along the lines of the seminal work on glycerol−protein interactions by Gekko and Timasheff (*13*). These workers discussed surface-dependent interactions and suggested that glycerol was attracted to the polar regions of (nonglycosylated) proteins while nonpolar areas interacted more favorably with water. The current finding of a sizable preferential binding of glycerol to the polar surface of the oligosaccharides corroborates the suggestion of Gekko and Timasheff. More recent work in this field by Bolen and coworkers found that the interaction of amino acid side chains and compatible solutes was typically weakly attractive, while the compatible solutes were strongly expelled from the interface of the peptide backbone (*9*, *54*, *55*). The preferential binding of polyols to glycans found here may point toward

an additional interrelationship of surface chemistry and preferential interaction. Thus, the subtle balance of water and polyol interactions with the interface of glycoproteins seems to result in a relative affinity (or preference) for water, which scales as peptide backbone > side chains > glycans. Elucidation of the validity and importance of this suggestion will rely on further systematic studies of preferential interactions of glycosylated proteins.

To summarize, we have found that the compatible solutes glycerol and sorbitol preferentially bind to the carbohydrate moiety of the glycoprotein phytase, while they are preferentially excluded from the peptide surface of this protein. The thermal stability of Phy is slightly reduced upon deglycosylation, but the stabilizing action of the compatible solutes is identical for Phy and dgPhy. It is suggested that the polyols accumulate in the hydration sphere of the oligosaccharides as a result of direct interactions and that these moieties are fully accessible to the solvent in the native protein. Because of the rather strong preferential binding, this high accessibility is suggested to be essential for the compatibility of polyols and glycoproteins. Further studies of glycosylated proteins including parallel investigations of native and denatured forms will be required to clarify these proposals.

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