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Glycoprotein-surfactant interactions: A calorimetric and spectroscopic investigation of the phytase-SDS system

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Abstract

The interactions of sodium dodecyl sulfate (SDS) and two glyco-variants of the enzyme phytase from *Peniophora lycii* were investigated. One variant (Phy) was heavily glycosylated while the other (dgPhy) was enzymatically deglycosylated. Effects at 24 °C of titrating SDS to Phy and dgPhy were studied by Isothermal Titration Calorimetry (ITC) and Synchrotron Radiation Circular Dichroism (SRCD) spectroscopy. Comparisons of results for the two variants were used to elucidate glycan–surfactant interrelationships.

The CD spectra suggested that both the native and the SDS-denatured states of the two variants were mutually similar, and hence that the denaturation process was structurally equivalent for the two glyco-variants. The denatured state was far from fully unfolded and probably retained a substantial content of native-like structure. Furthermore, it was found that the glycans brought about only a small increase in the resistance towards SDS induced denaturation. The SDS concentration required to denature half of the protein molecules differed less than 1 mM for the two variants.

The affinity for SDS of both variants was unusually low. The amount of bound SDS (w/w) at different stages of the binding isotherm was 3–10 times lower than that reported for the most previously investigated globular proteins. Analysis of the relative affinity of the glycan and peptide moieties suggested that the carbohydrates bind much less surfactant. At saturation, glycans adsorbed about half as much SDS (in g/g) as the peptide moiety of Phy and about five times less than average proteins.

Keywords: Surfactant-protein interactions; Thermodynamics; Glycosylation; Stability; Circular dichroism; Titration calorimetry

1. Introduction

Understanding the interactions of proteins and solutes affecting their physical properties is essential for improved formulation and application of industrial- and pharmaceutical proteins. Surfactants constitute an important and interesting class of such solutes. Thus, the broad range of chemical structures within this group of additives enables modulation of for example protein solubility and aggregation behavior [1]. Owing to their amphiphilic nature, surfactants tend to adsorb at interfaces or mutually associate (form micelles) to minimize the contact of their hydrophobic part with water. This tendency also leads to an unspecific binding to proteins in solution, which in turn underlies the increased solubility and conformational instability of proteins in the presence of surfactants [2]. This relationship between weak binding on the one hand, and solubility and instability on the other, is rigorously established through the so-called linkage theory by Wyman and Gill [3].

To date, the most studied surfactant is sodium dodecyl sulfate (SDS). This anionic compound, which is generally considered a potent denaturant [4], was investigated in many early works on bovine serum albumin (BSA)[5–10]and a number of other proteins[11–17]. This led to several general conclusions regarding the modes of interaction of SDS and proteins (for reviews see[18–20]). Thus, a certain pattern of binding events has been observed before the adsorption of surfactant eventually reached saturation at about 1.4 g SDS per g protein with reduced S–S bonds [15,21] and somewhat less (~1 g/g) in proteins with intact cystines [21]. Some exceptions

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from this picture, however, have been reported. Glucose oxidase, for example, binds very little SDS and (in a certain pH range) retains its catalytic activity at high SDS [12,22]. Also, we have recently found that the lipolytic enzyme cutinase binds only about 0.4 g SDS/g at saturation [23,24].

The current work investigates the interactions between SDS and the enzyme phytase (Phy) from *Peniophora lycii*. This is a glycoprotein (N-glycosylated on ten glycosylation sites) consisting of a 47.5 kDa peptide and a total of ~18 kDa carbohydrate, which mainly consists of oligomeric mannose [25–27]. To address the effects of glycosylation on the SDS-induced destabilization, and to determine the relative affinity of the detergent for the peptide and glycan moieties, we compared data for Phy with results for an enzymatically deglycosylated form (dgPhy). The importance of glycoprotein-surfactant interrelationships has been pointed out already in the seminal work of Pitt-Rivers and Impiomba [21], but none of the nearly 500 papers quoting this publication has addressed the problem. Recently, progress has been made in the understanding of surfactant interactions with soluble oligosaccharides[28–31], but protein conjugated glycans remain practically unexplored. Increased knowledge in this area may play a role in the analysis of glycoproteins in SDS gel electrophoresis [32,33]. More importantly, information on glycan-detergent interactions appears to be of particular interest in attempts to apply glycosylation as a tool to modify the properties of proteins and peptides in biotechnology. The purpose of the current work is to elucidate some thermodynamic and structural effects of the glycans on Phy during exposure to SDS. The work also revealed an unusually low SDS-affinity of this protein, which will be briefly discussed.

2. Methods

P. lycii phytase was expressed in *Aspergillus oryzae*, purified according to previously published procedures [27], and stored at −25 °C in buffer solution (20 mM Tris-acetate, pH 6). The molecular mass of the glycosylated phytase (~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 is described in details elsewhere [25]. To ensure that the deglycosylation was quantitative the reaction mixture was investigated by SDS–PAGE. This showed one narrow band at ~48 kDa in accordance with a deglycosylated protein. Before the experimental trials, Phy and dgPhy were dialyzed (molecular weight cut of 12–14 kDa), at 4 °C, against milliQ water and subsequently freeze dried. All analytical (CD and ITC) was conducted with pure water as the solvent. Parallel samples of both protein variants were freeze dried in small vials and analyzed for residual water content by overnight exposure to 80 °C. In accordance with previous work on lyophilized phytase [26] no systematic weight loss could be detected in either of the protein variants. Based on this and the purity derived from the SDS–PAGE it was considered appropriate to derive the protein concentration from weighing of the lyophilized powders. SDS (>98%) was purchased from BDH laboratory (Poole, UK).

2.1. Isothermal titration calorimetry (ITC)

The freeze dried protein was dissolved in milliQ water (Spectrapore, VWR Scientific, San Francisco, CA). The protein solution was then loaded into the cell (1.43 ml) of a MicroCal VP-ITC instrument (Northampton, MA) and titrated with 110 mM SDS (dissolved in milliQ) from a 250 μL syringe. Four different protein concentrations in the 17–67 μM range were investigated for both Phy and dgPhy. Both protein and detergent solutions were degassed by stirring under vacuum prior to the experiment. The experimental temperature was 24 °C. The molar enthalpy change associated with SDS-protein interactions was calculated by integrating the raw calorimetric data (heat flow vs. time) using the Origin software, supplied with the instrument.

2.2. Synchrotron radiation circular dichroism (SRCD) spectroscopy

Stock solutions of Phy (~2 mg/ml) and dgPhy (~1.7 mg/ml) were prepared by redissolving the lyophilized powders in milliQ water. Aliquots of 500 μl stock were quantified by mass in eppendorf tubes and freeze dried in the tube. Fifteen minutes prior to the CD measurement, the proteins were hydrated in 500 μl SDS solution, giving a concentration of respectively 32 μM and 35 μM for Phy and dgPhy. Fourteen different Phy solutions and 12 dgPhy solutions covering the 0–20.4 mM SDS range were investigated.

The SRCD spectra were recorded at the UV1 beam line on the storage ring ASTRID, Institute for Storage Ring Facilities (ISA), University of Aarhus, Denmark. The instrument was carefully calibrated with respect to CD signal using camphor sulfonic acid (CSA) after each beam fill (once a day) of the storage ring. All spectra were recorded using a 0.1 mm path length quarts SUPRASIL cell (Hellma GmbH, Germany), over a wavelength range of 176–265 nm in 1 nm steps and a dwell time of 3 s per wavelength point. A pure solvent baseline collected with the same cell was subtracted all spectra which were processed and analyzed using the CDtool software package [34]. The machine unit (mdeg) was converted into the per residue molar absorption unit, delta epsilon ($\Delta\varepsilon$) in $M^{-1}\cdot cm^{-1}$, by normalization with respect to polypeptide concentration and path length. Secondary structure content analysis of the spectra was based on the SP29 (29 water soluble proteins) reference set from the soft ware package CDPro [35].

CD spectra for Phy were also measured in 5 °C intervals from 24 °C to 84 °C. The sample was equilibrated 5 min at each temperature prior to the measurement.

3. Results

The SDS induced structural changes of *P. lycii* phytase are illustrated by the CD spectra in Fig. 1. This figure shows Phy spectra in 14 different solvents covering the 0–20.4 mM SDS

range. No significant changes could be detected in the seven spectra with a SDS concentration of 4.0 mM or less. Higher concentrations brought about spectral changes particularly a decreased ellipticity at about 189 nm and 206 nm and a concomitant increase in $\Delta\varepsilon$ around 222 nm. The changes around 206 nm observed at intermediate SDS concentrations resemble those reported earlier for other proteins [16,36]. The changes in the CD spectra of Phy continue until about 9–10 mM SDS. Hence, the CD data suggests that the native structure of Phy remains intact until the SDS concentration exceeds ~4 mM, and that the structural transition is completed when [SDS] ~ 10 mM.

Analogously the structural changes of the deglycosylated enzyme are studied by SRCD. Fig. 2 shows CD spectra for dgPhy in 12 solvents (0–20.4 mM SDS). The general trends in the spectral changes around 189 nm and 206 nm appear to be similar to those for Phy, however, the experimental scatter is larger in these trials and the increase in $\Delta\varepsilon$ around 222 nm is not clear for dgPhy (see below). Deviation from the native CD spectrum of dgPhy is observed at ~3 mM SDS, thus detectable structural changes of dgPhy starts at a lower SDS concentration compared to Phy. No significant changes in the four spectra from 8.1–20.4 mM SDS could be discerned.

Absolute values of $\Delta\varepsilon$ are somewhat smaller for dgPhy at the lower SDS concentrations (Fig. 2) and this behavior is paralleled by the UV-absorption of the same samples (measured simultaneously in the SRCD instrument). Thus, the effective protein concentration is lowered in the deglycosylated samples, most likely due to some aggregation and surface adsorption of dgPhy [37, 38]. As SDS increases solubility and inhibits aggregation, the differences in the spectral intensities decrease with increasing SDS concentration, and for the highest surfactant concentrations, no differences between the two enzyme variants were detected. In pure water, $\Delta\varepsilon$ for dgPhy was consistently lower than for Phy, but application of a scaling factor provided practically superimposed spectra (Fig. 3). This suggests that the structural transition associated with SDS-induced denaturation is similar for the two variants and that the (secondary) structure of both the native and the SDS denatured state of phytase is unaffected by glycosylation. For the native conformation, this observation is expected as the catalytic activity has been shown to remain unchanged following deglycosylation [25,26]. The spectra in Figs. 1 and 3 exhibit quasi isodichroic point around 212 nm suggesting a two-state transition. Owing to the scatter in

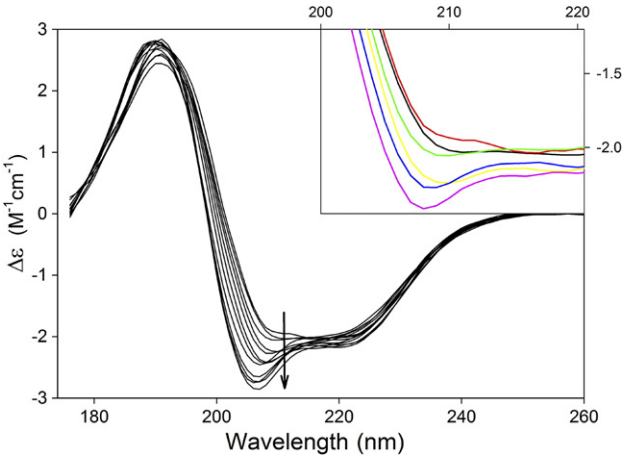

Fig. 2. CD spectra of dgPhy in twelve different solvents: 0, 0.2, 2.0, 3.0, 4.0, 5.1, 6.0, 7.1, 8.1, 10.1, 15.3 and 20.4 mM SDS. The $\Delta\varepsilon$ values change with increasing [SDS] in the direction of the arrow. [dgPhy] is 35 μM in all solutions. The inset is an enlargement of the 200–221 nm range. The spectra for 0 (black), 0.2 mM (red) and 2.0 mM (green) do not differ significantly. Structural changes induced by SDS can be detected in the spectra for higher SDS concentrations (3.0 mM (yellow), 4.0 mM (blue) and 5.1 mM (pink)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

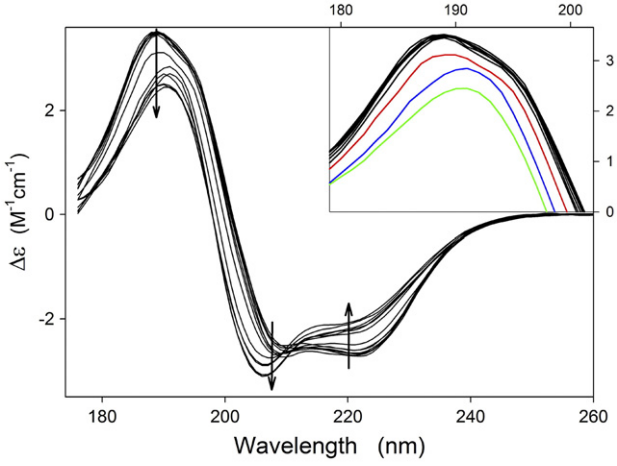

Fig. 1. CD spectra of Phy in fourteen different solvents ranging from pure water to 20.4 mM SDS. The actual concentrations: 0, 0.2, 0.5, 1.0, 1.5, 3.0, 4.0, 6.0, 8.1, 9.0, 10.1, 10.2, 15.3 and 20.4 mM SDS. The $\Delta\varepsilon$ values change with increasing [SDS] in the direction of the arrows in the main panel. [Phy] is 32 μM in all solutions. The inset represents an enlargement of the 179–202 nm range. The seven spectra (black) in the 0–4.0 mM SDS range are overlapping (to within the experimental precision). The red, blue and green curves represent respectively 6.0, 8.1 and 9.0 mM SDS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

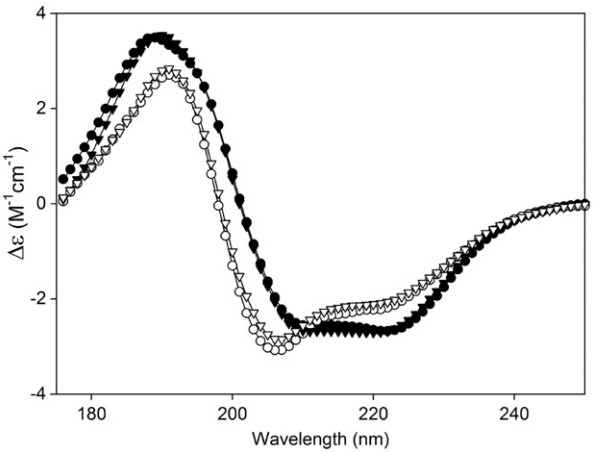

Fig. 3. CD spectra of Phy (circles) and dgPhy (triangles) in two different solvents: 0 mM SDS (filled) and 20.4 mM SDS (open). The recorded CD signal from dgPhy in the SDS free solution is multiplied by a factor of 1.3 (see main text). The superposition of SDS-free samples supports the conclusion that the native state of Phy is not affected by glycosylation. The scaling factor has only been applied in this figure (and only for dgPhy in pure water).

dgPhy spectra for low [SDS], a quasi isodichroic point is only found for SDS concentrations above 2 mM in Fig. 2.

To further illustrate the structural transition of Phy and dgPhy, and its cooperativity, the intensities of selected CD bands were plotted as a function of the total SDS concentration, $[SDS]_{tot}$, in Fig. 4. Panel A of this figure shows $\Delta\varepsilon$ for Phy at 189, 206 and 222 nm. This data is in accordance with a two state transition with a midpoint of about 7–8 mM SDS (*i.e.* half of the population of protein molecules denatured at this concentration). As discussed above, the CD data for dgPhy shows more scatter, but the results in Fig. 4B support a parallel interpretation with a midpoint value of 5–6 mM SDS.

In Fig. 5, isotherms from the ITC trials illustrate the enthalpic effects of SDS-protein interactions. The curve represented by crosses (panel A) is a reference experiment (SDS into pure water). This were used to assess the heat of dilution (and de-micellization) of the 110 mM SDS titrand and to determine the critical micelle concentration (CMC). The experimental temperature (24 °C) was chosen so that the injection of the first few aliquots of SDS was athermal ($\Delta H \sim 0$ in Fig. 5). Hence, the correction due to heats of dilution was as small as possible. Nevertheless, the heat flow associated with the dissolution of the micelles is concentration dependent and a small endothermic increase in the reference curve (crosses) builds up as the SDS concentration increases (Fig. 5A). The steeper endothermic increase in the ~7.1–9.2 mM SDS interval, designate the micellization process. Thus, the concentration of SDS in the calorimetric cell is raised to CMC and additional micelles will not be dissolved; c.f. [23,39]. The CMC value of SDS in the protein free solution is best described by the inflexion point of this part of the curve [39]. Four separate trials in pure water at 24 °C showed CMC = 8.1 ± 0.5 mM SDS, which is in good accordance with recent data based on other experimental techniques [40].

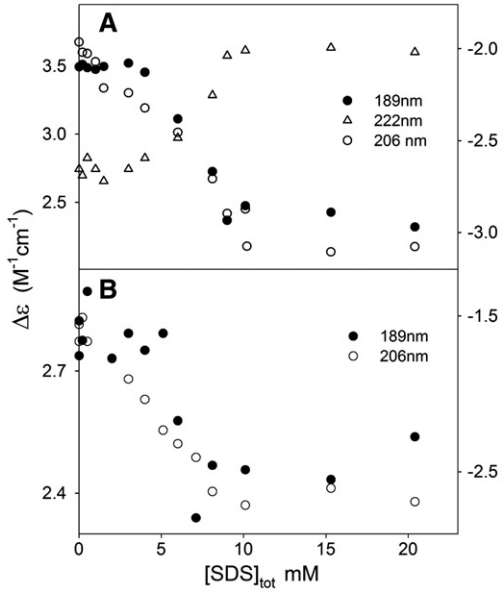

Fig. 4. $\Delta\varepsilon$ at selected wavelengths for Phy (A) and dgPhy (B) plotted as a function of the SDS concentration. Panel A shows the intensity of the 189 nm (filled circles, left ordinate), 206 nm (open circles, right ordinate) and 222 nm (triangles, right ordinate) bands. Panel B analogously depicts the 189 nm and 206 nm bands for dgPhy.

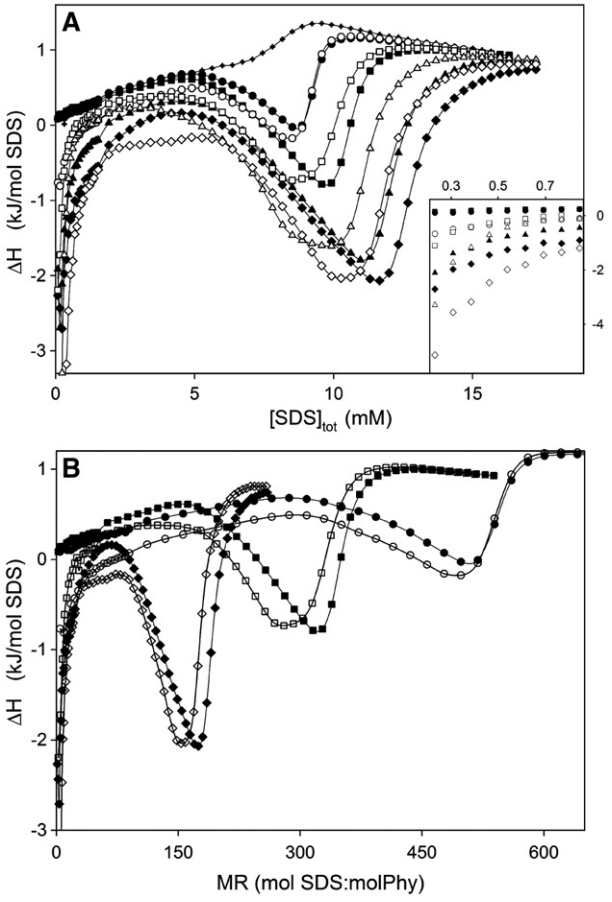

Fig. 5. The enthalpy change, ΔH (kJ/mol SDS) associated with the titration of 110 mM SDS into different enzyme solutions at 24 °C. In panel A, The abscissa is the total (bound + free) concentration of SDS in the calorimetric cell. The upper trace (crosses) is a reference experiment (SDS into pure water). Solutions of Phy are represented by filled symbols and dgPhy solutions by open symbols. Increasing protein concentration follows the sequence: circles, squares, triangles and diamonds. The actual concentrations were 17.1, 30.3, 50.1 and 66.6 μM for Phy, and 17.1, 30.4, 50.4 and 66.9 μM for dgPhy. The inset in panel A is an enlargement of the dilute region (0–0.8 mM SDS). In panel B, the data is shown as a function of the molar ratio MR = mol SDS/mol protein in the calorimetric cell (for clarity data for ~50 μM is not included in panel B).

Titrations of SDS into solutions with different concentrations of Phy (filled symbols) and dgPhy (open symbols) are also shown in Fig. 5. The enthalpy changes, ΔH, are plotted as a function of respectively the total SDS concentration (Panel A) and the protein:surfactant molar ratio, MR (Panel B). The latter representation is most commonly used in ITC work, but we have recently argued that isotherms with the total surfactant concentration on the abscissa provide some advantages for the analysis [23]. The results share a number of common traits. Thus, a conspicuous exothermic effect is observed at the lower SDS concentrations. The effect is limited to [SDS] < 1 mM and most likely reflects the specific binding of a few detergent molecules to the native protein[18,41–43]. To assess this further, we analyzed the data for 0–1 mM SDS by the conventional mass-action approach, using the Origin software package delivered with the ITC instrument [44]. The simple model accounted well for the measurements (below 1 mM) and

suggested that both Phy and dgPhy bound 3-4 SDS molecules with an affinity corresponding to a dissociation constant of 0.1-0.5 mM. The binding enthalpy was −5 to −10 kJ/mol SDS. Although binding in this affinity range can be measured only approximately at the current protein concentrations, we note that the derived parameters are typical for the specific binding of SDS to proteins [18,41]. As illustrated in the inset of Fig. 5A, the enthalpy changes for the specific binding of SDS to dgPhy is almost twice that for Phy which suggests more exothermic binding to the deglycosylated enzyme.

At intermediate SDS concentrations (~2–5 mM) we observe only weak thermal effects of SDS titration (Fig. 5). At higher concentrations (~5–10 mM), corresponding to the transition range determined by SRCD (Fig. 4), the heat signal decreases (becomes increasingly exothermic) in a near-linear fashion. We ascribe this change to the cooperative break-down of the native protein structure and the concurrent binding of detergent to the additionally exposed protein surface. This part of the isotherm is displaced to the left for dgPhy relative to Phy, thus illustrating (in accord with the SRCD data) that the denaturation occurs at slightly lower SDS concentration when glycans have been removed.

At still higher SDS concentrations (~9–12 mM SDS, depending on the enzyme concentration), the isotherms curve upwards in a sigmoid manner and eventually all merge with the control experiment (Fig. 5A). This behavior has been observed for other proteins [23,24,41] and ascribed to the completion of the surfactant adsorption process. This saturation is governed by the appearance of micelles in the aqueous bulk (see below) and thus essentially reflects an "apparent CMC" in the presence of the protein. Hence, in accordance with the suggestions of Paula et al. [39] we use the inflexion point (D in Fig. 6) to identify saturation. The molecular origins of other characteristic points in the isotherms were inferred from the SRCD data and identified in Fig. 6 as point A, B and C (reflecting respectively onset-midpoint- and completion of the denaturation process).

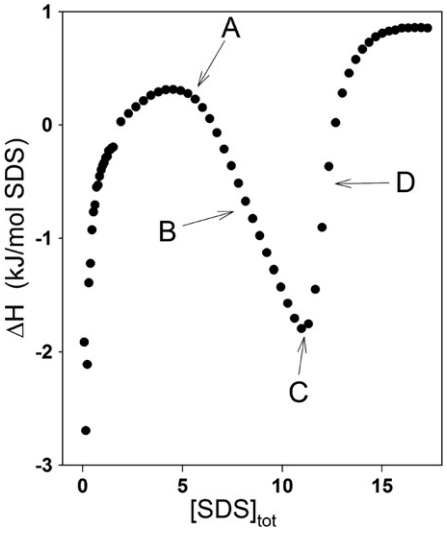

Fig. 6. Assignments of the molecular origins of certain characteristics in the enthalpograms discussed in the main text. A: onset of denaturation (a detectable fraction of the protein molecules is in the denatured form). B: denaturation midpoint (equal concentration of native and denatured protein). C: denaturation completed. D: saturation of SDS adsorption.

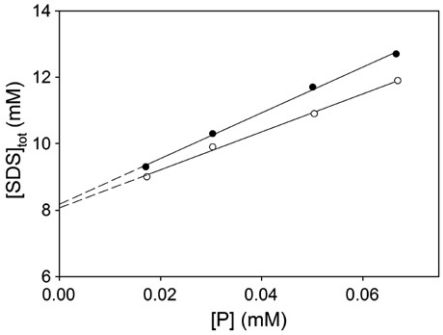

Fig. 7. Total (bound+free) concentration of SDS, $[SDS]_{tot}$, at the saturation point plotted as a function of the enzyme concentration [*P*]. Data for Phy and dgPhy are indicated by filled and open symbols respectively.

More quantitative data may be derived from the ITC results by applying a simple partitioning model which considers the equilibrium between dissolved (free) and protein bound detergent [23].

$$[SDS]_{tot} = [SDS]_{free} + [P] \cdot n \quad (1)$$

where $[SDS]_{free}$ is the concentration of SDS monomers in the aqueous bulk, n denotes the binding number (SDS/protein) and [*P*] is the concentration of protein (Phy or dgPhy). Eq. (1) is valid when $[SDS]_{free}$ does not exceed CMC. Values of $[SDS]_{tot}$ at the saturation point (*D* in Fig. 6) were read off enlarged copies of Fig. 5A and plotted as a function of [*P*] in Fig. 7. It appears that the partitioning scheme, which predicts a linear relationship (Eq. (1)), accounts well for the results. The parameters (*n*, $[SDS]_{free}$) were (69±2, 8.2±0.10 mM) and (57±2, 8.1±0.11 mM) respectively for Phy and dgPhy. Uncertainties are given as standard errors. It follows that Phy adsorbs 20% more SDS (in mol/mol) than dgPhy at saturation. Moreover, these parameters show that saturation is reached exactly when the bulk (free) concentration of SDS is equal to CMC (for [*P*]=0, the lines in Fig. 7 extrapolate to the CMC (8.2 mM) obtained in the reference experiments). This clearly indicates that saturation in both systems is governed by the activity of SDS in solution (which becomes practically constant

Table 1
Compilation of binding data derived from the ITC measurements

	Phy		dgPhy	
	n	$[SDS]_{free}$ (mM)	*n*	$[SDS]_{free}$ (mM)
D: Saturation of SDS adsorption	69±2	8.2±0.1	57±2	8.1±0.1
C: Denaturation complete	61±5	7.8±0.2	44±6	7.4±0.3
B: Denaturation midpoint	31±2	6.9±0.1	28±4	6.2±0.2
A: Initial denaturation	8±3	5.4±0.1	–	–

Based on the combined interpretation of the SRCD and ITC results, four different molecular assignments were made for the characteristic features in the enthalpograms in Fig. 5. These assignments (discussed in the main text) are listed in the first column. Values of $[SDS]_{tot}$ and [P] in Fig. 5A were used together with Eq. (1) to find the binding number, n (in mol SDS/mol protein), and the free concentration $[SDS]_{free}$ (in mM) as exemplified in Fig. 7. Uncertainties are standard error of the linear regressions.

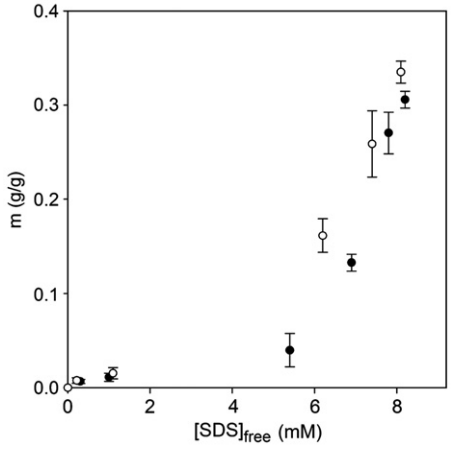

Fig. 8. Binding isotherm for Phy (filled symbols) and dgPhy (open symbols). The amount of adsorbed SDS (in g/g enzyme) is plotted as a function of the free (bulk) concentration of SDS, $[SDS]_{free}$.

above CMC) rather than other limitations such as full coverage of the protein interface (c.f. [20]).

A corresponding analysis of points A, B, and C (in Fig. 6) gave the parameters listed inTable 1. The binding number at point B (the transition midpoint) is an average of the number of SDS bound to respective native (N) and denatured (D) protein.

4. Discussion

4.1. Binding isotherms

Glycosylation appears to have a potential to modulate the physical properties of proteins without compromising enzymatic activity[25,38,45–48]. This makes controlled glycosylation an interesting avenue for the engineering and formulation of pharmaceutical peptides and industrial enzymes. Progress in this area, however, will rely on knowledge on the interactions of glycans and the surrounding solvent, and on how these interactions govern the properties of the glycosylated protein.

Interrelationship of SDS binding and deglycosylation of Phytase can be demonstrated by binding isotherms of the two glyco variants (Fig. 8). The binding isotherms are derived by combining the data in Table 1 and the results for the specific binding. The ordinate is the mass of bound SDS per mass unit of enzyme. If the affinity (per unit mass) of glycans and peptide were similar, the curves in Fig. 8 would be superimposed. It appears, however, that dgPhy adsorbs more SDS (in g/g) than Phy, *i.e.* that SDS interacts more favorably with the peptide. At saturation dgPhy binds 0.34 g/g whereas Phy binds 0.31 g/g. Under the assumption that the amount of SDS bound to the peptide moiety of Phy is equal to the amount adsorbed by dgPhy it appears that SDS adsorption by glycans corresponds to 0.20 g/g at saturation.

As mentioned in the Introduction, polypeptides typically adsorb 1–1.4 g SDS/g at saturation. Thus, dgPhy exhibits an unusual low affinity for this surfactant. Moreover, if the degree of SDS adsorption by glycans found here (0.2 g/g) is general, the difference between peptides and glycans might be more pronounced for other glycoproteins. This suggestion is in accord with the study by Pitt-Rivers and Impiombato [21], who found that saturation occurred at around 1.0 g/g peptide for a range of different proteins whether glycosylated or not. While the contribution from the glycans cannot be unambiguously discerned from the variability among the different proteins studied by these workers, this result indeed suggests very low SDS binding to glycans. A similar conclusion was reached by Russ and Polakova on the basis of mobility of glycoproteins in SDS–PAGE [33]. The current results are also in accord with recent data on the binding of SDS to soluble oligosaccharides. Thus, maltodextrin with 10 glucose units (i.e. a sizes comparable to the glycans on Phy) bound 1.5 mM SDS in 0.5% (w/v) maltodextrin [29,30], which translates into about 0.1 g SDS/g carbohydrate. Control ITC experiments (not shown) on solutions of pure (monomeric) mannose did not reveal any interaction with SDS, in line with more systematic work on this type of systems [30].

4.2. Protein structure

The combined analysis of CD and ITC data suggests that both forms of phytase specifically bind 3-4 SDS molecules

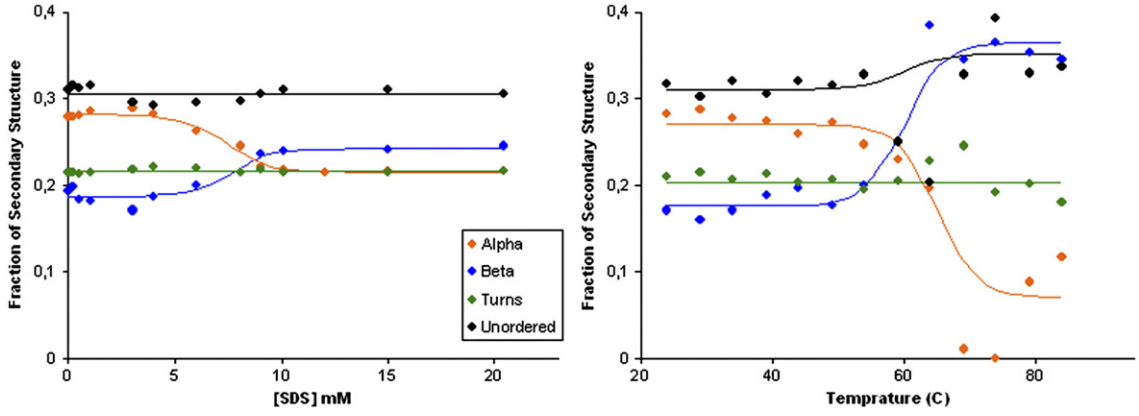

Fig. 9. Estimated amounts of α-helix, β-sheet, turns and unordered secondary structures in Phy as function of respectively the SDS concentration (left panel) and temperature (right panel). These determinations of secondary structure contents were based on a protein reference set from the soft ware package CDPro [35].

without detectable changes in the protein structure. This process most likely involves the interaction of the anionic surfactant and cationic groups on the protein, particularly if this binding is located around hydrophobic areas or clefts where the surfactant acyl chain can be lodged [18].

At intermediate SDS concentrations (1–5 mM) Phy interacts only weakly with SDS and the surfactants do not induce major changes in the protein structure. The binding number towards the high end of this interval corresponds to ~0.04 g/g which is about an order of magnitude less than typically observed values [15,19,41]. Above 5 mM SDS, the regime of weak interaction changes into another mode of interaction in which the heat signal becomes increasingly exothermic (Fig. 5A), the binding number increases steeply (Fig. 8) and the native structure changes (Figs. 1 & 2). This is interpreted as a typical denaturant induced transition, where the population of protein molecules is gradually brought from the *N* to the *D* state as the concentration of denaturant increases. Both methods suggest that the *N*–*D* transition is completed approximately as the free concentration of SDS reaches CMC, and this implies that formation of surfactant aggregates in the bulk promotes denaturation. This type of micelle-driven unfolding has been reported for a number of other proteins[43,49–51].

The change in secondary structure accompanying the SDS-denaturation was investigated by analysis of the CD data for secondary structure contents using a protein reference set from the soft ware package CDPro [35]. This analysis estimates the amount of α-helix, β-sheet, turns and unordered secondary structures, and suggested about 28% of α-helix and 19% of β-sheet in the native Phy (*i.e.* for [SDS]=0). These values are reasonably consistent with the crystal structure which has 33% helix and 11% sheet (Dr. L.DeMaria, Novozymes, personal communication). Denaturation by SDS only changes the secondary structure moderately (Fig. 9). Hence, the completely SDS-denatured protein (in>10 mM SDS) has a composition of ~22% helix and 24% sheet. No increase in the fraction of unordered chain was identified in the structural analysis, and it appears that the SDS-denatured state of Phy is far from disordered. Hence, it is suggested that increased SDS-binding upon denaturation primarily is coupled to changes in the tertiary protein structure. Residual structure (at room temperature) in SDS denatured states of proteins is not uncommon. Serum albumin [19] and a number of other proteins show different degrees of secondary structure, when exposed to high concentrations of ionic surfactants[11,16,52–56].

To compare the structural effects of SDS-denaturation with temperature induced denaturation SRCD data from temperature scans of Phy (data not shown) were also analyzed for secondary structure content.Fig. 9 shows the estimated amount of α-helix, β-sheet, turns and unordered secondary structures in Phy as function of respectively the SDS concentration (left panel) and temperature (right panel). It is clear that thermal denaturation induces more pronounced changes in the secondary structure. Large structural perturbations occur between 59–69 °C which is consistent with the measured denaturation temperature (T_d=62 °C) determined by DSC prior to the CD investigations and in previous work [25,38].

5. Conclusions

The thermodynamic and spectroscopic data collectively suggests that the glycan "mantle" of phytase only confers a limited positive effect on the resistance towards SDS induced destabilization. We suggest that this relies on rather weak carbohydrate–surfactant interactions, and on peptide-surfactant interactions, which are mostly unaffected by the presence of glycans. At saturation, the adsorbed amount of SDS to the carbohydrate moieties was about 0.2 g SDS/g glycan or about 5 times less than the value found for average polypeptides. Interestingly, binding of SDS to the peptide part of phytase is also weak throughout the investigated concentration range. The binding number for dgPhy at saturation is 3–4 times less than typically reported values. Together with a few other examples [12,22–24] this observation challenges the general view of an universal (mass/mass) saturation binding level for SDS. The low binding numbers are suggested to be a consequence of a strong potential of this protein to preserve secondary structure in SDS which limits the area accessible to the surfactant.

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