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Articles

Influence of the Carbohydrate Moiety on the Stability of Glycoproteins†

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ABSTRACT: To study the role of oligosaccharides on the properties of glycoproteins, five glycoproteins (yeast external invertase, bovine serum fetuin, glucoamylase from *Aspergillus niger*, and chicken egg white ovotransferrin and avidin) of previously established glycan patterns were purified to homogeneity and deglycosylated with endo- and exo-glycosidases in native conditions. Thermal stability and conformational changes were measured by high-resolution differential scanning microcalorimetry and circular dicroism spectroscopy before and after they were deglycosylated. It was found that deglycosylation decreases protein thermal stability, as judged by the decrease in denaturation temperature and denaturation enthalpy, while it does not affect substantially the conformation as indicated by the CD spectra in the far UV range. The destabilization effect of deglycosylation seems to depend on the carbohydrate content, i.e., the maximum effect was observed for the most heavily glycosylated protein, irrespective of the types (N-linked or O-linked) or patterns (mono- or multi-branched) of the covalently attached carbohydrate chains. In addition, studies of the reversibility to heat denaturation revealed that deglycosylated proteins have a poorer thermal reversibility in calorimetric scans than their native counterparts and tend to aggregate during thermal inactivation at acidic pH. These results suggest that carbohydrate moieties, in addition to the apparent stabilizing effect, may prevent the unfolded or partially folded protein molecules from aggregation. Our results support the hypothesis that the general function of protein glycosylation is to aid in folding of the nascent polypeptide chain and in stabilization of the conformation of the mature glycoprotein [Lis, H., & Sharon, N. (1993) *Eur. J. Biochem. 218*, 1−27].

Glycosylation is one of the major naturally occurring modifications of the covalent structure of proteins. Most secretory proteins become glycosylated as soon as the growing polypeptide chains enter the endoplasmic reticulum, before the final native-like folded state is reached (Rothman et al., 1975; Kiely et al., 1976). There are two different types of protein glycosylation: O-glycosylation at hydroxyl groups of serine and threonine residues and N-glycosylation at asparagine residues in the consensus sequence of Asn-X-Ser/Thr. Although much is known about the structure and biosynthesis of oligosaccharides in glycoproteins, the central question of how glycosylation contributes to the glycoprotein structure and function is not entirely clear. Many specific effects have been observed and ascribed to particular structural properties of the carbohydrate moieties, such as regulation of intracellular traffic and localization of glycoproteins (Kornfelt & Mellman, 1989; Moore et al., 1992), modulation of enzymatic and hormone activity (Marzuk et al., 1989; Yamaguchi et al., 1991), modification of immunological properties (Feizi & Childs, 1987), participation

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in cell−cell interactions (Kojima et al., 1992), and many others. However, the close similarity of the composition and peripheral sequences of N- and O-linked sugar chains, larger sugar chains especially, may suggest some general nonspecific effect of glycosylation on protein property. Studies on the native glycosylated, carbohydrate-depleted, and recombinant non-glycosylated proteins have revealed such effects as stabilization of protein conformation (Walsh et al., 1990), protection from proteolysis (Bernard et al., 1983; Rudd et al., 1994), and enhancement in nascent polypeptide solubility (Paul et al., 1986; Kern et al., 1993), but none of them was constantly observed for all glycoproteins. Moreover in some cases controversial results were obtained for the same glycoprotein (Chu et al., 1978; Schülke & Schmid, 1988a).

To elucidate if glycosylation has a general stabilization effect on protein thermal stability and conformation, and if it aids or participates in the protein folding process, a dozen glycoproteins with different content, type, and pattern of glycosylation were tested and five of them were chosen on the basis of availability, ease of deglycosylation, and calorimetric behavior.

YEI[1] is a glycoprotein associated with the cell wall and possesses on average nine N-linked oligosaccharide chains, all of which are high-mannose type (Reddy et al., 1988). BSF is a glycoprotein abundant in calf fetus serum. Each molecule carries six carbohydrate chains, of which three are N-linked and three are O-linked (Spiro, 1962; Spiro & Bhoyroo, 1974; Green et al., 1988). Studies on fetuin oligosaccharide structure also revealed the presence of *N*-acetylneuraminic acids attached to each end of the carbohydrate branches (Cumming et al., 1989). GA is an O-glycosylated protein to which more than thirty short oligomannose chains are attached at serine or threonine residues on the linker region separating the two functional domains of the enzyme molecule (Gunnarsson et al., 1984; Williamson et al., 1992a). AVD and OTF are only N-glycosylated and have lower carbohydrate content (Bruch et al., 1982; Dorland et al., 1979). The oligosaccharide characteristics of these proteins are summarized in Table 1.

Carbohydrate moieties of glycoproteins can be released by chemical or enzymatic methods. Enzymatic methods have the advantage of a high degree of specificity, but the potential target of glycosidases may be masked in the native state of glycoproteins and denaturation may be necessary before enzymatic release (Mellors & Sutherland, 1994). In the present study, various endo- and exo- glycosidases were used and different non-denaturing conditions were selected for individual protein deglycosylation. The heat stability of all the species was analyzed by differential scanning microcalorimetry (DSC) and by circular dichroism (CD).

MATERIALS AND METHODS

All glycoproteins were purchased from SIGMA. Sialidase and α-mannosidase were from Boehringer Mannheim; *O*-glycosidase, Endo H, and PNGase F were from Oxford Glycosystem (U.K.).

Table 1: Major Glycosylation Characteristics of the Five Glycoproteins

glycan properties	glycoproteins				
	YEI	BSF	GA	AVD	OTF
N-glycans	+[a]	+		+	+
O-glycans		+	+		
binary complex	+	+		+	+
triantennary complex		+			+
tetraantennary	+				
NeuNAc		+			
oligomannose	+		+		
carbohydrate content	50%	22%	19%	10%	2.2%
number of sugar chains	9	6	>30	1	1
native polymer form	dimer	monomer	monomer	tetramer	monomer
molecular weight[b]	240 000	48 000	82 000	68 000	80 000

[a] "+" indicates the presence of a particular glycan property. [b] Glycans included.

YEI (grade VII), GA, and BSF were purified to homogeneity according to Trimble and Maley (1977), Svensson et al. (1982), and Green et al. (1988), respectively; OTF and AVD were used without further purification.

Proteins were deglycosylated at the concentration of 1−2 mg/mL. Deglycosylation conditions were probed for each of the proteins as follows. First, the protein sample was incubated with one glycosidase at 37 °C. By changing the type and amount of glycosidase, the most "efficient" enzyme was determined. Second, the temperature and time of incubation were changed to find the conditions in which the glycoprotein was deglycosylated while the protein structure remained native. In this step each protein sample was divided in two fractions, one of which was deglycosylated while the other was treated exactly in the same way, except that no glycosydase was added, and thereby used as a control. This non-deglycosylated control fraction was then analyzed by CD and DSC, and its parameters were compared with those of the native untreated protein. When no difference was observed, the proper incubation temperature and time were established and all subsequent deglycosylations were performed under this condition. The control proteins thus incubated were considered identical to the native proteins. Comparison of parameters was made between the "coupled" control and deglycosylated fractions. The extent of deglycosylation was assessed electrophoretically by running the enzyme-treated samples on SDS−PAGE (Laemmli, 1970) followed by staining with Coomassie Blue (for BSF, YEI, and GA) or followed by transferring to nitrocellulose sheets and probing with lectin Con A (Faye et al., 1985).

Purified BSF, in 50 mM phosphate buffer, pH 8.0, was incubated with PNGase F (200 milliunits/mg) at 25 °C for 24 h. The protein was then separated from the released *N*-oligosaccharides and incubated with sialidase (50 milliunits/mg) at 30 °C, in 50 mM acetate, pH 5.0. Release of sialic acids was monitored by HPAEC-PAD equipped with a CarboPac PA1 column. De-O-glycosylation was performed by incubation of the asialic and N-carbohydrate-free sample with *O*-glycosidase (10 milliunits/mg) in 50 mM phosphate, pH 6.0. Oligomannose of YEI was released by

[1] Abbreviations: YEI, yeast external invertase; BSF, bovine serum fetuin; GA, glucoamylase from *Aspergillus niger*; OTF, chicken egg white ovotransferrin; AVD, chicken egg white avidin; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; Endo H, endo-*β*-*N*-acetylglucosaminidase H from *Streptomyces plicatus*; PNGase F, peptide *N*-glycosidase F from *Flavobacterium meningosepticum*; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DSC, differential scanning calorimetry.

incubation with Endo H (10 milliunits/mg) for 20 h at 30 °C, pH 5.5. GA was de-O-glycosylated with α-mannosidase in 20 mM sodium acetate for 18 h at 37 °C, pH 4.5. OTF and AVD were incubated with PNGase F in the presence of 1% octyl β-D-thioglucopyranoside for 24 h at 37 °C.

CD spectra were recorded in the far-UV region (190−250 nm) on a Jasco J-710 spectropolarimeter. A jacketed 1 mm cuvette was connected to a water bath for temperature control. Effects of temperature on CD spectra were examined as follows: samples were heated to a given temperature and maintained at that temperature for 1 min. Then CD spectra were recorded at 3 min intervals with a scan rate of 20 nm/min and a bandwidth of 1 nm. Measurements were performed at temperatures which correspond to pre-transition, transition, and post-transition. The protein concentrations were 400, 150, and 50 μg/mL, respectively for YEI, BSF, and GA.

DSC experiments were carried out with a MicroCal MC-2 differential scanning microcalorimeter (MicroCal Inc., Northampton, MA) interfaced to a personal computer. Protein concentrations were 2.0, 2.5, 1.8, 1.5, and 1.0 mg/mL, respectively, for YEI, BSF, GA, AVD, and OTF. No difference in T_m or in ΔH was noticed for YEI in the concentration range 1.0−5.0 mg/mL. The buffers used, if not otherwise stated, were 50 mM sodium phosphate for YEI, BSF, and GA; 0.5 M HEPES for OTF, and 50 mM phosphate in the presence of 1 M GuHCl for AVD. Before measurements, protein solutions were extensively dialyzed against the appropriate buffer. The sample cell was loaded with the protein solution, whereas the reference cell was filled with the dialysis buffer. A scan rate of 60 °C/h was used in all experiments.

Data acquisition and analysis were performed with the software package (Origin) also supplied by MicroCal. All the five glycoproteins showed only one peak, and therefore a unique maximum in the excess heat capacity function, in the temperature range 45−75 °C. By extrapolating the pre- and post-transitional base lines into the transition region, a progress base line was drawn. When precipitation occurred (as in the case of AVD, and also YEI and GA at low pH), a linear base line connecting the initial and final temperatures of the overall transition was used. Calculation of T_m (temperature of maximum heat capacity), ΔH (calorimetric enthalpy of denaturation), ΔH_{vH} (van't Hoff enthalpy of denaturation), and ΔC_p (difference in heat capacity between the native and the denatured states) were performed after normalizing the data for protein concentration and subtracting the appropriate base lines.

ΔH_{vH} was calculated using the formula

$$\Delta H_{vH} = ART_{1/2}^2 c_{ex,1/2}/\Delta H_{cal}$$

(Sturtevant, 1987) where $c_{ex,1/2}$ (C_p at $T_{1/2}$ in our notation) is the excess heat capacity (i.e., the distance from the base line to the curve) at $T_{1/2}$, $T_{1/2}$ is the temperature (K) at which the process is half completed, ΔH_{cal} corresponds to ΔH in our notation, and $A = 4$ for a nondissociating system undergoing a two-state transition.

The cooperativity of the heat transition was evaluated from the ratio $\Delta H_{vH}/\Delta H$ (Bertazzon & Tsong, 1990). The reversibility of the thermal transitions was checked by a second and, sometimes, a third heating cycle of the same sample immediately after ending and cooling the previous scan. Protein concentrations were measured spectrophotometrically using the following absorption coefficients: BSF, $E^{1\%}_{278} = 5.3$; YEI, $E^{1\%}_{280} = 22.5$; AVD, $E^{1\%}_{282} = 15.4$; GA, $E_{280} = 137\ mM^{-1}\ cm^{-1}$; OTF, $E_{280} = 92\ mM^{-1}\ cm^{-1}$. The molecular weights of all proteins (protein moiety plus glycidic moiety) are reported in Table 1. Only when Δh (cal/g) and ΔC_p (cal/g °C) values were determined for comparison with normal values for small globular proteins was the molecular weight used that of the protein moiety (i.e., the total molecular weight subtracted by the weight of the glycidic moiety).

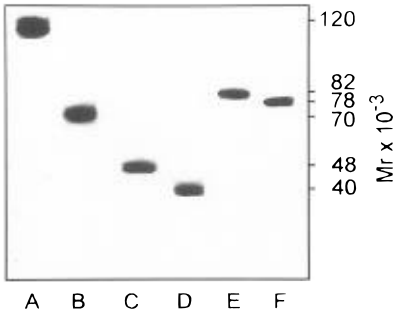

FIGURE 1: SDS−PAGE of YEI, BSF, and GA before and after deglycosylation. YEI was treated with Endo H; BSF was treated with PNGase F, sialidase, and O-glycosydase; and GA was treated with α-mannosidase. Deglycosylation conditions were described in Materials and Methods. The proteins were electrophoresed on a 10% polyacrylamide gel and subsequently stained with Coomassie Blue. Lanes A, C, and E, native glycosylated YEI, BSF, and GA; lanes B, D, and F, deglycosylated YEI, BSF, and GA. The molecular mass (M_r, kDa) markers are located to the right.

RESULTS

Deglycosylation of Glycoproteins. PNGase F is a potent endo-glycosidase, cleaving most common mammalian N-linked high-mannose-, hybrid-, and complex-type glycans at the N-glycosidic bond (Mellors & Sutherland, 1994) and was used for releasing *N*-oligosaccharides throughout the present study.

BSF is easily deglycosylated (*N*- and *O*-glycans and sialic acids) with glycosidases at 37 °C. However, incubation at this temperature resulted in structural perturbation and partial unfolding, as indicated by the distortion of CD spectra and DSC curves (data not shown). To overcome this problem we have tried, in a series of protocols, to perform deglycosylation at lower temperatures (25−30 °C) for 22−24 h. As shown in Figure 1, nearly all N- and O-linked glycans can be released.

YEI migrated as a diffused band around 120 kDa on SDS−polyacrylamide gel. Incubation with Endo H for 20 h decreased its molecular weight to a 70 kDa species, indicating that 80% of its oligomannose had been released. Higher mannosidase concentration or longer incubation time failed to release the remaining glycans, which represent the nine residual N-acetylglucosamines and perhaps the two short oligomannose chains which have been shown to be inaccessible to glycosidases under native conditions (Trimble et al., 1983).

GA carries more than 30 *O*-oligomannose chains with an average size of 2 mannoses. Incubation with α-mannosidase released 30% of its glycans (Figure 1).

OTF and AVD are thermally stable glycoproteins. Although they are less glycosylated, their resistance to gly-

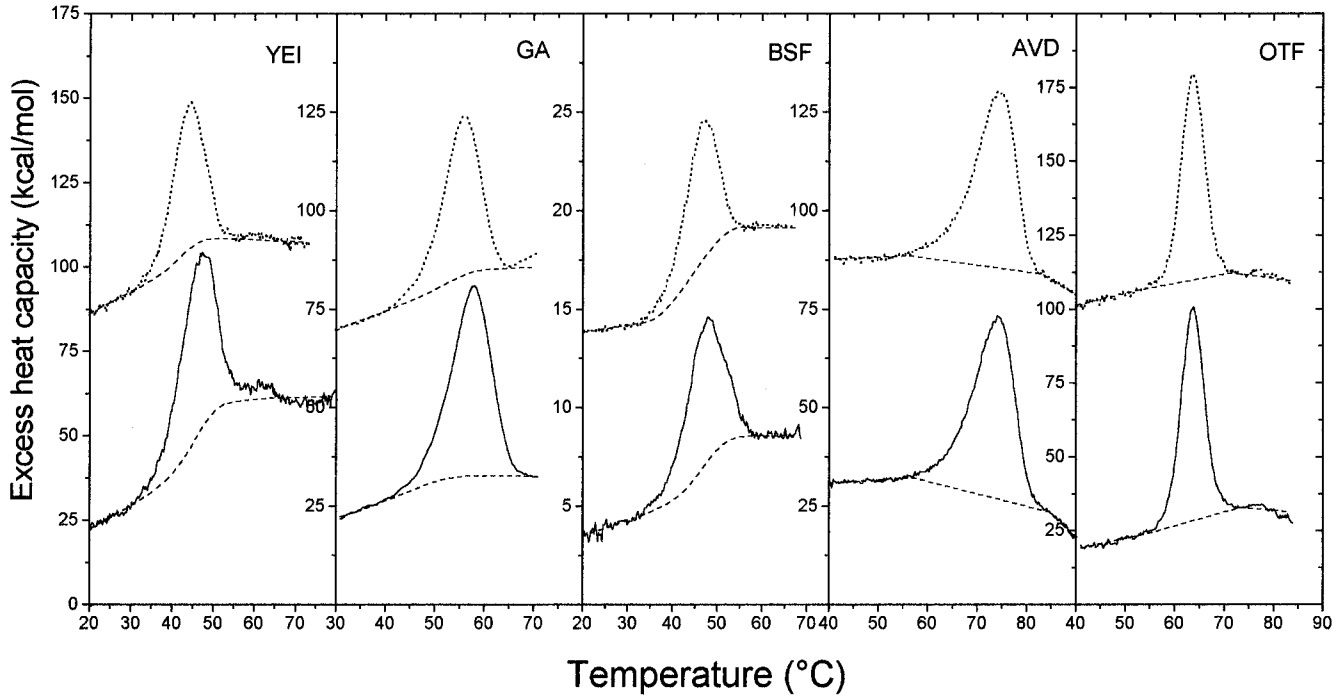

FIGURE 2: Differential scanning calorimetry of YEI, BSF, GA, OTF, and AVD before and after carbohydrate removal. Experiments were performed at pH 8 in 50 mM sodium phosphate for YEI, BSF, and GA, in 0.5 M HEPES, pH 7.5, for OTF, and in 50 mM sodium phosphate, pH 8.0, in the presence of 1 M GuHCl for AVD. The solid lines give the experimental heat capacity traces for the native glycosylated proteins, the dotted lines for the deglycosylated forms, and the dashed lines represent the relative base lines. Protein concentrations are 8.5 (dimer), 90.0, 17.8, 23.0, and 22.6 μM (tetramer), respectively, for YEI, BSF, GA, OTF, and AVD. Traces have been arbitrarily displaced on the *Y* axis for clarity.

cosidase action is apparently higher than that of YEI and BSF. This is probably due to the inaccessibility of the glycosidic bonds which might be masked and buried under the protein surface. Hence we tried to remove enzymatically the carbohydrate moieties in the presence of the non-ionic detergent octyl β-D-thioglucopyranoside (Saito, et al., 1984). As indicated by the negative staining with Con A, OTF and AVD could be completely deglycosylated if hydrolysis conditions were carefully controlled (data not shown).

Effect of Deglycosylation on the Unfolding Temperature of Glycoproteins. Figure 2 shows the temperature dependence of the excess heat capacity of YEI, BSF, GA, OTF, and AVD before and after deglycosylation at pH 8.0. The extensive heat absorption peak was associated with the heat denaturation which resulted, as in the case of most small globular proteins (Privalov, 1979), in a significant increase of excess heat capacity. For each protein, the shape of the two thermal profiles was essentially the same. The only difference was that the carbohydrate-depleted protein had a lower T_m than its native counterpart. At pH 8.0 native YEI unfolded at 47.9 °C. After carbohydrate removal its T_m was shifted to 44.4 °C. The same behavior was also observed for BSF and GA at pH 8.0: T_m was decreased by 1.9 °C in both cases. AVD is a highly stable glycoprotein. It is stable even for weeks in the presence of 3 M guanidine hydrochloride (Green, 1975). This property was employed to prevent aggregation upon heat denaturation of this protein. At pH 8.0 in the presence of 1 M guanidine hydrochloride, deglycosylated AVD showed a T_m of 72.2 °C. This value is only 0.2 °C lower than that of the native AVD and is within the limits of experimental uncertainty.

OTF is a Fe^{3+} binding protein. Its stability depends strongly on the bound ferric ions, with T_m 21 °C higher for the fully saturated form than for the apo-form (Lin et al., 1994). In order to make a reasonable comparison between T_m values, special attention has been paid in each step of sample preparation. In addition, a high concentration (0.5 M) of HEPES has been used to avoid precipitation which occurs at low ionic strength.

Glycosylated GA and OTF were also studied previously by Williamson et al. (1992b) and Lin et al. (1994) under similar conditions: our results were in good agreement with those reported by these authors.

The thermodynamic parameters and standard deviations obtained from replicated experiments for the five glycoproteins are listed in Table 2.

Effect of Deglycosylation on the Transitional Enthalpy of Glycoproteins. Table 2 also shows the molar transitional enthalpy of the five glycoproteins in the native and the deglycosylated forms. Specific heats of denaturation were obtained dividing the ΔH of each protein by its molecular weight. Inspection of the data at pH 8.0 revealed that all the specific enthalpy values, and particularly those of the more heavily glycosylated proteins, were considerably lower than those reported for other globular proteins (Privalov, 1979). If, however, the heat effect was referred to the protein component of the molecule, i.e., subtracting the contribution of the glycan moiety from the overall molecular weight, all the values (4.64 $\pm$ 0.08, 6.78 $\pm$ 0.22, 1.90 $\pm$ 0.05, 5.40, and 7.06 cal/g, respectively, for YEI, GA, BSF, OTF, and AVD) fell in the normal range with a single exception, BSF. This protein had a very low specific heat of denaturation, which might represent an uncomplete unfolding of the molecule.

The cooperative ratio ($\Delta H_{vH}/\Delta H$) of the five proteins was also calculated. The value of this ratio was close to 1 for BSF, while it was much smaller than unity, suggesting the presence of multidomain structures, for all the other proteins.

Table 2: Thermodynamic Parameters of YEI, BSF, GA, AVD, and OTF, before and after Carbohydrate Removal, as a Function of pH

		pH						
		4.5	5.0	5.5	6.0	7.0	8.0	8.8
YEI								
deglycosylated	T_m[a]			62.4 ± 0.05	60.9	50.0 ± 0.5	44.4 ± 0.3	43.8 ± 0.1
	ΔH[b]			(336)	(639)	550 ± 8	420 ± 6	400 ± 14
native	T_m			64.7 ± 0	62.9	51.7 ± 0.3	47.9 ± 0.2	46.2 ± 0.1
	ΔH			958 ± 6	916	686 ± 11	556 ± 11	461 ± 4
	N[c]			2	1	3	3	2
BSF								
deglycosylated	T_m				58.2 ± 0.1	53.0 ± 0.4	46.4 ± 0.2	45.5
	ΔH				84.5 ± 0.3	75.0 ± 2.6	68.9 ± 1.3	62.8
native	T_m			60.3	59.6 ± 0.2	54.2 ± 0.4	48.4 ± 0.3	46.8
	ΔH			89.6	87.7 ± 0.2	77.6 ± 2.6	71.0 ± 1.8	67.8
	N			1	2	3	8	1
GA								
deglycosylated	T_m	67.5	68.2	67.7 ± 0.1		60.0 ± 0.1	55.9 ± 0.4	
	ΔH	(180)	(452)	478 ± 1		439 ± 6	400 ± 7	
native	T_m	67.8	69.7	69.1 ± 0.1	64.0	62.4 ± 0	57.8 ± 0.1	
	ΔH	(190)	(190)	528 ± 6	482	490 ± 6	450 ± 6	
	N	1	1	2	1	2	4	
AVD[d]								
deglycosylated	T_m						74.2 ± 0.1	
	ΔH						422 ± 3	
native	T_m						74.4 ± 0.1	
	ΔH						432 ± 4	
	N						3	
OTF[e]								
deglycosylated	T_m						63.5 ± 0.1	
	ΔH						418 ± 3	
native	T_m						63.5 ± 0.1	
	ΔH						422 ± 6	
	N						4	

[a] Denaturation temperature in °C ± sd. [b] Denaturation enthalpy in kcal/mol ± sd. Data in parentheses are approximated values because of the lack of measurable post-transition base line due to the precipitation upon thermal unfolding. The buffers used were 50 mM sodium acetate, pH 4.5−5.5; 50 mM sodium phosphate, pH 6.0−8.0, and 50 mM glycine-sodium hydroxide, pH 8.8. [c] Number of experiments. [d] In 50 mM sodium phosphate in the presence of 1 M GuHCl. [e] In 500 mM HEPES, pH 7.5.

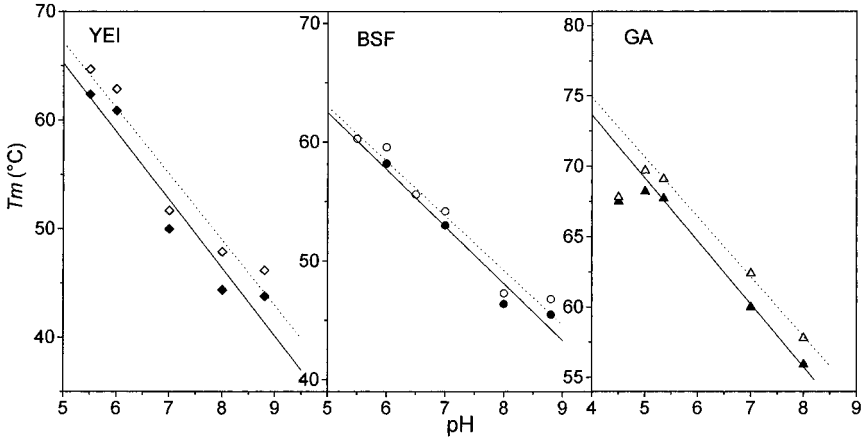

FIGURE 3: pH dependence of the transition temperature (T_m) of native glycosylated YEI (◇), BSF (○), and GA (△), and deglycosylated YEI (◆), BSF (●), and GA (▲). The straight lines (broken for native proteins and solid for deglycosylated ones) are linear regressions of the data. Protein concentrations and buffers used were indicated in Table 2.

Deglycosylation did not appreciably influence the $\Delta H_{vH}/\Delta H$ ratio: the values for the native and deglycosylated forms were 0.13 and 0.19 for YEI, 0.20 and 0.23 for GA, 1.00 and 0.98 for BSF, 0.35 and 0.35 for OTF, and 0.18 and 0.17 for AVD.

Thermal Unfolding of Glycosylated and Deglycosylated Proteins As a Function of pH. As deglycosylation did not induce significant changes in the thermal stability of the less glycosylated AVD and OTF, we have concentrated our study on the more heavily glycosylated YEI, BSF, and GA. The effect of deglycosylation on the thermal unfolding of these three glycoproteins was studied in the pH range 4.5−8.8. As seen in Table 2, an increase in pH led to a decrease in both T_m and ΔH. At pH 6.0, native BSF unfolded at 59.6 °C with a ΔH of 87.7 kcal/mol. When pH was increased to 8.8, T_m was shifted to 46.8 °C and ΔH lowered to 67.8 kcal/mol. In a similar way, the deglycosylated BSF gave values of 58.9 °C, 84.5 kcal/mol, and 45.5 °C, 62.8 kcal/mol respectively at pH 6.0 and 8.8. When T_m was plotted against pH (Figure 3), an apparently linear function was obtained for each of the two species. Figure 3 also shows the DSC results for YEI and GA: the pH dependence of T_m again

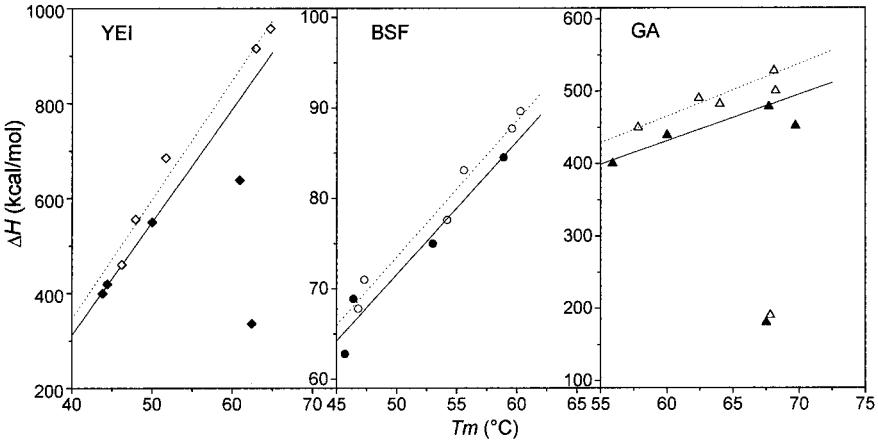

FIGURE 4: Temperature (T_m) dependence of the calorimetric enthalpy (ΔH) of native YEI (◇), BSF (○), and GA (△) and deglycosylated YEI (◆), BSF (●) and GA (▲). The straight lines (broken for native proteins and solid for deglycosylated proteins) are linear regressions of the data (data of experiments in which precipitation occurred have not been used). The slope of the straight lines (broken for native proteins, and solid for deglycosylated proteins) gives the heat capacity change, ΔC_p. Experimental conditions were indicated in Table 2.

approximates a linear function. The scatter of the points in the graphs reflects the variability of experimental conditions, since there are differences in ionic strength in buffers of different pH. If, however, ΔT_m is calculated from the data in Table 2 and a parallel pH dependence is assumed for both forms, the average decrease in T_m for the deglycosylated proteins is 2.8 ± 0.5 °C for YEI, 1.8 ± 0.4 °C for GA, and 1.3 ± 0.1°C for BSF, over the entire pH range examined.

The enthalpy values of denaturation of YEI, BSF and GA, in both glycosylated and deglycosylated forms, are presented as a function of T_m in Figure 4. The slope of the plots represents the difference in the heat capacity of the protein in the native folded and denatured states (ΔC_p) and is considered a more accurate and reliable value than that obtained from the thermal profiles (Privalov, 1979; Becktel & Schellman, 1987). In fact, ΔC_p data calculated from the thermograms are of the same order of magnitude but affected by greater error. ΔC_p is an important thermodynamic parameter as it characterizes the thermal transition and provides a measure of the hydrophobic stabilization of the proteins. In the temperature range where ΔH is a linear function of T_m, ΔC_p is calculated to be 0.21, 0.10, and 0.04 cal/g (of protein, excluding glycans) °C, respectively, for YEI, GA, and BSF. No significant change of ΔC_p was noticed after carbohydrate depletion.

A remarkable feature of the data in Figure 4 is that while the ΔH of native YEI increases linearly with T_m, that for the deglycosylated form decreases sharply at pH ≤ 6.0. This is due to the occurrence of aggregation and precipitation upon thermal denaturation. Similar results were obtained with GA. Above pH 5.0, both de-O-glycosylated and native GA unfolded without aggregation. At pH 5.0, precipitation occurred for deglycosylated GA but not for the native one. When pH was further decreased to 4.5, approaching the isoelectric point of the protein (pI = 4.0, Svensson et al., 1982), both forms precipitated (Figure 4).

Effect of Deglycosylation on the CD Spectra. To see if carbohydrate depletion influences the secondary structure of glycoproteins, the same samples used for DSC study were diluted and their CD spectra were measured in the amide region at 25 °C. YEI and BSF are characterized by minima at 218 and 208 nm, respectively. The essential absence of the 222 nm transition in the spectra suggests that the two proteins contain a low amount of α-helical structure (Chang

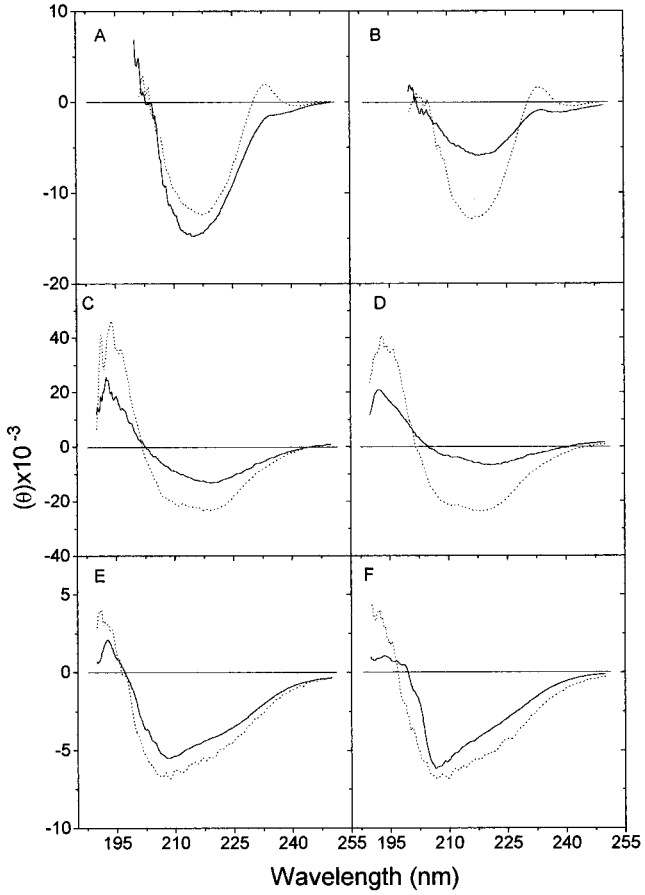

FIGURE 5: CD spectra of YEI (A, B), GA (C, D), and BSF (E, F), with left panels for native and right panels for deglycosylated proteins. Experiments were performed in 50 mM sodium acetate, pH 5.5, for YEI, in 50 mM sodium acetate, pH 5.0, for GA, and in 50 mM sodium phosphate, pH 8.0, for BSF. Protein concentrations were 150, 400, and 50 μg/mL, respectively. Experimental conditions were described in the text. Dotted lines represent the spectra obtained at pre-transitional temperatures, and solid lines represent the spectra obtained at the transition temperature (post-transitional temperature for BSF) after 15 min incubation. θ values in deg cm^2 dmol^{-1}.

Table 3: Comparison of the Thermal Denaturation Reversibility of YEI, BSF, and GA before and after Deglycosylation[a]

		native					deglycosylated				
		I scan		II scan			I scan		II scan		
	pH	T_{mI}	ΔH_I	T_{mII}	ΔH_{II}	$\Delta H_{II}/\Delta H_I$	T_{mI}	ΔH_I	T_{mII}	ΔH_{II}	$\Delta H_{II}/\Delta H_I$
YEI	6.0	62.9	916	61.2	114	12.4%	61.2	640	precipitated		0%
	8.8	46.2	461	46.6	172	37.1%	43.8	400	43.2	120	30.0%
BSF	6.0	59.6	87.7	59.5	83.8	95.3%	58.2	84.5	58.5	15.9	18.8%
	8.0	47.3	71.0	48.2	64.4	90.0%	46.4	68.9	45.0	34.3	49.8%
GA	7.0	62.4	490	58.5	65.7	13.0%	60.0	439	57.4	54.9	12.5%
	8.0	57.8	470	57.2	153	32.5%	55.9	400	55.7	57.8	14.5%

[a] The quantities T_m and ΔH are defined as in Table 2. The buffers used were 50 mM sodium phosphate pH 6.0−8.0 and glycine-sodium hydroxide pH 8.8. The subscripts I and II to T_m and ΔH indicate the first and the second cycles of scanning, respectively.

et al., 1978; Murray et al., 1969). GA has a double minimum at 209 and 220 nm. The shape of the spectra indicates that the protein adopts predominantly α-helical conformation. For all the three glycoproteins tested, no significant change was observed in the spectra before and after deglycosylation. These results are also consistent with those obtained by other authors on the same proteins (Schülke & Schmid, 1988b; Murray at al., 1969; Takegawa et al., 1988).

Although carbohydrate depletion has little effect on the protein secondary structure at low temperature, this does not mean that it has no effect in any condition. The higher T_m values for native glycosylated proteins in the DSC scans suggest that carbohydrate moieties stabilize protein structure in some way. At first we compared the CD spectra of the native and carbohydrate-depleted glycoproteins at 25 °C under different pH conditions. A variation in the range of 4.5−8.0 did not affect significantly the protein structure either in the native or in the deglycosylated form (data not shown). Further studies were performed with the two forms of proteins preincubated at higher temperatures for 15 min. In 50 mM acetate buffer, pH 5.5, heating to 47 °C did not change the structure of either form of YEI. As the temperature was increased, the CD spectra changed. At 58 °C, the negative band at 218 nm was decreased by half of its original intensity for the deglycosylated form while it remained nearly unchanged for the native one. Further heating to 64 °C, which corresponds to the T_m of native YEI in the DSC scan, resulted in the complete unfolding of the deglycosylated protein while the native form retained most of its original structural features (Figure 5A,B). GA was studied in 50 mM acetate buffer at pH 5.0. Below 50.0 °C, neither the glycosylated nor the deglycosylated GA showed conformational changes. Heating to T_m (67.0 °C) resulted in partial denaturation of both forms as indicated by the time dependent decrease of the two negative bands. Inspection of the data confirms that GA becomes less stable after carbohydrate depletion (Figure 5C,D). Figure 5 also shows the CD spectra of BSF at transitional temperature (47.0 °C), because no difference was noticed in the spectra run at lower temperatures, and at the post-transitional temperature (70.0 °C) (Figure 5E,F). A small effect was observed upon carbohydrate removal in the α-helix region of the spectrum while the predominent β structure of this protein appears to be nearly unaffected in both forms. This indicates that the calorimetric transition represents an incomplete unfolding of this protein, in accordance with its low ΔH value.

Reversibility and Renaturation of the Unfolded Glycoprotein by Rapid Cooling. For a reliable thermodynamic description of protein unfolding, it is necessary that the experimentally measured unfolding process be reversible. This was checked by rescanning the sample after rapid cooling and calculating T_m and ΔH of the second scan. The percent of area recovery ($\Delta H_{II}/\Delta H_I \times 100$) is the measure of reversibility. Depending on proteins and the buffer conditions employed, the extent of reversibility differs significantly. Thermal denaturation of BSF is highly reversible. At pH 7.0, if the first scan does not exceed by much the temperature at which the unfolding is completed, reversibility can reach nearly 100%. The high reversibility of this glycoprotein might be due to the high content of disulfide bonds (six per molecule) present in the protein molecule (Spiro 1963). YEI and GA have relatively poorer reversibility than BSF. However, their reversibility depends on buffer conditions and can be substantially improved by increasing pH. AVD and OTF undergo aggregation after denaturation, and no rescan was therefore performed.

Reversibility is crucial in analyzing the thermal unfolding process of proteins. We are interested in studying the reversibility because we found it closely related to the glycosylation state of glycoproteins. In the present study, protein solutions, both in glycosylated and deglycosylated forms, were prepared at the same concentration and were scanned twice through the transition region. As BSF is highly reversible even if it is exposed to high temperature for a long time, the first scan was stopped at 80 and 90 °C, respectively, at pH 8.0 and 6.0. These temperature values are practically 30 °C higher than T_m values and are particularly useful in determining the heat capacity of the protein in the denatured state.

The extent of reversibility, seen on the second scan for the native and the carbohydrate-depleted BSF at pH 8.0 and 6.0, is compared in Table 3. Both forms showed a reduced peak at the same temperature where the transition occurred in the first scan, but the recovered area was substantially larger for the native than for the carbohydrate-depleted form. At pH 8.0, the reversibility was more than 90% for the former and only 50% for the latter. At pH 6.0, the former recovered 95% area of its first scan while the latter less than 20%. Considering the fact that the comparative scans were performed under the same conditions, e.g., protein concentration, scan rate, pH, etc., the difference in reversibility is apparently due to the difference in the glycosylation state of the proteins. A difference in reversibility was also observed with the two forms of YEI and GA. At acidic pH, renaturation occurred for the two native species after cooling but not for the carbohydrate-depleted ones (Table 3).

DISCUSSION

The main scope of this study was to elucidate the roles of carbohydrate moieties in the thermal stability and in the denaturation process of glycoproteins. Five glycoproteins, with carbohydrate content ranging from 2.2% of OTF to 50% of YEI were comparatively studied with DSC and CD. Previously Chu et al. (1978), in studying the Endo H-treated YEI, have shown that although the carbohydrate did not influence the conformation of the polypeptide backbone, its presence considerably enhanced protein stability toward heat and resistance to proteolysis. On the contrary, Schülke and Schmid (1988a) found that the thermal stabilities of yeast external (glycosylated) and internal (not glycosylated) invertases were essentially identical. The controversial results of these authors could be explained by the different methodologies adopted. In the study of Chu et al. (1978), the stability was evaluated by measuring the residual enzymatic activity after the proteins were treated with heat or denaturant, while Schülke and Schmid (1988a) measured the recovered enzyme activity after the treated samples were diluted and cooled. In the present work, native and deglycosylated YEI were comparatively studied by DSC. To our knowledge, this represents the first direct measurement of yeast invertase stability toward heat. Table 2 clearly shows that removal of carbohydrate decreases its T_m by 2.8 ± 0.5 °C. This difference is also observed for BSF (1.3 ± 0.1°C) and GA (1.8 ± 0.4 °C). Although the difference is small, it was consistently observed for all the heavily glycosylated proteins throughout the whole pH region tested. The less glycosylated OTF and AVD did not show measurable variations in T_m at pH 8 and were not further analyzed.

Table 2 also gives the denaturation enthalpy of the five glycoproteins. At any given pH, deglycosylated protein denatured with a lower ΔH than their native form. Such a decrease in ΔH is in part due to its lower T_m (Privalov, 1979), and in part due to the depletion of oligosaccharides. In fact, the plots in Figure 4 permit to calculate and compare the ΔH values of the native and the deglycosylated proteins at the same temperature. It revealed that the denaturation enthalpies were decreased by 9.5% ± 2.0%, 3.6% ± 1.6%, and 6.9% ± 1.5%, respectively, for YEI, BSF, and GA after deglycosylation. The cooperativity of the system was instead practically unchanged in all cases by deglycosylation. It should also be noted that the $T_m - \Delta H$ plots of the native and deglycosylated proteins in Figure 4 give the same slope, i.e., the same ΔC_p, showing that the number of contacts between nonpolar groups at pre-transitional temperatures has not changed (Privalov & Gill, 1988).

The results of CD studies on native and deglycosylated proteins closely parallel the finding of the DSC studies and offer additional evidence that glycosylation enhances the protein conformational stability against heat.

Recently, Olsen et al. (1991) examined the relationship between stability and glycosylation with the two heavily glycosylated β-1,3/1,4-glucanases. They found that the glycosylated enzymes expressed in *Saccharomyces cerevisia* were considerably more heat stable than their unglycosylated counterparts which are expressed in *Escherichia coli*.

It is important to note that all the heavily glycosylated proteins, whether N-glycosylated or O-glycosylated or both, are thermally destabilized by carbohydrate removal. If the results from the present study can be generalized, then we may expect that stabilization of protein conformation and thermal stability are common general properties conferred by the covalent attachment of carbohydrate to the polypeptide backbone and are closely related to the extent of glycosylation.

While distinct differences in heat stability were found, these results do not explain the molecular basis by which carbohydrate affects the glycoprotein properties. One possible way for the attached carbohydrate moieties to stabilize protein conformation is to form hydrogen bonds with the polypeptide backbone. Hecht et al. (1993), in studying the crystal structure of glucose oxidase, have shown that the N-linked mannose residues form strong hydrogen bonds with the backbone nitrogen and the carbonyl oxygen of glutamic acid. Computer simulation of molecular dynamics of RNase B also revealed the possible hydrogen bonding of the nitrogen atom of lysine side chain with the ring oxygen and the hydroxymethyl group of glucosamine (Woods et al. 1994). In addition, carbohydrate moieties may stabilize protein conformation simply by steric interactions of the carbohydrate with the adjacent peptide residues (Gerken et al., 1989; Rudd et al, 1994).

In view of the apparent stabilizing effect of carbohydrate on glycoproteins in the native folded state, it was of interest to determine if carbohydrate affects the protein in the unfolded state and if it participates in the process of protein renaturation. The most dramatic effect was observed when DSC scans were performed with the two forms of YEI at acidic pH. While the native form still showed some area recovery in the second scan, the carbohydrate-depleted form precipitated irreversibly. As the two forms of proteins differ only in their carbohydrate content, it is reasonable to assume that the carbohydrate moiety is responsible for the observed difference.

Our studies on the native and deglycosylated BSF and GA gave similar results. As shown in Table 3, the native forms show a greater recovery of peak area in a second heating cycle than their carbohydrate-depleted counterparts. Previously, experiments on the *in vitro* refolding of carbohydrate-free and carbohydrate-containing proteins were performed by a number of authors. They concluded that the carbohydrate moiety is not involved in the refolding process nor does it affect the refolding kinetics (Grafl et al., 1987; Schülk & Schmid, 1988b). If this holds true, then the poorer reversibility in the absence of carbohydrate would simply indicate that aggregation occurs and competes with efficient renaturation of the denatured protein.

In other words, the presence of carbohydrate moieties improves the solubility of proteins in the denatured or partially denatured states, renders the random polypeptides less likely to form aggregates, and leads to a higher yield of renaturation after cooling.

An improved solubility of the denatured protein molecules may also be important during the *in vivo* folding of the nascent glycoproteins. As the newly synthesized polypeptides may be transiently concentrated in the endoplasmic reticulum, glycosylation may help to maintain the solubility and prevent nonspecific aggregation until the polypeptide chains are correctly folded and transported to the secretory pathway.

While there is much evidence to show that glycosylation helps in protein renaturation in a nonspecific way, recent studies have revealed that N-glycosylation is involved

directly in the folding of nascent glycopolypeptides (Fiedler & Simons, 1995). When a newly synthesized peptide chain enters the lumen of the endoplasmic reticulum, it is attached with the 14-saccharide core unit ($Glc_3Man_9GlcNAc_2$), trimmed by glucosidases, bound to the membrane chaperone calnexin, folded, and secreted (Ou et al., 1993; Hammond et al., 1994). In this process, N-glycosylation occurs in the early stage and plays a central role in protein folding. Our question is, however, why do proteins undergo further modifications or additions of sugar residues to reach the final glycosylation state? One of the answers would be that early co-translational glycosylation serves to prevent aggregation of partially structured protein chains and to allow proper folding. Once the protein is correctly folded, glycosylation may have a general nonspecific effect of stabilization toward proteolytic and denaturing agents.

REFERENCES

Becktel, W. J., & Schellman, J. A. (1987) *Biopolymers 26*, 1859−1877.

Bernard, E. R., Sheila, A. N., & Olden, K. (1983) *J. Biol. Chem. 258*, 12198−12202.

Bertazzon, A., & Tsong, T. Y. (1990) *Biochemistry* 29, 6447−6452.

Bruch, R. C., & White, H. B. (1982) *Biochemistry 21*, 5334−5341.

Chang, C. T., Wu, C.-S., & Young, J. T. (1978) *Anal. Biochem. 91*, 13−31

Chu, F., Watorek, W., & Maley, F. (1983) *Arch. Biochem. Biophys. 223*, 543−555.

Chu, F. K., Trimble, R. B., Maley, F. (1978) *J. Biol. Chem. 253*, 8691−8693.

Cumming, D. A., Hellerqvist, C. G., Harris-Brandts, M., Michnick, S. W., Carver, J. P., & Bendiak, B. (1989) *Biochemistry 28*, 6500−6512.

Dorland, L., Haverkamp, J., & Vliegenthart, J. F. G. (1979) *Eur. J. Biochem. 100*, 569−574.

Faye, L., & Chrispeels, M. J. (1985) *Anal. Biochem. 149*, 218−224.

Feidler, K., & Simons, K. (1995) *Cell 81*, 309−312.

Feizi, T., & Childs, R. A. (1987) *Biochem. J. 245*, 1−11.

Gerken, T. A., Butenhof, K. J., & Shogren, R. (1989) *Biochemistry 28*, *5536*−5543.

Goldberg, M. E. (1985) *Trends Biochem. Sci. 10*, 388−391.

Grafl, R., Lang, K., Vogl, H., & Schmid, F. X. (19887) *J. Biol. Chem. 22*, 10624−10629.

Green, E. D., Adel, G., & Baenziger, J. U. (1988) *J. Biol. Chem. 263*, 18253−18268.

Green, N. M. (1975) *Adv. Protein Chem. 29*, 85−133.

Gunnarsson, A., Svensson, B., Nilsson, B., & Svensson, S. (1984) *Eur. J. Biochem. 145*, 463−467.

Hammond, G., Braakman, I., & Helenius, A., (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 913−917.

Hecht, H. J., Kalisz, H. M., Hendle, J., Schmid, R. D., & Schomburg, D. (1993) *J. Mol. Biol. 229*, 153−172

Kern, G., Kern, D., Jaenicke, R., & Seckler, R. (1993) *Protein Sci. 2*, 1862−1868.

Kiely, M. L., McNight, G. S., & Schimke, R. T. (1976) *J. Biol. Chem. 251*, 5490−5495.

Kojima, N., Shiota, M., Sadahira, Y., Handa, K., & Hakomori, S. (1992) *J. Biol. Chem. 267*, 17264−17270.

Kornfeld, S., & Mellman, I. (1989) *Annu. Rev. Cell Biol. 5*, 483−525.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Lin, L.-N., Mason, A. B., Woodworth, R. C., & Brandts, J. F. (1994) *Biochemistry 33*, 1881−1888.

Lis, H., & Sharon, N., (1993) *Eur. J. Biochem. 218*, 1−27.

Marzuk, M. M., Keene, J. L., & Boime, I. (1989) *J. Biol. Chem. 264*, 2409−2414

Mellors, A., & Sutherland, D. R. (1994) *Trends Biotechnol. 12*, 15−18

Moore, K. L., Stults, N. L., Diaz, S., Smith, D. F., Cummings, R. D., Varki, A., & McEver, R. P. (1992) *J. Cell Biol. 118*, 445−456.

Murray, A. C., Oikawa, K., & Kay, C. M. (1969) *Biochim. Biophys. Acta 175*, 331−338.

Olsen, O., & Thomsen, K. K. (1991) *J. Gen. Microbiol. 137*, 579−585.

Ou, W.-J., Cameron, P. H., Thomas, D. Y., & Bergeron, J. J. M. (1993) *Nature 364*, 771−776.

Paul, G., Lottspeich, F., & Wielland, F. (1986) *J. Biol. Chem. 261*, 1020−1024.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*,167−241.

Privalov, P. L., & Gill, S. J. (1988) *Adv. Protein Chem. 39*, 191−234.

Reddy, V. A., Johnson, R. S., Biemann, K., Williams, R. S., Ziegler, F. D., Trimble, R. B., & Maley, F. (1988) *J. Biol. Chem. 263*, 6978−6985.

Rothman, J. E., & Lodish, H. F. (1975) *Nature 269*, 775−780.

Rudd, P. M., Joao, H. C., Coghill, E., Fiten, P., Saunders, M. R., Opdenakker, G., & Dwek, R. A. (1994) *Biochemistry 33*, 17−22.

Saito, S., & Tsuchiya, T. (1984) *Biochem. J.* 222, 829−832.

Schülke, N., & Schmid, F. X. (1988a) *J. Biol. Chem. 263*, 8827−8831.

Schülke, N., & Schmid, F. X. (1988b) *J. Biol. Chem. 263*, 8832−8837.

Spiro R. G. (1962) *J. Biol. Chem. 237*, 382−388.

Spiro, R. G. (1963) *J. Biol. Chem. 238*, 644−649.

Spiro, R. G., & Bhoyroo, V. D. (1974) *J. Biol. Chem. 249*, 5704−5717.

Sturtevant, J. (1987) *Annu. Rev. Phys. Chem. 38*, 463−488.

Svensson, B., Perdersen, T. G., Svendsen, I., Sakai, T., & Ottesen. M. (1982) *Carlsberg Res. Commun. 47*, 55−69.

Takegawa, K., Inami, M., Yamamoto, K., Kumagai, H., Tochihura, T., Mikami, B., & Morita, Y. (1988) *Biochim. Biophys. Acta 955*, 187−193.

Trimble, R. B., & Maley, F. (1977) *J. Biol. Chem. 252*, 4409−4412.

Trimble, R. B., Maley, F., & Chu F. K. (1983) *J. Biol. Chem. 258*, 2562−2567.

Williamson, G., Belshaw, N. J., & Williamson, M. P. (1992a) *Biochem. J. 282*, 423−428.

Williamson, G., Belshaw, N. J., Noel, T. R., Ring, S. G., & Williamson, M. P. (1992b) *Eur. J. Biochem. 207*, 661−670.

Woods, R. J., Edge, C. J., & Dwek, R. A. (1994) *Nat. Struct. Biol. 1*, 499−501.

Yamaguchi, K., Akai, K., Kawanishi, G., Ueda, M., Masuda, S., & Sasaki, R. (1991) *J. Biol. Chem. 267*, 7703−7709.

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