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Effect of Oligosaccharides and Chloride on the Oligomeric Structures of External, Internal, and Deglycosylated Invertase†

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ABSTRACT: External invertase exists in an oligomeric equilibrium of dimer, tetramer, hexamer, and octamer, the concentrations of which vary with pH, time, and concentration of enzyme [Chu, F. K., Watorek, W., & Maley, F. (1983) *Arch. Biochem. Biophys. 223*, 543-555; Tammi, M., Ballou, L., Taylor, A., & Ballou, C. E. (1987) *J. Biol. Chem. 262*, 4395-4401]. To assess the influence of carbohydrate on this equilibrium, we investigated the self-association of external invertase (10 oligosaccharides per subunit), deglycosylated external invertase (2 oligosaccharides per subunit), and internal invertase (no carbohydrate) under various conditions. In addition, the effect of carbohydrate on the interaction of the subunits of these various invertases to form heterooligomers was studied. Chloride ion was found to promote subunit association in the various invertases irrespective of their glycosylation status. However, external invertase was less responsive to chloride ion relative to the internal and deglycosylated invertases. The higher oligomers of deglycosylated invertase were stable at 47 °C whereas those of external invertase dissociated rapidly into dimers, suggesting that the additional oligosaccharides in external invertase destabilize subunit interaction. Hybridization experiments with the various invertases showed that the dimers of internal invertase formed heterooligomers with either external or deglycosylated invertase. By contrast, the monomers of external and internal invertases formed their respective homodimers, but not heterodimers. These results suggest that the oligosaccharide content of invertase not only influences the extent of self-association but also affects heterooligomer formation.

The influence of oligosaccharides on the structure and function of glycoproteins has long been a subject of wide interest. Oligosaccharides are implicated in such functions as protein folding, stability, sorting, bioactivity, and protein–protein recognition. Invertase (EC 3.2.1.26, β-D-fructofuranoside fructohydrolase) from *Saccharomyces cerevisiae* is ideally suited to study the function of oligosaccharides since it exists as an external invertase with 50% (w/w) carbohydrate and an internal invertase with no carbohydrate. Both enzyme forms are derived from the SUC2 gene but from different start codons (Perlman & Halvorson, 1981; Carlson & Botstein, 1982). Because internal invertase does not contain a signal sequence, it cannot enter the endoplasmic reticulum and as a consequence is not glycosylated. By contrast, external invertase contains a signal sequence which directs it into the endoplasmic reticulum where it is glycoslyated to about 10 oligosaccharides per subunit (Trimble & Maley, 1977; Reddy et al., 1988), about 80% of which can be removed by endo-β-*N*-acetylglucosaminidase (Endo H)[1] (Tarentino et al., 1974). Eight of the oligosaccharides are from 8–14 mannoses in length while the remainder contain over 50 mannoses (Ziegler et al., 1988). Although oligosaccharides are not essential for enzyme activity, they protect the enzyme from proteases (Chu et al., 1978; Brown et al., 1979) and maintain external invertase in an oligomeric equilibrium of dimer, tetramer, hexamer, and octamer (Chu et al., 1983). More recently, it has been shown that freezing (Tammi et al., 1987) or the addition of poly-

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[1] Abbreviations: buffer A, 0.05 M KH_2PO_4, pH 4.5; buffer B, 0.05 M sodium acetate, pH 5.5; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Endo H, endo-β-*N*-acetylglucosaminidase H; GuHCl, guanidine hydrochloride; PEG, poly(ethylene glycol); RM-invertase, reductively methylated invertase.

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(ethylene glycol) (Esmon et al., 1987) promotes the self-association of external invertase subunits, but not those of internal invertase.

In the present study, we have investigated further the role of oligosaccharides in subunit association. The self-association of invertase subunits bearing varying amounts of oligosaccharides was reexamined, and attempts were made to hybridize the glycosylated, partially glycoslyated, and nonglycosylated subunits. In addition, the effect of oligosaccharides on the heat stability of oligomers was studied. The results show that the self-association of internal and deglycosylated invertase subunits into higher oligomers is more sensitive to Cl^- than that of external invertase. Evidence is presented to show that glycosylation affects the subunit association at the level of both monomer and dimer.

Materials and Methods

Enzymes. Cloned endo-β-*N*-acetylglucosaminidase H (Endo H) was purified from *Escherichia coli* as described (Trumbly et al., 1985). External invertase was purchased from Boehringer and Mannheim (specific activity about 300 units/mg of protein) and separated from internal invertase and contaminating carbohydrate material by DE-52 (Schleicher & Schuell) column chromatography. Further purification on a Sephacryl S-300 (Pharmacia) column yielded the final preparation (specific activity 4500 units/mg of protein) which was homogeneous on SDS–PAGE (Trimble & Maley, 1977; Williams et al., 1985).

Cloned internal invertase was purified from yeast cells by ion-exchange, hydrophobic, and gel filtration chromatographies on DE-52, phenyl-Sepharose CL-4B (Pharmacia), and Ultrogel AcA44 (LKB) columns, respectively (Williams et al., 1985). The final preparation had a specific activity comparable to external invertase and was homogeneous on SDS–PAGE.

Deglycosylated external invertase was prepared by incubation of external invertase (20 mg) with Endo H (200 milliunits) in 0.05 M sodium citrate, pH 5.5 (2 mL), at 37 °C for 20 h. The released oligosaccharides were removed by Bio-Gel A1.5m (Bio-Rad) column chromatography (2.5 × 44 cm) using 0.05 M potassium phosphate, pH 4.5, as the eluant. The Endo H treated enzyme retained about 20% of its native oligosaccharides and was fully active.

Reductive Methylation of Internal Invertase. Internal invertase (0.5 mg) was incubated at 23 °C for 5 min with 50 μCi of [^{14}C]formaldehyde (57 mCi/mmol, New England Nuclear) in 0.3 mL of 0.02 M potassium phosphate, pH 7.0. A solution of sodium cyanoborohydride was added to a final concentration of 13 mM, and the reaction was continued for 30 min. The pH was lowered to 5.5 by addition of sodium acetate, and the excess reagents were removed by dialysis in 20 mM sodium acetate, pH 5.5. Labeled enzyme (5500 cpm/μg of protein) was fully active.

HPLC and FPLC. Invertase oligomers were routinely separated by size-exclusion chromatography at 23 °C either on a TSK G4000SW column (0.5 × 60 cm; fractionation range 5K–900K; LKB Instruments) or on a Superose-6 column (1.0 × 30 cm; fractionation range 5K–5000K; Pharmacia Co.) using a Beckman Model 110A HPLC system equipped with Chromatopac C-R3A (Shimadzu Corp.) data processor. The flow rates were 0.9 and 0.5 mL/min for the TSK G4000SW column and Superose-6 column, respectively. Protein was detected at 229 nm, and when necessary, the column effluent was monitored continuously for radioactivity on a Radiomatic Flo-One detector using Ready Flow III scintillation cocktail (Beckman Instruments). The chromatograms and radiograms were stored in the memory of the C-R3A and radiometric detector, respectively, and replotted at a chart speed of at least 0.5 cm/min to spread out the peaks. To determine the relative concentration of oligomers, the peaks were cut out and weighed. The error factor in these determinations was no more than ±10%.

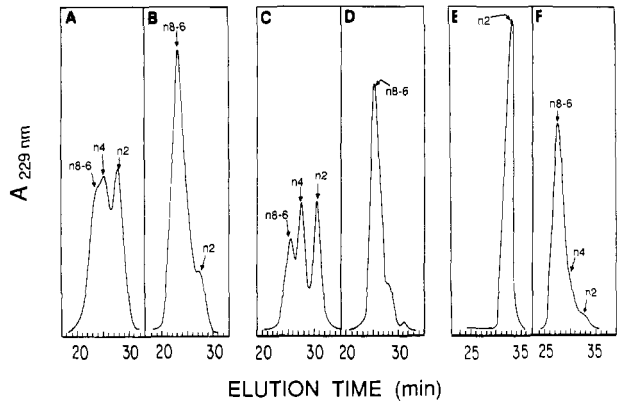

FIGURE 1: Effect of chloride on the oligomeric structure of invertases. The respective invertases (10 μg of each) were taken in about 100 μL of either buffer A or buffer A containing 0.8M NaCl and analyzed by FPLC on a Superose-6 column using either buffer A or buffer A containing 0.8M NaCl as eluant. External invertase, deglycosylated invertase, and internal invertase were eluted in the absence of NaCl (panels A, C, and E, respectively) or in the presence of 0.8 M NaCl (panels B, D, and F, respectively). Oligomer concentrations were as follows: panel A, *n*8–6 (35%), *n*4 (31%), *n*2 (34%); panel B, *n*8–6 (74%), *n*4 (15%), *n*2 (11); panel C, *n*8–6 (28%), *n*4 (36%), *n*2 (36%); panel D, *n*8–6 (86%), *n*4 (12%); panel E, *n*2 (100%); panel F, *n*8–6 (78%), *n*4 (15%), *n*2 (7%).

Ultracentrifugation. Sedimentation studies were performed with a Beckman Model E analytical ultracentrifuge. Schlieren optics were employed on solutions having higher protein concentrations. Lower protein concentrations were monitored by using the photoelectric scanner with its absorption optics at 280 nm.

Other Methods. Invertase concentration was determined by the absorbance at 280 nm (1 mg/mL = 2.25). Invertase activity was assayed according to Goldstein and Lampen (1975). Carbohydrate was estimated by the phenol–sulfuric acid method (Dubois et al., 1956).

Results

Self-Association of Invertases into Higher Oligomers. External invertase exists as an oligomeric mixture of dimer, tetramer, hexamer, and octamer at pH 4.5–5.0 where the enzyme is maximally active (Chu et al., 1983). Size-exclusion HPLC separated the oligomers into three peaks corresponding to dimer, tetramer, and a mixture of hexamer and octamer (Figure 1, panel A). However, in the presence of 0.8 M NaCl, the subunits of external invertase associated to a predominant mixture of octamer and hexamer (74% of the total) (panel B). Potassium chloride also facilitated the formation of higher oligomers, but salts such as sodium acetate, ammonium sulfate, potassium phosphate, and sodium fluoride at concentrations up to 0.4 M had no effect, indicating that the chloride ion was primarily responsible for promoting the self-association. Interestingly, in the presence of 0.8 M GuHCl,[1] external invertase existed mostly as tetramers, reflecting the opposing effects of dissociation and association by guanidinium and chloride ions, respectively (data not shown).

Deglycosylated invertase was resolved on the size-exclusion column into an equilibrium mixture of oligomers with sharper peaks than those of external enzyme (Figure 1, panel C), but in the presence of 0.8 M NaCl, it existed mainly as an oc-

Table I: Effect of Varying Concentrations of NaCl on the Oligomeric Structure of Different Types of Invertase[a]

enzyme	[NaCl] (M)	relative oligomer concentration		
		*n*8–6	*n*4	*n*2
external invertase	0.0	32	32	36
	0.05	38	32	30
	0.10	50	29	21
	0.20	51	29	20
deglycosylated invertase	0.0	12	33	55
	0.05	33	36	31
	0.10	48	32	20
	0.20	81	15	4
internal invertase	0.0	0	0	100
	0.05	83[b]	0	17
	0.10	85[b]	0	15
	0.20	88[b]	0	12

[a] Invertase (10 μg) was taken up in buffer A containing the indicated concentration of NaCl and analyzed on a Superose-6 column (see Materials and Methods). Buffer A containing the same concentration of NaCl as in the sample was used for elution. [b] Represents a poorly resolved mixture of octamer, hexamer, and tetramer.

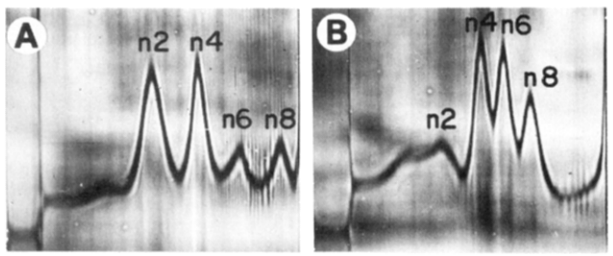

FIGURE 2: Analytical ultracentrifugation of external invertase. (A) External invertase (4.2 mg in 0.6 mL of buffer A) was sedimented at 40000 rpm, 5 °C, in a Beckman Model E ultracentrifuge. The sedimentation profile was recorded by using schlieren optics. The oligomer concentrations were 40% dimer, 31% tetramer, 13% hexamer, and 12% octamer. (B) The same conditions as in (A) were used except that the enzyme was taken up in buffer A containing 0.7 M NaCl. The relative state of subunit association was calculated by using the equation $S1/S2 = (M1/M2)^{2/3}$ where S is the sedimentation coefficient and M the molecular mass (Cantor & Schimmel, 1980). The oligomer concentrations were 13% dimer, 28% tetramer, 27% hexamer, and 22% octamer. It is unclear whether the small diffuse peak to the left of dimeric peak represents monomer.

tamer–hexamer mixture (86% of the total), although small amounts of tetramer and dimer were still evident (panel D). Unlike the external and deglycosylated invertases, internal invertase eluted as a dimer, which in the presence of NaCl yielded an oligomeric mixture consisting mostly of octamer and hexamer (78% of the total) (compare panel E with panel F).

Table I shows the effect of low concentrations of NaCl on the oligomeric structure on invertases. In general, external invertase was less sensitive to Cl^- than deglycosylated and internal invertases, as evidenced by the formation of a smaller amount of the octamer–hexamer mixture in 0.2 M NaCl. However, at 0.8 M NaCl, all of the invertases regardless of their carbohydrate content yielded the maximal octamer–hexamer mixture (see Figure 1). The decreased sensitivity of external invertase to the lower levels of Cl^- may signify that oligosaccharides shield the subunit from interacting with Cl^-.

Unlike HPLC or FPLC, ultracentrifugation resolves all four oligomers of external invertase (7 mg/mL) into distinct bands, the major species being the dimer and tetramer (Figure 2, panel A). However, in the presence of 0.7 M NaCl, the dimer band was reduced significantly with a concomitant increase in hexamer and octamer bands (panel B), confirming that Cl^- promotes the formation of higher oligomers. Interestingly, both deglycosylated invertase (6.2 mg/mL) and internal invertase (6.4 mg/mL) migrated as single octomeric species with an $S_{20,w}$ of 11.3 and 10.0 S, respectively. These results suggest that at higher protein concentrations the subunits of deglycosylated and internal invertase self-associate into octamers more efficiently than external invertase at a comparable concentration. The above findings contrast with those of Tammi et al. (1987) which revealed that at a comparable concentration external invertase subunits associate to a greater degree than those of the underglycosylated form of invertase. Information on which of the sequons are glycosylated may help in clarifying this apparent discrepancy.

Table II: Dissociation of External and Internal Invertases to Monomers[a]

enzyme treatment	no. of subunits	% of total
external invertase (buffer A-3 M GuHCl, pH 4.5)	1.0	67
external invertase (pH 11.0)	1.0	89
	3.8	10
internal invertase (buffer A–3 M GuHCl, pH 4.5)	1.0	100
internal invertase (pH 11.0)	1.0	60
	3.9	38

[a] enzyme samples (0.5 mg/mL) were centrifuged in a Model E Beckman analytical ultracentrifuge at 52000 rpm, 20 °C. The sedimentation was followed by absorption optics. The relative state of subunit association was calculated as described in Figure 2. Dilute NaOH was used to adjust the pH of enzyme samples to 11.0.

Hybridization of Different Glycosylation Forms of Invertase. The monomer of external invertase is twice as large as that of internal invertase (M_r 120000 versus 60000) owing to the presence of an average of 10 oligosaccharides (Trimble & Maley, 1977; Reddy et al., 1988). To determine whether the oligosaccharides influence the monomer–monomer interaction, we attempted to promote the hybridization of external and internal invertase monomers into heterodimers and oligomers. Earlier attempts to dissociate the enzyme into monomers using such conditions as acidic pH and GuHCl were unsuccessful due to the formation of inactive aggregates. However, at pH 11.0, both external and internal invertases (0.5 mg/mL) dissociated mostly into monomers (Table II), which then reassociated at pH 5.0 into oligomers in the former case and dimers in the latter, both with nearly full activity (data not shown). The hybridization experiment consisted of incubating a mixture of external and ^{14}C-reductively methylated (RM) internal invertases at pH 11.0, followed by lowering the pH to allow reassociation. Analysis of the sample on a Superose-6 column (Figure 3) showed that external invertase eluted mostly as a dimer with some tetramer, but no radioactivity was associated with these peaks. The single radioactivity peak coincided with the RM-internal invertase dimer, indicating that the nonglycosylated monomers failed to associate with the glycosylated monomers.

As a logical extension of the experiment above, we investigated the influence of the oligosaccharides on the interaction between dimers, the smallest enzymically active units of invertase. External invertase was mixed with [^{14}C]RM-internal invertase in buffer A, and NaCl was added to 0.8 M NaCl to promote the formation of higher oligomers. Figure 4, panel B, shows an HPLC analysis of the resulting oligomeric mixture using buffer A containing NaCl as eluant. Mixing the enzymes resulted in a decrease in the absorbances of external invertase dimer and the internal invertase octamer–hexamer mixture, and a concomitant increase in higher oligomers (compare panel B with control profiles in panel A). Also, a major portion (66%) of the radioactivity eluted earlier than

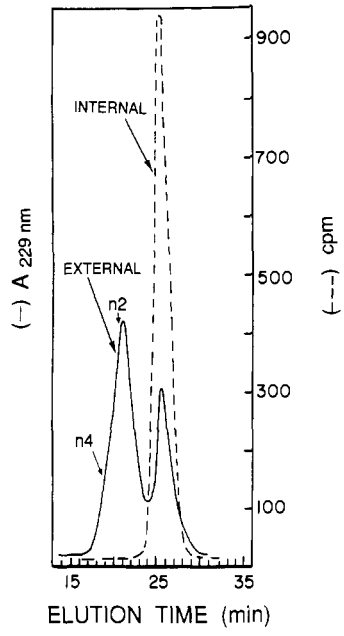

FIGURE 3: Attempt at hybridizing external and internal invertase monomers into a heterodimer. External invertase (7 μg) and [^{14}C]RM-internal invertase (3.5 μg) were incubated at pH 11.0, 23 °C, for 30 min. The pH was lowered to 5.0 by addition of 0.5 M sodium acetate, pH 5.0. After an additional 30 min, the sample (90 μL) was analyzed on a TSK G4000SW column using buffer A as eluant. For controls, external invertase and [^{14}C]RM-internal invertase were treated as above, but separately.

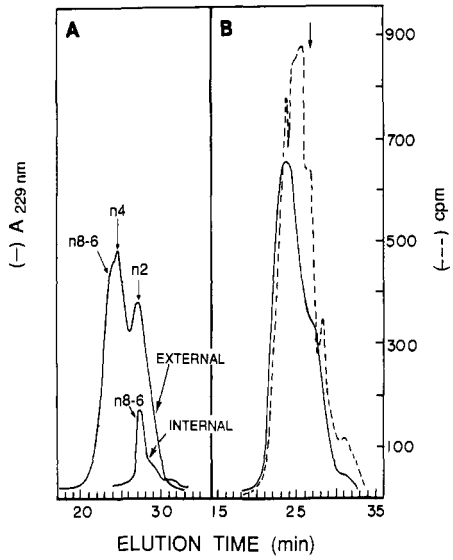

FIGURE 4: Hybridization of external and internal invertases into heterooligomers. (A) Control profiles of external invertase and [^{14}C]RM-internal invertase obtained from separate runs. External invertase oligomers (10 μg) or [^{14}C]RM-internal invertase dimer (3 μg) was taken up in 0.05 M potassium phosphate–0.8 M NaCl, pH 4.5, and analyzed on a Superose-6 column using 0.05 M potassium phosphate–0.8 M NaCl, pH 4.5, as eluant. External invertase dimer (240 kDa) eluted at about the same time point as the internal invertase octamer–hexamer mixture (480–360 kDa) because of the attached oligosaccharides. (B) External invertase oligomers (10 μg) and [^{14}C]RM-internal invertase dimer (3 μg) were mixed in the presence of 0.8 M NaCl and analyzed as in (A). For comparison, the position of the radioactive peak of labeled internal invertase alone is marked by an arrow.

expected of the octamer–hexamer mixture of [^{14}C]RM-internal invertase (panel B, dashed line), indicating that internal invertase subunits must have associated with external invertase subunits to yield the faster eluting labeled heterooligomer. Increasing the concentration of external invertase in the hybridization mixture by 2–3-fold resulted in a corresponding enhancement in heterooligomer formation. It is difficult to determine the composition of the heterooligomers since HPLC did not adequately resolve the heterooligomeric mixture. Attempts to obtain heterooligomers by incubating external and [^{14}C]RM-internal invertases at pH 5.0 in the absence of NaCl, or by freezing the enzyme mixture, were not successful.

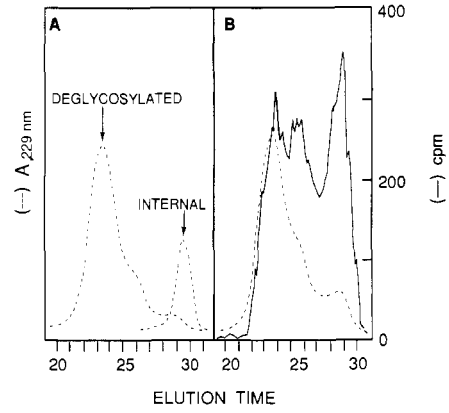

FIGURE 5: Hybridization of deglycosylated and internal invertases to heteroologiomers. (A) Individual absorbance profiles of deglycosylated invertase and [^{14}C]RM-internal invertase. Deglycosylated invertase (16 μg) or [^{14}C]RM-internal invertase (4 μg) was incubated for 30 min in 0.05 M CHES buffer, pH 9.2. The pH was lowered to 5.0 by addition of 0.5 M sodium acetate, pH 5.0, and NaCl was added to a final concentration of 0.8 M. The sample (90 μL) was analyzed on a Superose-6 column using buffer B as eluant. (B) A mixture of deglycosylated invertase oligomers (16 μg) and [^{14}C]-RM-internal invertase (4 μg) was treated and analyzed as in (A). The radioactivity is distributed among dimer (38%), heterotetramer (30%), and heterooctamer–hexamer (32%). This distribution varied with the concentration of deglycosylated invertase used. Replacement of deglycosylated enzyme with bovine serum albumin resulted in a single radioactivity peak, ruling out nonspecific protein association.

A similar experiment was conducted to investigate the association between the dimers of deglycosylated and internal invertases (Figure 5). Deglycosylated invertase was taken up in alkaline buffer (pH 9.2) to dissociate it into dimers. Following the addition of [^{14}C]RM-internal invertase, the pH was lowered to 5.0, and NaCl was added to 0.8 M to promote subunit association. The oligomeric mixture was then analyzed by HPLC using buffer A as eluant. A comparison of the absorbance profile in Figure 5B with those of control profiles in Figure 5A reveals that mixing the enzymes resulted in a decrease in the concentration of internal invertase dimer and an increase in higher oligomer concentration. The radioactivity was distributed among three distinct peaks corresponding to dimer (32%), heterotetramer (30%), and heterohexamer–heterooctamer (32%), providing evidence that internal invertase is capable of forming heterooligomers with deglycosylated invertase also. A significant amount of heterooligomers was also obtained by incubation of the enzyme mixture in buffer A alone, indicating that Cl^- is not an absolute requirement for heterooligomer formation.

Heat Stability of Oligomeric Forms of External and Deglycosylated Invertases. To determine whether the degree of glycosylation influences the stability of the higher oligomers, external and deglycosylated invertases were incubated at 47 °C in buffer B, and their state of association was analyzed by HPLC at different time intervals. External invertase which exists mostly as an octamer–hexamer mixture at 23 °C (Figure 6, panel A) dissociated predominantly to a tetramer in 6 min (Figure 6, panel B) and then to the dimeric state (panels C and D), until by 120 min the enzyme was fully dissociated to a dimer (data not shown). This result is in agreement with the finding of Esmon et al. (1987), that following incubation

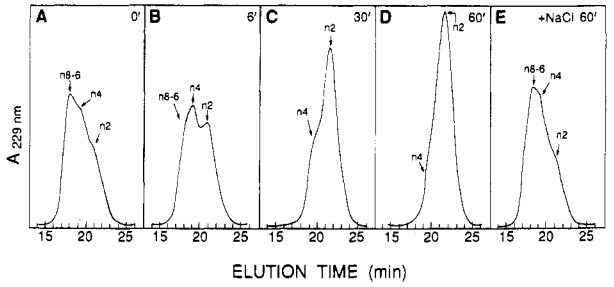

FIGURE 6: Effect of temperature on the oligomeric structure of external invertase. External invertase (1 mg/mL in buffer B) was incubated at 47 °C. At nine invervals from 0 to 120 min, 10 μL of the enzyme solution was cooled to room temperature by dilution into 80 μL of buffer A and analyzed on a TSK G4000SW column using buffer A as eluant. Only the analyses at 0, 6, 30, and 60 min are presented (A–D). The relative concentration of oligomers was determined as described under Materials and Methods. (E) represents external invertase that was incubated for 60 min in the presence of 0.8 M NaCl.

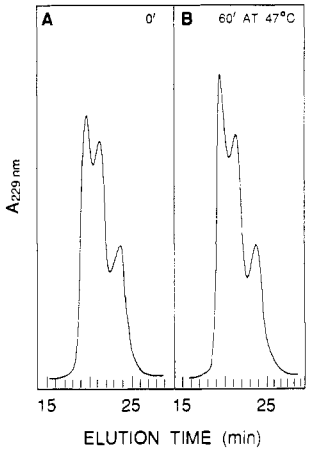

FIGURE 7: Heat stability of deglycosylated invertase oligomers. (A) and (B) represent deglycosylated invertase incubated at 47 °C for 0 and 60 min, respectively. Other conditions are identical with those in Figure 6.

at 30 °C for 12 h invertase from a secretory mutant existed mostly as dimers and tetramers on nondenaturing PAGE. Interestingly, incubation of external invertase for 60 min at 47 °C in the presence of 0.8 M NaCl resulted in little change in the oligomeric equilibrium (compare panel A of Figure 6 with panel E), indicating that Cl^- protects against heat dissociation. In contrast to external invertase, incubation of deglycosylated enzyme in buffer B for 60 min at 47 °C resulted in no appreciable change in the oligomeric structure (Figure 7A,B). No significant change in the oligomeric equilibrium was noted even after 2 h of incubation (data not shown). These results demonstrate that the higher oligomers of external invertase are less stable to heat than those of deglycosylated invertase, possibly because the additional oligosaccharides in external invertase weaken subunit interactions.

Discussion

The role of carbohydrate associated with external invertase has been the subject of several investigations, which in essence have revealed that the oligosaccharides are not essential for enzyme activity (Trimble & Maley, 1977; Tarentino et al., 1974) or for the maintenance of the peptide backbone conformation (Williams et al., 1985). In addition, denaturation studies have shown that the oligosaccharides do not afford protection against GuHCl or heat (Trumbly et al., 1985; Shulte & Schmid, 1988a,b). However, the oligosaccharides appear to be involved in protecting external invertase against proteolytic degradation (Chu et al., 1978; Brown et al., 1979) and in maintaining an oligomeric equilibrium among the subunits (Chu et al., 1983; Esmon et al., 1987; Tammi et al., 1987). Esmon et al. (1987) have reported that some salts stabilze the oligomeric structure of external invertase while others such as $CaCl_2$ and $MgCl_2$ have no effect. The present study shows that in addition to stabilizing the oligomeric structure (Figure 6E), chloride ion promotes subunit association. It is possible that Esmon et al. used too low a concentration of chloride in their study to have observed its effect on subunit association in external invertase. The mechanism by which Cl^- promotes association is apparently different from that of PEG or freez-promoted subunit association (Esmon et al., 1987; Tammmi et al., 1987), as the association of internal invertase is facilitated by Cl^- but not by PEG or freezing. The observation that heterooligomer formation between the subunits of external and internal invertase is promoted by Cl^- but not by freezing is in agreement with this proposal. One mechanism by which Cl^- may promote association is by neutralization of the repulsive forces between dimers.

Whether internal invertase exists in an oligomeric equilibrium is not clearly borne out by the published literature, although electron micrographs of internal invertase show octamers at sufficiently high concentration (Esmon et al., 1987). Analytical ultracentrifugation indicates that internal invertase migrates as an octamer, but HPLC analyses of samples at much lower concentrations (0.1–0.2 mg/mL in buffer A) reveal the dimer only. Collectively, these observations indicate that the octameric form of internal invertase dissociates rather quickly into dimer upon dilution. However, as shown in Figure 1F, internal invertase exists in an oligomeric equilibrium in the presence of Cl^-, and by manipulating the Cl^- concentration, it is possible to obtain a substantial amount of stable tetramers. Since internal invertase begins to form higher oligomers at Cl^- concentrations as low as 25 mM (unpublished observation), it is likely that this enzyme exists as an oligomeric mixture inside the yeast cell.

Esmon et al. (1987) and Tammi et al (1987) have also presented convincing evidence that oligosaccharides are involved in promoting the association of invertase into higher oligomers. Failure of the carbohydrate-free internal invertase to form higher oligomers in the presence of PEG or by freezing is in agreement with this conclusion. It is unclear which, if any, of the 13 oligosaccharides are involved in promoting the association. However, the observation that deglycosylated invertase, which has an average of only two oligosaccharides per monomer, exists in an oligomeric equilibrium (Figure 1, panel C), and also forms higher oligomers on freezing (unpublished data), suggests that specific oligosaccharides are involved. Previous studies (Zeigler et al., 1988) have shown that the oligosaccharides on sequon 2 (Asn45) and sequon 10 (Asn337) are completely resistant to Endo H and those on sequon 4 (Asn92), sequon 8 (Asn247), and sequon 11 (Asn350) are partially resistant. Sequon 8 is unlikely to play a role since it is found to be poorly glycosylated (Reddy et al., 1988). A systematic elimination of sequons 2, 4, 10, and 11 by site-directed mutagenesis could possibly reveal which oligosaccharide(s) are essential for maintaining the oligomeric equilibrium.

Electron microscopy has shown that the basic structural unit of the external and internal invertases is a dimer (Esmon et al., 1987; Tammi, et al., 1987). These basic units appear to associate with each other irrespective of their glycosylation status, as evidenced by heterooligomer formation in a mixture of internal and external invertases or internal and de-

glycosylated invertases (Figure 4 and 5). However, monomers of external and internal invertases failed to associate into heterodimers (Figure 3), but instead formed their respective homodimers, indicating that monomer–monomer interactions are subtly different in these enzyme forms. It is possible that oligosaccharides constitute part of an intermonomer contact region as in the case of influenza virus haemagglutinin (Wilson et al., 1981). If so, the likely candidates are the two oligosaccharides on sequons 2 and 10, which are inaccessible to Endo H and probably are buried in the interior of the molecule (Ziegler et al., 1988). Experiments are underway to investigate the role of these oligosaccharides by site-directed mutagenesis.

The extent of glycosylation of invertase appears to determine its oligomeric state, particularly when incubated at 47 °C. Thus, it can be seen on comparing the results in Figure 6 with those in Figure 7 that external invertase is converted almost completely to its dimeric form from an octamer–hexamer mixture in 60 min while deglycosylated invertase is not affected by this treatment. It is tempting to suggest from these results that the protruding oligosaccharides in external invertase partially obstruct the dimer–dimer binding, thus weakening the higher oligomeric structure. This view is compatible with ultracentrifugation analyses which show that deglycosylated invertase at high concentrations migrates as an octamer whereas external invertase at a comparable concentration associates only partially to its octameric state (see Figure 2, panel A). The observation that external invertase failed to form heterooligomers with internal invertase in the absence of Cl^-, while deglycosylated invertase could do so under similar conditions, is also in agreement with this suggestion. Tammi et al. (1987) have drawn a similar inference from their electron microscopic study of external and internal invertases. External invertase is composed of four spherical dimers arranged roughly in the form of an open-sided square. The authors suggest that sterical interference by oligosaccharides might cause the open-sided conformation. In this connection, it is interesting to note that oligosaccharides have been reported to decrease the binding affinity of fibronectin (Zhu & Laine, 1986; Jones et al., 1986). Thus, highly glycosylated fetal fibronectin possesses a lower binding affinity for gelatin relative to a less glycosylated form of fibronectin, which in turn binds to gelatin or fibroblast surface receptor even less tightly than deglycosylated fibronectin.

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