IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ZYMOGENETICS, INC., a Washington Corporation, Plaintiff, v. BRISTOL-MYERS SQUIBB CO., a Delaware Corporation, and DOES 1 through 100, Defendants.	)	Civil Action No: 06-500-SLR-MPT **REDACTED PUBLIC VERSION**

DECLARATION OF DANA LAU IN SUPPORT OF BRISTOL-MYERS SQUIBB COMPANY'S RESPONSE TO PLAINTIFF ZYMOGENETICS, INC.'S STATEMENT OF MATERIAL FACTS AS TO WHICH THERE IS NO GENUINE ISSUE TO BE TRIED AND LEGAL ISSUES IN SUPPORT OF PLAINTIFF'S MOTION FOR SUMMARY JUDGMENT OF VALIDITY OF PATENTS-IN-SUIT WITH RESPECT TO THE CAPON PATENTS AND SEED PATENT

I, Dana Lau, hereby declare the following:

1. I am an associate with the law firm of Fitzpatrick, Cella, Harper & Scinto, attorneys for Defendant Bristol-Myers Squibb Company ("Bristol") in this action. I submit this Declaration in support of Bristol's Response to Plaintiff ZymoGenetics, Inc.'s Statement of Material Facts As To Which There Is No Genuine Issue To Be Tried And Legal Issues In Support Of Plaintiff's Motion For Summary Judgment of Validity Of Patents-In-Suit With Respect To The Capon Patents And Seed Patent.

2. Attached hereto as BMS Exhibit 1 is a true and correct copy of excerpts of Wayne Kindsvogel Deposition Transcript, August 8, 2007, at 177-184 and 189-192.

3. Attached hereto as BMS Exhibit 2 is a true and correct copy of excerpts of Anne Bell Laboratory Notebook at ZG040369, ZG040452, ZG040464-65, ZG040479, ZG040510-12, ZG040518-525, ZG040527, and ZG040550-51.

4. Attached hereto as BMS Exhibit 3 is a true and correct copy of excerpts of Steven Levin Deposition Transcript, November 8, 2007, at 73-76.

5. Attached hereto as BMS Exhibit 4 is a true and correct copy of Kern et al., *Stability, quaternary structure, and folding of internal, external, and core-glycosylated invertase from yeast*, PROTEIN SCIENCE, 1:120-131 (1992).

6. Attached hereto as BMS Exhibit 5 is a true and correct copy of Esmon et al., *Structure, assembly and secretion of octameric invertase*, JOURNAL OF BIOLOGICAL CHEMISTRY, 262:4387-4394 (1987).

7. Attached hereto as BMS Exhibit 6 is a true and correct copy of oligonucleotide request forms for ZC1776, ZC1777, ZC1846 and ZC1847, at ZG047284-87.

8. Attached hereto as BMS Exhibit 7 is a true and correct copy of excerpts of Paul Edward Purdue Deposition Transcript, May 5, 2008, at 237-244.

9. Attached hereto as BMS Exhibit 8 is a true and correct copy of excerpts of Nicholas R.J. Gascoigne, Ph.D. Deposition Transcript, April 29, 2008, at 57-60 and 65-68.

10. Attached hereto as BMS Exhibit 9 is a true and correct copy of excerpts of David A. Ostrov, Ph.D. Deposition Transcript, May 6, 2008, at 101-108.

I declare under penalty of perjury under the laws of the United States of America that the forgoing is true and correct.

Executed this 2nd day of June, 2008, in New York, New York.

Dana Lau

EXHIBIT 1

REDACTED IN ITS ENTIRETY

EXHIBIT 2

REDACTED IN ITS ENTIRETY

EXHIBIT 3

REDACTED IN ITS ENTIRETY

EXHIBIT 4

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Stability, quaternary structure, and folding of internal, external, and core-glycosylated invertase from yeast

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(RECEIVED August 22, 1991; ACCEPTED September 5, 1991)

Abstract

The role of carbohydrate chains for the structure, function, stability, and folding of glycoproteins has been investigated using invertase as a model. The protein is encoded by several different genes, and its carbohydrate moiety is heterogeneous. Both properties complicate physicochemical comparisons. Here we used the temperature-sensitive sec18 secretion mutant of yeast with a single invertase gene (SUC2). This mutant produces the carbohydrate-free internal invertase, the core-glycosylated form, and, at the permissive temperature, the fully glycosylated external enzyme, all with identical protein moieties. The core-glycosylated enzyme resembles the nascent glycoprotein chain that folds in the endoplasmic reticulum. Therefore, it may be considered a model for the in vivo folding of glycoproteins. In addition, because of its uniform glycosylation, it can be used to investigate the state of association of native invertase.

Glycosylation is found to stabilize the protein with respect to thermal denaturation and chaotropic solvent components; the stabilizing effect does not differ for the external and the core-glycosylated forms. Unlike the internal enzyme, the glycosylated forms are protected from aggregation.

Native internal invertase is a dimer (115 kDa) whereas the core-glycosylated enzyme is a mixture of dimers, tetramers, and octamers. This implies that core-glycosylation is necessary for oligomerization to tetramers and octamers. Dimerization is required and sufficient to generate enzymatic activity; further association does not alter the specific activity of core-glycosylated invertase, suggesting that the active sites of invertase are not affected by the association of the dimeric units.

Reconstitution of the glycosylated and nonglycosylated forms of the enzyme after preceding guanidine denaturation depends on protein concentration. The maximum yield (≈80%) is obtained at pH 6–8 and protein concentrations ≤4 μg/mL for the nonglycosylated and ≤40 μg/mL for the glycosylated forms of the enzyme. The lower stability of the internal enzyme is reflected by a narrower pH range of reactivation and enhanced aggregation. As indicated by the sigmoidal reactivation kinetics at low protein concentration both folding and association are rate-determining.

Keywords: association; folding; glycosylation; invertase; quaternary structure; stability; yeast

N-glycosylation is a cotranslational process (Sharon & Lis, 1982). As soon as the nascent polypeptide chain enters the lumen of the endoplasmic reticulum (ER), prefabricated oligosaccharide units are transferred from dolicholphosphate to asparagine residues in Asn-X-Ser/Thr sequons (Marshall, 1972, 1974; Mononen & Karjalainen, 1984). In the yeast Golgi apparatus, a varying number of extended polymannose outer chains are added, yielding high-mannose-type glycosylated proteins that are strongly heterogeneous in carbohydrate content (Kornfeld & Kornfeld, 1985). Many different biological functions have been ascribed to glycosylation, so far without an unambiguous result regarding the structure-function relationship (Tarentino et al., 1974; Trimble & Maley, 1977; Chu et al., 1978; Gibson et al., 1979; Man-

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junath & Sairam, 1983; Nose & Wigzell, 1983; Sharon, 1984; Olden et al., 1985). Physicochemical analyses devised to approach this problem are severely complicated by the heterogeneity of the oligosaccharide chains. In order to alleviate this problem, the carbohydrate moiety should preferably be as uniform as possible.

Yeast invertase has been used as a model for investigating the role of glycosylation in the stability and folding of proteins (Schülke & Schmid, 1988a,b). The enzyme occurs in both a nonglycosylated cytoplasmic form and a secreted form with a high amount of polymannose chains. The comparison of the two is hampered by the common heterogeneity of carbohydrate moieties in glycoproteins and by the presence of at least six different genes that code for invertase in yeast (Mortimer & Hawthorne, 1969; Carlson et al., 1980). To circumvent these problems, we used a mutant (sec18) that possesses only one single gene coding for both forms of the enzyme (SUC2). Furthermore, in this strain, protein transport from the ER to the Golgi is blocked at the nonpermissive temperature (Novick et al., 1980; Schekman, 1982). At the permissive temperature, the mutant produces internal and external invertase as does the wild type. However, at the restrictive temperature the addition of outer chains is blocked, and core-glycosylated invertase with a fairly homogeneous carbohydrate moiety accumulates in the ER (Novick et al., 1980; Esmon et al., 1981). This form is well suited for physicochemical studies; at the same time, it resembles the form of invertase that actually folds in the ER after glycoprotein synthesis.

The three invertase forms have been shown to be identical regarding their chemical properties, apart from (1) their degree of glycosylation and (2) one or two additional amino acid residues at the N-terminal end of the external and core-glycosylated enzymes, which are not found in internal invertase (Taussig & Carlson, 1983; Williams et al., 1985).

In the present study, the three forms of invertase were isolated from the sec18 mutant, and their spectral and hydrodynamic characteristics were compared with respect to the effect of glycosylation on stability, structure, and protein folding. Differences in the quaternary structure were examined by size exclusion chromatography and sedimentation analysis. The results show that glycosylation increases the stability of the protein and inhibits aggregation. It is a prerequisite for the oligomerization of the enzyme beyond the state of the active dimer: core-glycosylation is necessary for tetramer and octamer formation.

Upon refolding (after preceding guanidine denaturation), the glycosylated proteins show a low tendency to aggregate, leading to high yields of reconstitution; the nonglycosylated enzyme can only be recovered at exceedingly low protein concentrations where folding rather than aggregation becomes rate-determining in the overall renaturation reaction.

Results

Long-term stability of the various forms of invertase

SUC2-encoded internal, core-glycosylated, and external invertase were incubated at pH values ranging from 2.5 to 7.0 at 30 °C. After 42 h, enzyme activity was assayed at pH 5.0 under standard conditions. Figure 1 shows that all forms retained full activity when incubated between pH 4.5 and 6.0. The decrease in stability at low pH values was inversely related to the extent of glycosylation. Between pH 6 and 7, the three invertases lost activity, but did not exhibit pronounced differences.

pH of maximum stability

In order to determine the pH of maximum stability, experiments similar to those depicted in Figure 1 were performed at elevated temperature. The different forms of invertase were incubated at pH 2.5–7.0 for 90 min at 55 °C, and then the residual activity was determined at pH 5.0 and 30 °C under standard conditions. Figure 2 shows a common maximum of stability at pH ≈5.0 for all three forms. Both SUC2-encoded glycosylated enzymes retained full activity at pH 5.0, whereas the residual activity of internal invertase decreased to 67%.

Thermal stability

Thermal inactivation of invertase is an irreversible process. Therefore, equilibrium transitions and thermodynamic data cannot be obtained. However, both the kinetics of thermally induced inactivation and the transitions of thermally induced inactivation and unfolding

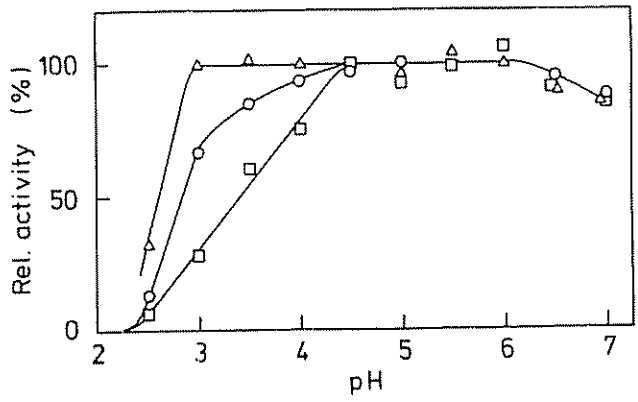


Fig. 1. Effect of pH on the long-term stability of internal (□), core-glycosylated (○), and external (△) invertase at 30 °C after 42 h. Samples of 10 μg/mL of invertase were incubated in 0.05 M citrate/0.05 M phosphate buffer plus 0.4 mg/mL NaN_3 at pH values ranging from 2.5 to 7.0. Activity was determined at pH 5.0 under standard conditions after 20-fold dilution

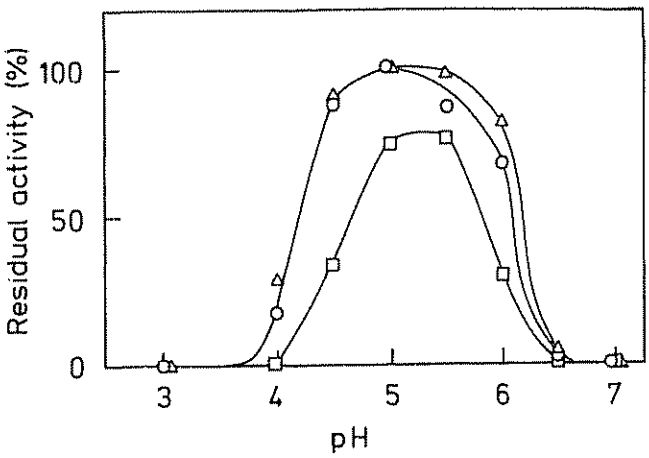


Fig. 2. Effect of pH on the stability against thermal inactivation of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase. Aliquots of 5 μg/mL invertase were incubated in 0.05 M citrate/0.05 M phosphate buffer at pH values ranging from 3.0 to 7.0 at 55 °C for 90 min. Fifty microliter samples were withdrawn and, after 20-fold dilution, the residual activity was determined at 30 °C under standard conditions.

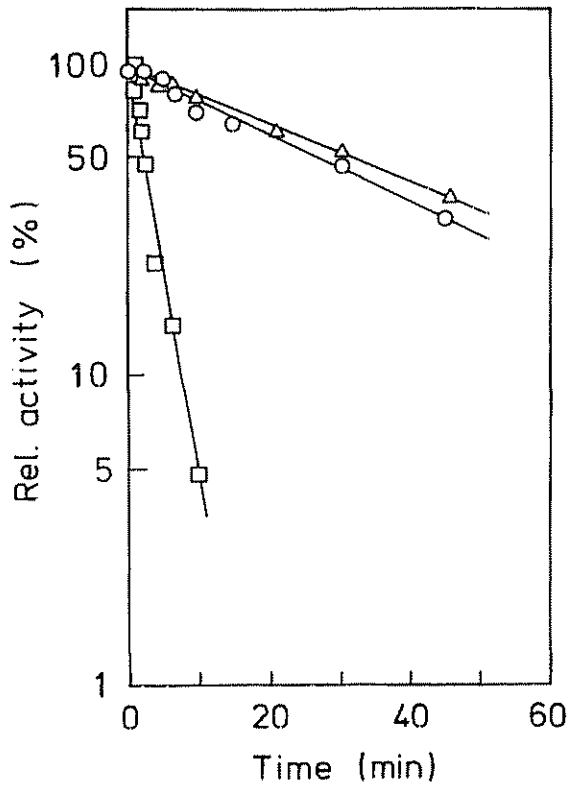


Fig. 3. Kinetics of thermal inactivation of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase at 65 °C and pH 5.0. Inactivation was started by adding 10 μL of invertase solution (1 mg/mL) to 990 μL 0.05 M sodium acetate, pH 5.0 (65 °C). At times t, residual activity was measured under standard conditions (30 °C). Halftimes of the first-order inactivation reaction for the external, core-glycosylated, and external enzyme are 32, 30, and 2.5 min, respectively.

can be measured and used as sensitive probes to compare the stability of related proteins.

Kinetics of thermal inactivation

Nonglycosylated invertase was 50% inactivated within 2.5 min when incubated at 65 °C and pH 5.0 (Fig. 3). Glycosylation provided significant stabilization: the halftimes of inactivation were increased to 30 and 32 min for core-glycosylated and external invertase, respectively.

Heat-induced unfolding and aggregation

Thermal unfolding was followed by a decrease in fluorescence at 325 nm. Solutions of the different invertases were heated from 20 to 75 °C and the thermal unfolding was monitored by the decrease of fluorescence emission at 325 nm. As taken from parallel measurements of fluorescence and light scattering, only the case of internal invertase unfolding was accompanied by aggregation (Fig. 4). The glycosylated forms remained soluble under these conditions. Core-glycosylated invertase showed a weak tendency to form aggregates at higher concentrations ($C > 0.4$ mg/mL) (data not shown), whereas the external form remained soluble. Under the given conditions, the midpoints of transition were 63.5 and 65 °C for core-glycosylated and external invertase, respectively. For the carbohydrate-free internal enzyme, aggregation (at $T \geq 57$ °C) did not allow the transition to be measured.

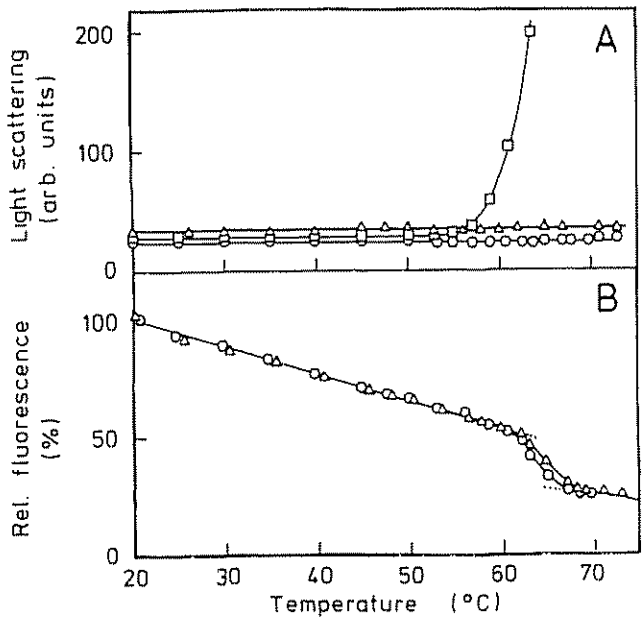


Fig. 4. Thermally induced unfolding of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase; 10 μg/mL of invertase in 0.05 M sodium acetate buffer, pH 5.0, were heated from 20 to 75 °C at a heating rate of 0.25 °C/min using a temperature-controlled cuvette. **A:** Light scattering at 500 nm. **B:** Fluorescence emission at 325 nm ($\lambda_{exc} = 280$ nm).

Denaturant-induced inactivation and unfolding transitions

Guanidinium chloride (GdmCl)-induced inactivation and unfolding

The three different invertase forms were incubated at 20 °C in the presence of 0–6 M GdmCl in 0.05 M sodium acetate buffer, pH 5.0. After 24 h incubation, the residual invertase activity was assayed, and the fluorescence emission was monitored at 325 nm. Inactivation and unfolding occurred at identical GdmCl concentrations (Fig. 5). The midpoints of transition of external and core-glycosylated invertase were observed at 2.0 and 1.9 M GdmCl, respectively. Internal invertase showed decreased stability with a transition midpoint at 1.6 M GdmCl, in agreement with data obtained by Schülke and Schmid (1988a). The small differences are caused by the lower temperature (10 °C) used by these authors.

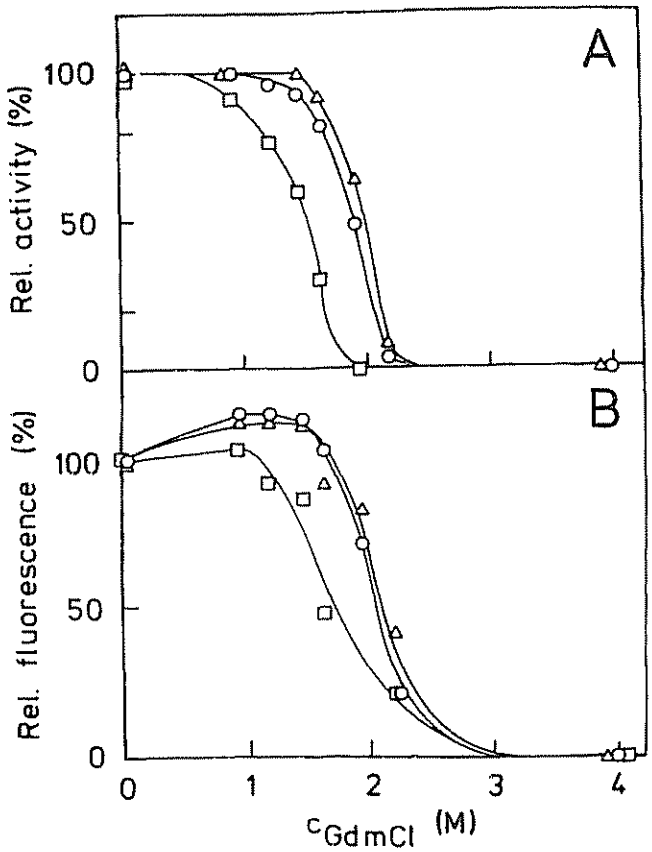


Fig. 5. GdmCl-induced inactivation (A) and unfolding (B) of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase. Aliquots of 30 μg/mL of protein were incubated in 0.05 M sodium acetate, pH 5.0, in the presence of 0–4 M GdmCl at 20 °C for 24 h. Activity was monitored under standard conditions; fluorescence emission at 325 nm (λ_{exc} = 280). To correct for long-term instability, a GdmCl-free sample, incubated for the same time, served as a reference. The fluorescence emission of the enzyme in its native and denatured states was taken to be 100 and 0%, respectively

Urea-induced unfolding and inactivation

The three different invertase forms (5 μg/mL each) were incubated at 20 °C in urea solutions ranging from 0 to 9 M in 0.05 M sodium acetate, pH 5.0. After 40 h, residual invertase activity was assayed and fluorescence emission was measured at 325 nm. Again, the glycosylated forms of invertase were found to be more stable than the carbohydrate-free protein. Inactivation and unfolding coincided (Fig. 6); the midpoints of transition were 5.25, 7.0, and 7.2 M for internal, core-glycosylated, and external invertase, respectively.

Kinetics of GdmCl- and urea-induced unfolding

The increased stability of the glycosylated forms of invertase (cf. Figs. 5, 6) is also reflected in decreased rates of unfolding in the presence of 4 M GdmCl or 9 M urea. In both cases, the glycosylated forms unfold slower than the nonglycosylated enzyme (Fig. 7). Unfolding is a complex reaction; its time course cannot be linearized according to a single first-order reaction.

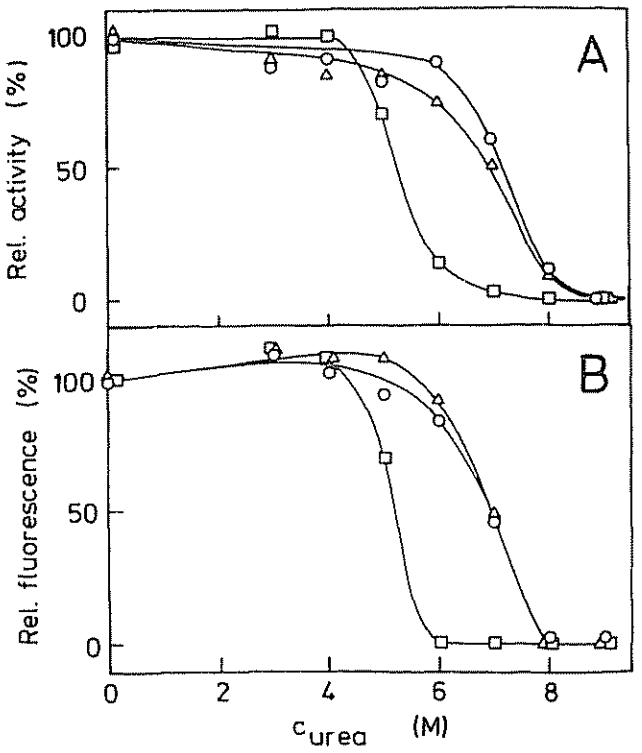


Fig. 6. Urea-induced inactivation (A) and unfolding (B) of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase. Aliquots of 5 μg/mL of protein were incubated in 0.05 M sodium acetate buffer, pH 5.0, in 0–9 M urea at 20 °C for 40 h. Activity was monitored under standard conditions; fluorescence emission at 325 nm (λ_{exc} = 280 nm). To correct for long-term instability, a urea-free sample, incubated for the same time, served as a reference. Activity was corrected for the inhibitory effect of the residual urea concentration after 50-fold dilution in the test. The fluorescence emission of the enzyme in its native and denatured states was taken to be 100 and 0%, respectively.

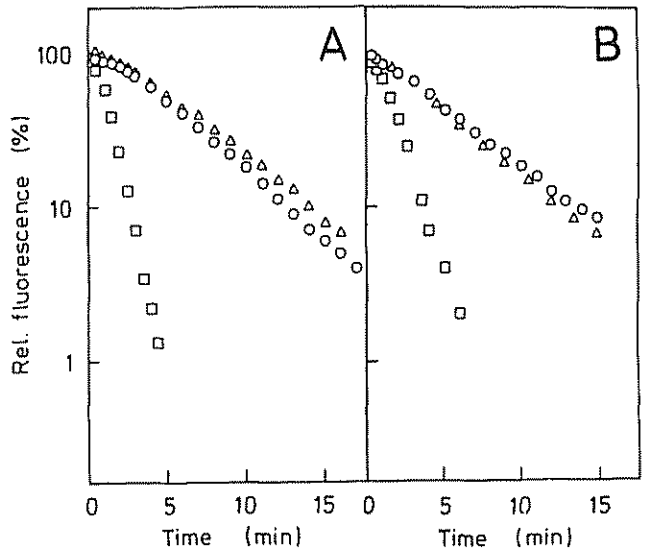


Fig. 7. Kinetics of GdmCl- (A) and urea-induced (B) unfolding of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase. Aliquots of 10 μL of invertase (1 mg/mL) were added at a final volume of 1 mL of 4 M GdmCl in 0.05 M sodium acetate buffer, pH 4.8, at 20 °C or 9 M urea in 0.05 M sodium acetate buffer, pH 5.0 at 40 °C. Unfolding was followed by a decrease in fluorescence emission at 325 nm (λ_{exc} = 280 nm).

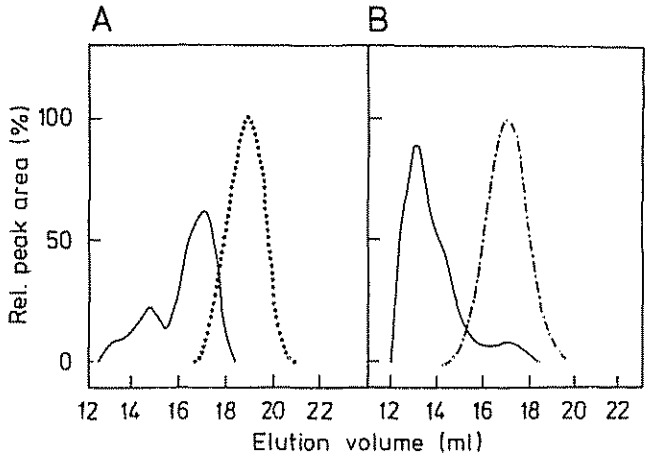


Fig. 8. Quaternary structure of internal and core-glycosylated invertase as determined by gel-permeation chromatography (TSK-3000). Aliquots of 20 μL of a 20-μg/mL protein solution in 0.05 M sodium acetate buffer, pH 5.0, 20 °C. Flow-rate was 0.5 mL/min, pressure was 6 bar, and running buffer was 0.2 M Na_2SO_4 in 0.05 M sodium acetate buffer, pH 5.0. Fluorescence was detected at 330 nm (λ_{exc} = 280 nm) A: Internal (· · ·) and core-glycosylated (—) invertase after preincubation in 0.05 M sodium acetate buffer, pH 5.0. B: Core-glycosylated invertase in 4 M urea (— · — · —) and 1 M GdmCl (—) in the same buffer. Incubation time was 24 h.

Quaternary structure

High performance liquid chromatographic (HPLC) gel-permeation chromatography

The quaternary structure of internal and core-glycosylated invertase was investigated by HPLC gel-permeation chromatography.[1] After preincubation in 0.05 M sodium acetate, pH 5.0, internal invertase eluted as a single peak with an apparent molecular mass of 135 kDa, corresponding to the native dimer. The core-glycosylated enzyme showed three distinct peaks. This heterogeneity suggests that the basic dimeric units may associate to form native higher oligomers (Fig. 8A). The same samples were applied to a nondenaturing polyacrylamide gel (8–25%). After staining for activity (Gabriel & Wang, 1969), only one band was observed for the internal enzyme whereas core-glycosylated invertase showed three bands, in accordance with the HPLC results.

Nondenaturing concentrations of urea and GdmCl had distinctly different effects on the quaternary structure of core-glycosylated invertase. As shown in Figure 8B, the dimeric form dominates in the presence of 4 M urea. In contrast, incubation in 1 M GdmCl favored the form with the highest molecular mass, presumably the octamer. Internal invertase showed no tendency to form higher oligomers after preincubation in 1 M GdmCl or 1 M NaCl (data not shown).

Ultracentrifugation

Sedimentation analysis was applied in order to obtain independent information regarding the native quaternary structure of internal and core-glycosylated invertase. Sedimentation velocity and sedimentation equilibrium runs were performed at 20 μg/mL protein concentration in 4 M urea and 1 M GdmCl (0.05 M sodium acetate, pH 5.0), after preincubation for 12 h under the same conditions. The sedimentation coefficients, $s_{20,w}$, in 4 M urea and 1 M GdmCl were found to be 7.1S and 17.1S, respectively; the molecular masses resulting from the corresponding equilibrium experiments were 142 kDa and 542 kDa. This clearly indicates that, in the presence of 4 M urea, core-glycosylated invertase is a dimer; at nondenaturing GdmCl concentrations (≤1 M), however, the equilibrium is shifted to the octamer. The third oligomeric form, detected by HPLC and native polyacrylamide gel electrophoresis (PAGE), was assigned to the tetramer (310 kDa), using the elution volumes of the dimeric (142 kDa) and octameric (542 kDa) forms as calibration standards. Fitting the radial distribution under meniscus depletion conditions with a population of dimers, tetramers, and octamers, this assignment is found to be compatible with the experimental profiles observed at sedimentation equilibrium.

Attempts to determine the molecular mass or the mass distribution of the external enzyme by sedimentation ve-

[1] The external enzyme, due to its excessive heterogeneity and retardation effects, did not provide unequivocal results that could be interpreted in quantitative terms.

locity or sedimentation equilibrium experiments failed due to the uncertainty of the correct value of the partial specific volume of the heterogeneous system.

Influence of the quaternary structure on the specific activity of core-glycosylated invertase

In order to evaluate the effect of association on enzyme activity, 0.2 mg/mL of core-glycosylated invertase was incubated in 0.05 M sodium acetate buffer, pH 5.0, in the absence and presence of 1 M NaCl or 4 M urea. Subsequent measurements of the distribution of oligomers by HPLC gel-permeation chromatography, and the respective specific activities are summarized in Table 1. These data show that the specific activity remains constant, independent of the quaternary structure of the core-glycosylated enzyme. In addition, this result allows the conclusion that the formation of oligomers higher than the dimer in the presence of 1 M GdmCl may be ascribed to the increase in salt concentration rather than to the chaotropic properties of guanidine.

The fact that the specific activity for the unglycosylated, internal enzyme confirms the result obtained for the various oligomeric forms stresses the fact that the different forms of invertase must be structurally closely similar if not identical.

Unfolding kinetics of core-glycosylated invertase preincubated at moderate denaturant concentration

The previous results show that core-glycosylated invertase is dimeric in 4 M urea but favorably octameric in 1 M GdmCl. This difference in quaternary structure is clearly reflected by the kinetics of unfolding in 4 M GdmCl: The dimeric form undergoes inactivation and unfolding in a single first-order reaction, whereas unfolding of the octamer appears to be a sequential process. An initial lag phase (probably originating from the dissociation of the octamer to dimers) is followed by an unfolding reaction that resembles the unfolding of the dimer (Fig. 9). This interpretation of the unfolding data obtained after preincubation in 1 M GdmCl is supported by the fact that the time constant of the late unfolding reaction ($k = 0.19\ min^{-1}$) is very close to the time constant of the unfolding reaction of the dimers obtained after preincubation in 4 M urea ($k = 0.24\ min^{-1}$). The difference in the inactivation and unfolding rates indicates that subunit dissociation or small changes in tertiary structure, accompanying the initial phase of denaturation, result in complete loss of enzymatic activity.

Table 1. *Quaternary structure and specific activity of core-glycosylated invertase after 24 h incubation at 20 °C*

Solvent condition	State of association			Specific activity[a] (U/mg)
	Octamer (%)	Tetramer (%)	Dimer (%)	
0.05 M sodium acetate, pH 5.0	23	34	43	3,900
4 M urea in 0.05 M sodium acetate, pH 5.0	–	–	100	3,840
1 M GdmCl in 0.05 M sodium acetate, pH 5.0	60	35	5	4,000
1 M NaCl in 0.05 M sodium acetate, pH 5.0	58	32	10	4,092

[a] In all activity assays the residual concentration of urea was kept constant (0.08 M). As taken from HPLC, the state of oligomerization did not change during the assay. The corresponding value of the specific activity observed for the unglycosylated, internal enzyme is 4,100 U/mg

Reactivation

The reconstitution of proteins after preceding denaturation is known to depend on a wide variety of solvent pa-

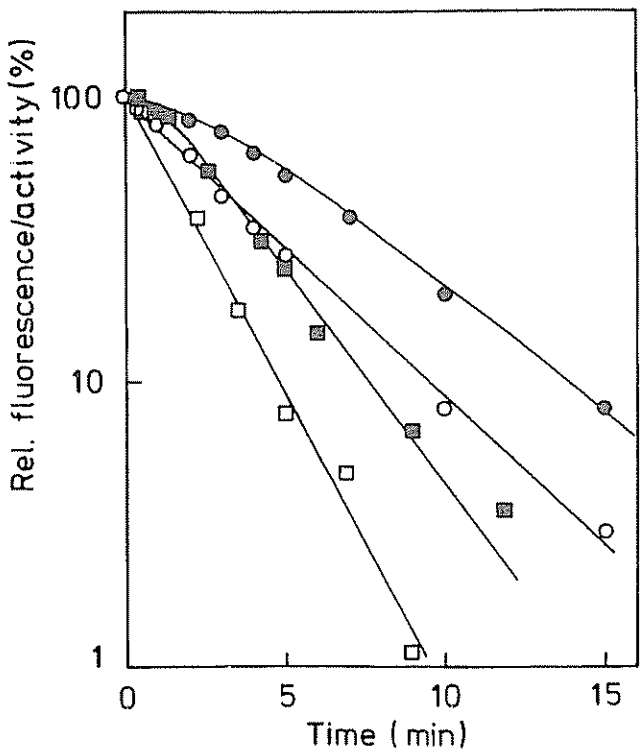


Fig. 9. Unfolding (○, ●) and inactivation (□, ■) kinetics of core-glycosylated invertase in 4 M GdmCl at 20 °C after 24 h preincubation in 4 M urea (○, □) or 1 M GdmCl (●, ■) in 0.05 M sodium acetate buffer, pH 5.0; protein concentration during preincubation was 0.5 mg/mL. Unfolding and inactivation at a final concentration of 10 μg/mL was initiated by a 1:50 dilution in 4 M GdmCl. In order to maintain constant solvent conditions, in the case of preincubation in GdmCl, 0.08 M urea (1:50 dilution of 4 M) was added to the denaturation buffer. Unfolding was monitored by fluorescence emission at 325 nm ($\lambda_{exc} = 280$ nm); inactivation was assayed under standard conditions.

rameters (Jaenicke & Rudolph, 1989). In the present context, aggregation as the most significant difference between the glycosylated and nonglycosylated forms of invertase suggests the effects of protein concentration and pH on the yield of renaturation to be of major importance.

Dependence on protein concentration

The renaturation of oligomeric proteins requires both proper folding of subunits and "docking" of structured monomers. Because association of (inactive) monomers to form (active) dimers is a bimolecular process, reactivation at sufficiently low protein concentration has often been shown to obey second-order kinetics. At high protein levels, first-order folding may become rate-determining. As a side reaction, aggregation (caused by wrong interactions of incompletely folded monomers) may compete with proper reconstitution (Jaenicke, 1987). In the case of invertase, at 0.5–3.0 μg/mL all three forms of the enzyme show an increase in the yield of reactivation with increasing concentration (Fig. 10A). At higher concentrations, an exponential increase in light scattering reflects the formation of inactive aggregates for the nonglycosylated enzyme (Fig. 10B), whereas the glycosylated forms do not form aggregates at concentrations as high as 40 μg/mL, yielding up to 80% reconstitution.

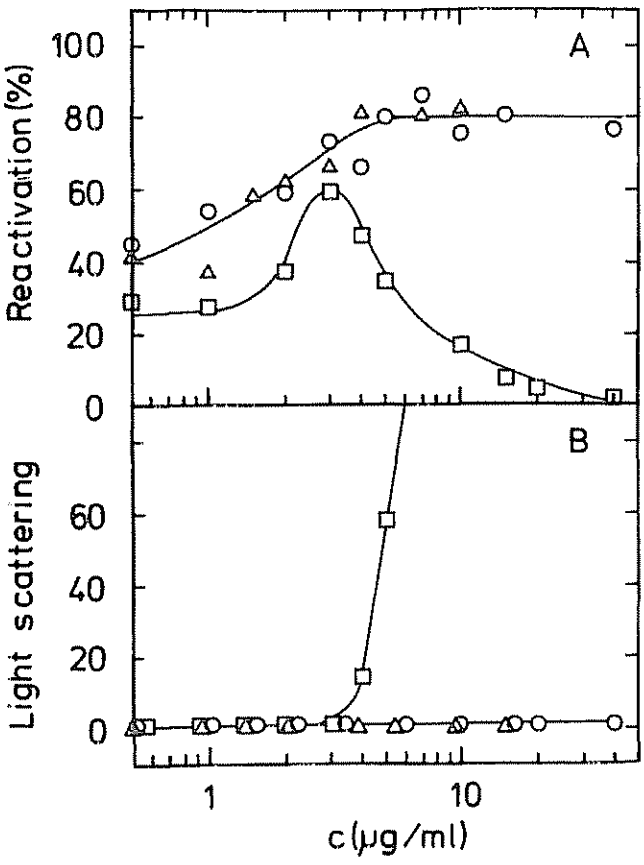


Fig. 10. Influence of protein concentration on the reactivation yield and aggregation of internal (□), core-glycosylated (○), and external (△) invertase. **A:** Reactivation after 1 h denaturation in 0.05 M sodium phosphate/citrate buffer, pH 7.0, in the presence of 6 M GdmCl at 20 °C. Protein concentration was 1.2 mg/mL for the external, and 3.2 mg/mL for the internal and core-glycosylated enzymes. Prior to refolding, protein concentration was adjusted by adding aliquots of 6 M GdmCl. This way, various concentrations of invertase during renaturation were obtained by a 1:80 dilution with renaturation buffer (0.05 M sodium citrate/phosphate, pH 7.0). The yield of reactivation after 48 h was determined relative to native controls (diluted with renaturation buffer without 6 M GdmCl). Controls contained 0.075 M GdmCl (1:80 dilution of 6 M). **B:** Relative light scattering at 320 nm. Scattering values obtained for the native controls were subtracted from the final values, observed for the renatured protein at the respective concentrations.

Dependence on pH

As shown in Fig. 11, nonglycosylated and glycosylated invertase differ in their pH profiles. Both glycosylated forms show high reactivation yields at pH 5.0–8.2, whereas the nonglycosylated enzyme is not reactivated at pH <5.5. The difference may be correlated with the shift of the isoelectric points from <3.5 (for the glycosylated forms) to 4.5 (for the nonglycosylated form). In the latter case, isoelectric aggregation of imperfectly folded molecules outruns proper folding, this way competing with correct subunit association to form active dimers. Maximum yields for all three forms are obtained at pH 6.5–8.2.

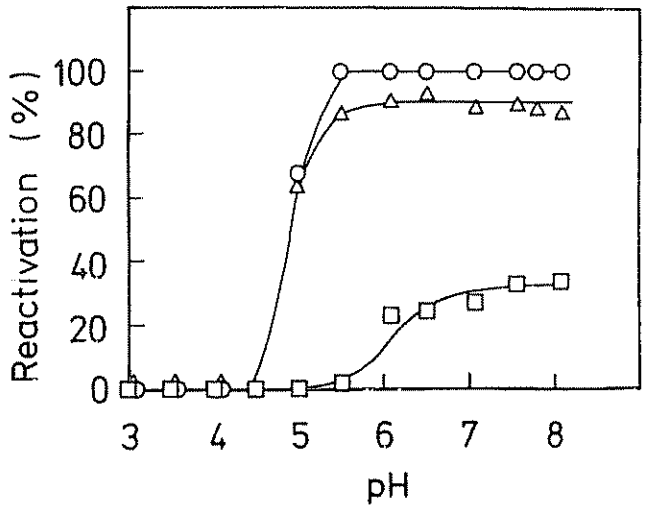


Fig. 11. Effect of pH on the yield of reactivation of internal (□), core-glycosylated (○), and external (△) invertase after 1 h denaturation with 6 M GdmCl in 0.05 M sodium phosphate/citrate buffer, pH 6.5, at 20 °C. Final protein concentration was 5 μg/mL. Reactivation was initiated by a 1:80 dilution of denatured protein with renaturation buffer, adjusted to the indicated pH values. The reactivation yield after 48 h was determined relative to native controls diluted with renaturation buffer without 6 M GdmCl. Controls contained 0.075 M GdmCl (1:80 dilution of 6 M).

Kinetics of reactivation

The kinetics of reactivation were determined in a wide range of protein concentration: 0.5–4.0 μg/mL for the nonglycosylated, and 0.5–10 μg/mL for the glycosylated forms of the enzyme. Figure 12 shows that in all three cases sigmoidal kinetics are observed; the rate of reactivation is enhanced with increasing protein concentration. Both sigmoidicity and concentration dependence are in agreement with a uni-bimolecular mechanism, in accordance with consecutive folding and association of the enzyme (Jaenicke, 1987). Below the concentration range where nonglycosylated invertase shows aggregation, the reactivation kinetics of the glycosylated and nonglycosylated forms of the enzyme do not differ significantly. Obviously, glycosylation does not affect the pathway and kinetics of folding; rather, it blocks the off-pathway reaction that competes with proper folding and association by solubilizing aggregation sites involved in wrong subunit contacts.

Discussion

Data presented in this study show that core-glycosylation as well as high mannose glycosylation stabilize yeast invertase against thermal and denaturant-induced inactivation and unfolding. The results show that core-glycosylation, as it occurs during protein biosynthesis and translocation in the ER, is sufficient for maximum stabilization of invertase. Apparently, this carbohydrate moiety contributes to the overall stability of folded invertase by noncovalent interactions. Apart from its stabilizing effect, core-glycosylation strongly suppresses aggregation of partially or completely unfolded invertase. During biosynthesis, rapid N-glycosylation in the ER could help to avoid nonspecific aggregation of the folding polypeptide chains. The tendency to form aggregates decreases with the amount of glycosylation. As reported earlier, the hydrophilic carbohydrate units increase the solubility of partially unfolded protein (Schülke & Schmid, 1988a). This also explains the increased long-term stability of the glycosylated invertase forms at pH values close to their isoelectric point (pH < 4.5), where the solubility of proteins generally is at a minimum. The pH of maximum stability was identical for all invertase forms.

External invertase (isolated from the sec18 secretion mutant that harbors a single SUC gene only) exhibits exceedingly higher stability compared to the commercially available yeast enzyme, which resembles internal invertase in its stability properties (Schülke & Schmid, 1988a,b).

Association of glycosylated yeast invertase dimers to native higher oligomers was postulated by Neumann and Lampen (1967). Chu et al. (1983), using commercially available external invertase, and Esmon et al. (1987), employing core-glycosylated invertase, corroborated that the carbohydrate moiety allows formation of higher oligomers. We used core-glycosylated invertase to characterize the quaternary structure of glycosylated invertase in the presence of high salt (1 M GdmCl or 1 M NaCl) or nondenaturing concentrations of urea (4 M). In the presence of GdmCl at nondenaturing concentrations, the octameric state is favored. This effect was attributed to the presence of chloride ions by Reddy et al. (1990). In the presence of nondenaturing concentrations of urea, only dimers were present at equilibrium. For glycoproteins with sugar chains of different length and structure, it has been difficult to find calibration standards for HPLC gel-permeation chromatography, or to determine the correct specific volume for ultracentrifugation experiments. Based on ultracentrifugation and HPLC gel-permeation chromatography experiments, we were able to assign the oligomeric forms of core-glycosylated invertase to the dimer, tetramer, and octamer. Evidently, the state of oligomerization has an effect on the kinetics of unfolding. Dissociation of higher oligomers precedes the unfolding of the active dimers in a sequential manner. On the other hand, the comparison of the dimeric core-glycosylated enzyme and its nonglycosylated counterpart clearly shows that the carbohydrate moiety has an intrinsically stabilizing effect on the dimeric protomer. Core-glycosylation enhances protein stability, first, through weak intermolecular interactions of the carbohydrate moiety with the protein surface and, second, through the formation of tetramers and octamers, which show a decreased rate of unfolding compared with the dimer.

During the process of (re-)folding, glycosylation clearly favors proper folding and association by inhibiting aggre-

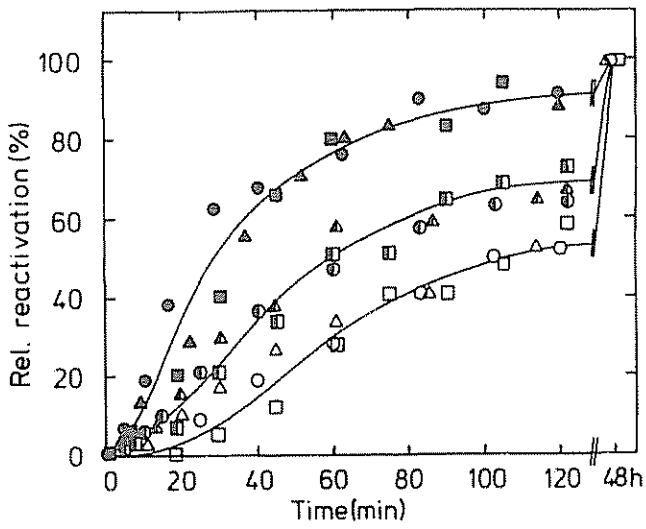


Fig. 12. Effect of protein concentration on the reactivation of internal (□, ■, ▩), core-glycosylated (○, ◐, ●), and external (△, ▲, ▲) invertase. Protein concentrations were 0.5, 2.0, and 4.0 μg/mL. Unfolding was accomplished by 1 h incubation in denaturation buffer, pH 7.0, at 20 °C. Reactivation was started by a 1:80 dilution in renaturation buffer. Activity assayed under standard conditions was compared to native controls kept under identical conditions, omitting the unfolding step; yields of reactivation after 48 h were taken as 100%.

gation of incompletely or incorrectly folded protein, even at high protein concentration. The fact that core-glycosylation is sufficient to serve this purpose may be important in connection with the in vivo folding reaction. Because core-glycosylation occurs in the ER, the solubilization of the nascent polypeptide chain may provide a salvage mechanism thereby avoiding misfolding and misassembly at high local protein levels (Fischer & Schmid, 1990; Jaenicke, 1991).

It is still an open question whether all carbohydrate chains are equally important for protecting the polypeptide chain from aggregation and in determining its state of association. Two out of the nine carbohydrate chains of invertase (linked to Asn 45 and Asn 337) cannot be removed by endoglycosidases (Ziegler et al., 1988), possibly because they interact strongly with the polypeptide scaffold. It will be interesting to find out whether glycosylation at these two sites is already sufficient to mediate the observed effects. Experiments to answer this question are under way.

Materials and methods

Materials

External, core-glycosylated, and internal invertase from yeast were purified from *Saccharomyces cerevisiae*, strain SEY 5188, which carries the SUC2 gene on a plasmid exclusively. This strain was kindly provided by Dr. L. Lehle (University of Regensburg). Yeast nitrogen base (without amino acids) was from Difco and uracil from Sigma. Q-Sepharose fast flow, chelating-Sepharose fast flow, phenyl-Sepharose CL-4B, and the MonoQ HR5/5 column were purchased from Pharmacia LKB Biotechnology Inc.; urea and GdmCl were ultrapure reagents from Schwarz/Mann (Orangeburg, New York). The glucose oxidase (GOD)-assay kit for the determination of glucose was from Boehringer Mannheim. All other chemicals were reagent grade from Merck (Darmstadt). Centricon microconcentrators were from Sartorius (Göttingen) and microtiter plates from Merck (Darmstadt). Quartz-bidistilled water was used throughout.

Methods

Isolation of internal invertase from Saccharomyces cerevisiae

The yeast strain SEY 5188/pRT 51.83 was grown on a minimal medium (6.7 g/L yeast nitrogen base without amino acids, 3% glucose, and 20 mg/L uracil) at 23 °C. At $OD_{600} = 5$, cells were incubated with 10 mM NaN_3 for 10 min and harvested by centrifugation (10 min at 680 × *g*). Cells were washed once with water and resuspended in 0.05 M sodium acetate, pH 5.0, and 40 μM phenylmethylsulfonylfluoride. All subsequent steps were carried out at 4 °C. Cells were broken in a French press at 900–1,000 psi in 3 cycles. The lysate was centrifuged (100 min at 20,000 × *g*) and the supernatant brought to 70% ammonium sulfate saturation. After incubation for 12 h, the precipitate was centrifuged (120 min at 20,000 × *g*), redissolved in 0.05 M sodium acetate, pH 5.0, and dialyzed against the same buffer. Subsequently, protein was loaded on a Q-Sepharose fast flow column (1 × 5 cm) and eluted with a linear gradient from 0 to 0.5 M NaCl in 70 mL of 0.05 M sodium acetate, pH 5.0. Internal invertase eluted between 0.15 and 0.3 M NaCl. Active fractions were pooled, dialyzed against 0.02 M sodium phosphate, pH 7.0, and applied to a Mono-Q HR5/5 column (0.5 × 5 cm), which was subsequently developed with 25 mL of a linear gradient of 0–0.5 M NaCl in 0.02 M sodium phosphate, pH 7.0. Internal invertase eluted as a sharp peak at 0.35 M NaCl. For desalting, the enzyme was subjected to ultrafiltration through a Centricon-30 microconcentrator tube at 5,000 × *g*, and the buffer exchanged to 0.05 M sodium acetate, pH 5.0. The purified enzyme was stored at −20 °C.

Purification of core-glycosylated and external invertase

For the production of the two glycosylated invertase forms, cells were grown and kept at 23 °C throughout as described before. At $OD_{600} = 5$, cells were harvested by centrifugation (10 min at 680 × *g*), washed with minimal medium without glucose, and then incubated in minimal medium containing 0.1% glucose for 2 h. Growth was stopped by adding 10 mM NaN_3. After 10 min incubation, cells were broken as described before and subsequently incubated for 2 h with DNase and RNase (10 μg/mL each) at 4 °C. The membranes were solubilized by 0.1% Triton-X-100 and the crude extract was dialyzed against 0.05 M sodium acetate, pH 5.0.

To obtain core-glycosylated invertase, cells were transferred to minimal medium with 0.1% glucose (as described before) and simultaneously the temperature was shifted to 37 °C. This treatment induces the sec18 mutation, which blocks transport from the ER to the Golgi. As a consequence, core-glycosylated invertase accumulates in the ER. Cells were broken and lysed as described before. After adding 0.1% Triton-X-100, the lysate was brought to 100% ammonium sulfate saturation and incubated for 12 h. After centrifugation (100 min at 20,000 × *g*), the precipitate was treated as described for internal invertase.

External and core-glycosylated invertase were purified by identical chromatographic procedures. The dialyzed crude extracts were loaded on a Q-Sepharose fast flow column (1 × 6 cm) equilibrated with 0.05 M sodium acetate, pH 5.0. The column was developed with 70 mL of a linear gradient from 0 to 0.5 M NaCl in 0.05 M sodium acetate, pH 5.0. The different glycosylated forms of invertase were identified by native PAGE after staining for activity (Grabriel & Wang, 1969). External invertase eluted

from 0.07 to 0.18 M NaCl; the core-glycosylated enzyme eluted from 0.1 to 0.25 M NaCl. Fractions with invertase activity were pooled and dialyzed against 0.02 M sodium phosphate buffer plus 1 M NaCl, pH 7.0. These fractions were further purified by metal affinity chromatography. A chelating-Sepharose fast flow column (1 × 16 cm) was loaded with 2 mg/mL aqueous $CuSO_4$ until 90% of the column was colored blue. The column was washed with 40 mL H_2O and subsequently equilibrated with 0.02 M sodium phosphate plus 1 M NaCl, pH 7.0. The pooled and dialyzed fractions were loaded, and the column was washed with buffer. External and core-glycosylated invertase eluted with the buffer whereas residual internal invertase and the majority of other proteins were retained. Fractions with invertase activity were pooled and dialyzed against 0.05 M sodium acetate, pH 5.0. Both glycosylated forms of invertase were applied to a fast performance liquid chromatography (FPLC) MonoQ HR 5/5 column and eluted with 20 mL of a linear gradient of 0–0.5 M NaCl in 0.05 M sodium acetate, pH 5.0. Pure external and core-glycosylated invertases were obtained after this step. The glycosylated invertases were desalted and concentrated in a microconcentrator. All three invertase forms had specific activities higher than 3,800 units/mg and were stored at −20 °C. Determinations of the carbohydrate content (Dubois et al., 1956) yielded 34% (w/w) for core-glycosylated invertase, and 62% (w/w) for the external enzyme.

Activity measurements

A modified version of the procedure of Goldstein and Lampen (1975) was used. Twenty-microliter samples were added to 980 μL of a 3.57% sucrose solution in 0.05 M sodium acetate, pH 5.0, to obtain standard conditions of 3.5% sucrose and 0.2–0.5 μg/mL of invertase at 30 °C in the assay. After 0.5–1 min incubation, invertase activity was inhibited by addition of 125 μL 1 M Tris/HCl, pH 8.8, and subsequent heating to 100 °C for 2 min. The amount of released glucose was determined by transferring 20 μL of the assay solution to a Nunc microtiter plate, and adding 380 μL of 0.1 M potassium phosphate 0.1 M Tris/HCl, pH 7.0 (containing 5 U/mL glucose oxidase, 0.4 U/mL peroxidase, and 0.5 mg/mL 2,2 azinodi-[3-ethylbenzthiazoline]-6 sulfonate) at 37 °C. After incubation for 15 min, absorbance at 405 nm was determined. The absorbance produced by 0.01 μM glucose in the same test was used for quantitation. One unit of invertase activity is defined as 1 μmol of sucrose hydrolyzed in 1 min at 30 °C. Because GdmCl and urea decrease the activity of invertase (Myrbäck, 1965; Schülke, 1988), all activity assays which served for determination of GdmCl- or urea-induced inactivation transitions were adjusted to an identical final concentration of 0.12 M GdmCl (resulting from the 50-fold dilution of 6 M GdmCl into the assay) or 0.18 M urea (resulting from the 50-fold dilution of 9 M urea into the assay).

Spectroscopic measurements

Cary 118 C, Perkin Elmer Lambda 5 UV-vis absorption, and Hitachi Perkin Elmer MPF 44A fluorescence spectrophotometers were used for spectroscopic measurements. The GdmCl- and heat-induced unfolding transitions were monitored by fluorescence emission at 325 nm (λ_{exc} = 280 nm, band width 5 nm). For heat-induced unfolding, a temperature-controlled cuvette holder was applied and the heating was followed in a reference cuvette, placed in the same holder. The heating rate was 0.25 °C.

Determination of denaturant-induced inactivation and unfolding transitions

GdmCl-induced inactivation and unfolding. Invertase solutions (30 μg/mL) were incubated at 20 °C in 0.05 M sodium acetate, pH 5.0, containing 0–6 M GdmCl. After 24 h, residual invertase activity was assayed as described before. Relative activities were calculated by normalizing to a sample incubated under the same conditions but in the absence of GdmCl. Unfolding was monitored by the decrease in fluorescence at 325 nm (λ_{exc} = 280 nm).

Urea-induced inactivation and unfolding. Invertase solutions (5 μg/mL) were incubated at 20 °C in urea solutions ranging from 0 to 9 M in 0.05 M sodium acetate, pH 5.0. After 40 h, remaining invertase activity was determined relative to a sample incubated under the same conditions but in the absence of urea. Unfolding was monitored by the decrease in fluorescence emission at 325 nm (λ_{exc} = 280 nm).

Kinetic measurements. GdmCl- and urea-induced unfolding was accomplished by diluting the native protein to a final concentration of 4 M GdmCl (20 °C) or 9.5 M urea (40 °C) in 0.05 M sodium acetate, pH 5.0. Unfolding was monitored by the decrease in fluorescence emission at 325 nm (λ_{exc} = 280 nm). The final protein concentration was kept constant at 5 μg/mL.

Thermal inactivation was initiated by adding 20 μL of native protein (0.5 mg/mL) to 980 μL of 0.05 M sodium acetate, pH 5.0, which was kept at 65 °C. Twenty-microliter samples were withdrawn after varying time intervals, and residual invertase activity was determined at 30 °C as described.

Heat-induced unfolding and aggregation

The thermal unfolding transition in 0.05 M sodium acetate, pH 5.0, was followed by the decrease in fluorescence emission at 325 nm (λ_{exc} = 280 nm). Solutions of 10 μg/mL of invertase were heated at a constant rate (0.25 °C/min) from 20 °C to 75 °C, and the fluorescence emission at 325 nm of the sample was measured. Aggregation was monitored by light scattering at 500 nm.

HPLC

Invertase oligomers were separated by size exclusion chromatography at 20 °C on a TSK-3000 (7.5 × 300 mm) or TSK-4000 column (7.5 × 600 mm), using an LKB HPLC system equipped with a Spectra Physics SP4290 integrator. The flow rate for both columns was 0.5 mL/min and the pressure 6 bar (TSK-3000) and 13 bar (TSK-4000), respectively. The running buffer was 0.2 M Na_2SO_4 in 0.05 M sodium acetate, pH 5.0. Eluting protein was detected by fluorescence emission at 330 nm ($\lambda_{exc} = 280$ nm) with a Merck/Hitachi F1000 fluorescence spectrophotometer. The quaternary structure of core-glycosylated invertase was studied by incubating 0.2-mg/mL solutions in 0.05 M sodium acetate buffer, pH 5.0, and 1 M NaCl or 4 M urea in the same buffer for 24 h at 20 °C, respectively.

Ultracentrifugation

Sedimentation velocity and sedimentation equilibrium measurements were performed in a Beckman Spinco Model E analytical ultracentrifuge. Sedimentation velocity experiments were run at 24,000 rpm. Sedimentation coefficients were corrected to 20 °C and water viscosity. Equilibrium runs were performed at 6,000 rpm, applying the meniscus depletion technique (Yphantis, 1964) at a scanning wavelength of 230 nm. For internal and core-glycosylated invertase, partial specific volumes of 0.735 and 0.692 cm^3/g were used (Durchschlag, 1986). The increase in viscosity at 4 M urea or 1 M GdmCl was corrected by using the factors tabulated by Nozaki and Tanford (1971). Protein concentration was 66 μg/mL. The state of association for core-glycosylated invertase was determined after preincubation in 1 M GdmCl or 4 M urea for 12 h.

Kinetics of unfolding and inactivation of core-glycosylated invertase preincubated at moderate denaturant concentration

Core-glycosylated invertase (1 mg/mL) was incubated in 4 M urea or 1 M GdmCl in 0.05 M sodium acetate, pH 5.0. After 12 h, 10-μL samples were added to 990 μL of 4 M GdmCl in 0.05 M sodium acetate, pH 5.0, and unfolding/inactivation at 20 °C were monitored by fluorescence emission at 325 nm or determination of residual activity. The concentrations of urea and GdmCl during renaturation were kept constant in all samples.

Effect of protein concentration on the yield of reactivation

The three forms of invertase were unfolded in denaturation buffer (6 M GdmCl in 0.05 M sodium phosphate/citrate) pH 7.0 at 20 °C. Protein concentrations were 3.2 mg/mL for the internal and core-glycosylated enzymes and 1.2 mg/mL for the external enzyme. Prior to refolding, protein concentrations were adjusted by dilution with denaturation buffer, thus allowing refolding experiments at varying protein concentration by a constant 1:80 dilution with 0.05 M sodium phosphate/citrate buffer (renaturation buffer) pH 7.0 at 20 °C. The yield of reactivation was measured after 48 h; for the native reference, GdmCl was omitted during the elution steps; for the sake of normalization, 0.075 M GdmCl was added.

Effect of pH on the yield of reactivation

The three forms of invertase were incubated for 1 h in denaturation buffer pH 6.5 at 20 °C. Renaturation was started by 1:80 dilution with renaturation buffer adjusted to pH values between 4.0 and 8.5 (20 °C). Final protein concentration was 5 μg/mL. Reactivation after 48 h, measured under standard conditions, was normalized relative to native controls containing 0.075 M GdmCl.

Concentration dependence of the reactivation kinetics

Invertase (unfolded in denaturation buffer pH 7.0 at 20 °C for 1 h) was adjusted to 0.05–1.0 mg/mL in the same buffer. A 1:100 dilution with renaturation buffer pH 7.0 (20 °C) yielded protein concentrations between 0.5 and 10 μg/mL during reconstitution. Reactivation kinetics (under standard conditions) were compared to native controls containing 0.06 M GdmCl.

Acknowledgments

The Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the BAP Program of the European Community provided financial support. R.J. thanks the Fogarty International Center, NIH, Bethesda, for generous support and hospitality. The excellent technical assistance of Heidi Blaschek, Anja Jacobsen, and Nils Kröger is gratefully acknowledged.

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EXHIBIT 5

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1987 by The American Society of Biological Chemists, Inc.

Vol. 262, No. 9, Issue of March 25, pp. 4387-4394, 1987
Printed in U.S.A.

Structure, Assembly, and Secretion of Octameric Invertase*

(Received for publication, November 21, 1986)

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Yeast invertase forms a homo-octamer of core glycosylated subunits during assembly in the lumen of the endoplasmic reticulum. This form has been purified from mutant cells (*sec18*) in which transport of secreted proteins from the endoplasmic reticulum is blocked. No heterologous protein subunits are found in the purified material. Analysis of invertase derived from wild type cells or from mutant cells blocked at subsequent stages in secretion demonstrates that invertase remains a homo-octamer throughout the pathway even though the extent of subunit glycosylation increases. Purified octameric invertase is dissociated into dimer units that reassociate in the presence of polyethylene glycol. Negatively stained preparations show the dissociated enzyme as individual spheres, whereas octameric invertase appears as four associated spheres. Assembly of the octamer *in vitro* and *in vivo* is facilitated by the presence of *N*-linked carbohydrate. Selective release of dimeric glycosylated invertase from intact yeast cells suggests that oligomerization helps retain the enzyme in the periplasmic space.

Secreted invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) resides in the periplasmic space of the yeast *Saccharomyces cerevisiae*, where it functions to cleave extracellular sucrose to fructose and glucose, sugars that yeast can import (1). The molecular structure of secreted invertase has been well characterized; it consists of 60-kDa polypeptide chains to which heterogeneous oligosaccharide units are attached (2). The quaternary associations of this protein have also been studied; secreted invertase, examined under stabilizing conditions, is an octamer (3). Earlier studies employed lysis conditions that caused octamer decay, resulting in appearance of dimeric secreted invertase (2, 4). Invertase dimer is quite stable, requiring protein denaturation before dissociation will occur (2).

A second form of invertase that shares many features with secreted invertase is found in the yeast cytoplasm. Cytoplasmic invertase is produced from the same gene as secreted invertase, but its translation product lacks the leader sequence required for translocation into the endoplasmic reticulum (ER)[1] (5). This unglycosylated form of invertase is expressed constitutively, whereas synthesis of secreted invertase is subject to catabolite repression (6). Although cytoplasmic invertase exists as a dimer and is less stable than its periplasmic counterpart, an octameric species is formed at high protein concentrations (3, 7).

Export of secreted invertase has been characterized using conditionally lethal secretory (*sec*) mutants that block secretion and cause accumulation of active secretory proteins within the cell in distinct secretory organelles (8). Invertase is inserted cotranslationally into the ER, transported to the Golgi apparatus, packaged into secretory vesicles, and finally is discharged into the periplasmic spaces (9, 10). In wild type cells, very little secreted invertase is released from the periplasmic space into the culture supernatant. Since invertase release from the periplasmic space may be attained by cell wall fracture, invertase retention within this compartment is thought to result from entrapment behind the cell wall (1). Glucan and a reducible cell wall component seem to be specifically involved in invertase retention in the periplasmic space because a portion of periplasmic invertase is released following cell wall reduction, proteolysis, or enzymatic glucan degradation. Complete invertase release is achieved by glucan degradation in concert with either cell wall reduction or proteolysis (11–13).

We report here the purification and characterization of glycosylated invertase octamer accumulated within the endoplasmic reticulum of *sec18* mutant cells as well as *in vivo* and *in vitro* studies that address conditions required for octamer formation. Evidence is presented that supports a role for glycosylation and octamer formation in retention of invertase in the periplasmic space.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Materials—*S. cerevisiae sec* mutant strains were derived from X2180-1A (wild type) and were described previously (8). SEY2102 (14) was provided by S. Emr (Division of Biology, California Institute of Technology, Pasadena, CA). LB344-1b (*mnn9*) was provided by Lun Ballou (this Department), 108-3Cα (*MEL1*) was obtained from B. Adams (University of Hawaii, Honolulu), and PEY8-5A (*mnn9. MEL1*) was constructed by standard techniques. The yeast multicopy plasmid pRB58, containing the *SUC2* gene, was obtained from M. Carlson (Department of Human Genetics and Development, Columbia University, New York).

YP medium contained 1% Bacto-yeast extract and 2% Bactopeptone plus the designated carbon source. Wickerham's minimal medium (15) was used with different levels of glucose, as indicated. The absorbance of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm in a Gilford 2451A spectrophotometer. Liquid cultures were grown in flasks or tubes with agitation to an OD_{600} of 1–5.

Reagents were obtained as indicated: o-dianisidine, dithiothreitol, glucose oxidase, invertase, peroxidase, polyethylene glycol (PEG)

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[1] The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate.

6000, QAE-cellulose, saponin, sodium azide, 2,3,5-triphenyltetrazolium chloride, and tunicamycin were from Sigma; Ultrogel AcA 22 was from LKB; and ferritin, phenyl-Sepharose, Sepharose 4B, and thyroglobulin were from Pharmacia P-L Biochemicals. Fraction II lyticase was used for spheroplast formation at a concentration of 20 units of lyticase per OD_{600} unit of cells (13). β-Galactosidase was provided by W. Kimmerly (this department).

sec18 and mnn9 Cell Lysis—HMSF176 (*sec18*) cells were grown overnight in YP plus 2% glucose at 25 °C. Cells (20 OD_{600} units) were harvested and resuspended at the restrictive temperature (37 °C) in YP plus 0.1% glucose for 2 h. These conditions caused invertase to accumulate in the ER. NaN_3 was added to 10 mM, and after 10 min cells were harvested, washed once with 0.1 M Tris-HCl, pH 9.4, 10 mM NaN_3, resuspended in 0.2 ml of the same buffer containing 10 mM dithiothreitol, and incubated 20 min at 30 °C. This procedure facilitated cell wall removal. Treated cells were sedimented, resuspended in 0.4 ml of 1.5 M sorbitol, 50 mM Tris, pH 7.5, 2 mM $MgCl_2$, 10 mM NaN_3, 40 mM β-mercaptoethanol, lyticase (Buffer A) and incubated at 30 °C for 30 min with occasional agitation. Spheroplasts were centrifuged from solution (3,000 × *g*, 5 min) and lysed by suspension in 0.2 ml of 50 mM Tris, pH 7.5. After 10 min on ice, the lysate was cleared by centrifugation at 12,000 × *g* for 10 min. The cleared supernatant was heated at 30 °C; after various lengths of time 20-μl aliquots were taken and placed on ice, and NaCl was added to 0.1 M.

For Ferguson analysis (16), a *sec18* cell lysate was prepared as above, omitting the 30 °C heating step.

Invertase-containing extracts from *mnn9*, *mnn9/sec*, and *sec* mutant cells were prepared in a similar manner, except that cells were derepressed for invertase for 1 h at 37 °C. Cells were then washed in 50 mM Tris, pH 7.5, and were converted to spheroplasts in Buffer A. The spheroplasts were sedimented (3,000 × *g*, 5 min), and the spheroplast pellet was resuspended in 50 mM Tris, pH 7.5, 0.1% Triton X-100.

Purification of Octameric Invertase (I-8)—SF610-4C (*sec18*) cells (30,000 OD_{600} units) transformed with pRB58 (contains *SUC2*) were derepressed for invertase expression in 12 liters of Wickerham's minimal medium containing 0.1% glucose for 2 h at 37 °C. Use of the multicopy plasmid pRB58 caused a 10-fold increase in invertase specific activity detected in the crude lysate. Cells were harvested using a Sharples centrifuge and were converted to spheroplasts as described earlier for *sec18* cells, but with only 5 units of lyticase per OD_{600} unit of cells. After 70 min at 30 °C, spheroplasts were harvested at 5,000 × *g* for 10 min. The spheroplast pellet was resuspended in 30 ml of 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5% saponin, incubated on ice for 5 min, and centrifuged at 12,000 × *g* for 10 min. The lysis supernatant was applied to a 250-ml QAE-cellulose column which had been equilibrated with 10 mM Tris-HCl, pH 7.5, 75 mM KCl. The column was washed with equilibration buffer until the A_{280} of the effluent was less than 0.01. Invertase activity was then eluted with 10 mM Tris-HCl, pH 7.5, 0.16 M KCl. Peak fractions were pooled and concentrated to 6.5 ml using a Millipore concentrator. The concentrate was adjusted to 25% ammonium sulfate, 0.1 M KCl, 0.1 M sodium acetate, pH 5.1, in a final volume of 10 ml and applied to a 2.2 × 50-cm Sepharose 4B column equilibrated with the same buffer. Peak fractions were pooled, adjusted to 40% ammonium sulfate, and applied to a 12-ml phenyl-Sepharose column equilibrated with 40% ammonium sulfate, 0.1 M KCl, 0.1 M sodium acetate, pH 5.1. The column was washed with 3 column volumes of equilibration buffer, and activity was eluted with a 60-ml linear gradient of buffer containing 40–0% ammonium sulfate. The column was finally washed with 1.5 volumes of 0.1 M KCl, 0.1 M sodium acetate, pH 5.1. Peak fractions of invertase activity were not pooled since fractionation of different forms occurred over one continuous peak of activity. The purification obtained at each step of this procedure is outlined in Table I.

Ultracentrifugation—The sedimentation coefficient of I-8 invertase was determined by sedimentation velocity ultracentrifugation using a Beckman Model E ultracentrifuge. Purified I-8 (0.21 mg/ml in 0.1 M KCl, 0.1 M NaAc, pH 5.1) was sedimented at 34,000 rpm at 10 °C for 111 min. Periodically (at least every 8 min) the position of the solute boundary was determined by measuring the absorbance at 280 nm across the cell. The natural log of the boundary position was plotted *versus* time, the slope of this line being the sedimentation coefficient, which was then converted to $s^0_{20,w}$. The specific volume of 0.7 for I-8 was calculated based on the relative contributions of invertase protein and mannan (9, 17, 18). Since I-8 appeared to be relatively spherical, a frictional coefficient of 1.3 was assigned (19). If I-8 is actually asymmetrical, the calculated molecular weight will be an underestimate of the actual molecular weight.

Electron Microscopy—For examination of purified invertase from *sec18*, samples were either diluted to 30 μg/ml in 0.1 M KCl, 0.1 M sodium acetate, pH 5.1, and kept at 30 °C for 12 h (heat-treated) or were diluted just prior to use and kept on ice. Commercially obtained invertase was suspended in the same buffer at a similar protein concentration. Samples were applied to carbon-coated Pelco 8HGC 400 mesh grids using a dual spray negative staining method, filling one micropipette with sample and the other with a saturated solution of uranyl acetate (20). Samples were photographed using a Philips 301 electron microscope.

Heat and PEG Treatment—Purified invertase was diluted to 20 μg/ml in 10 mM sodium acetate, pH 5.1, 10 mM KCl and was dissociated by heating. Dissociated invertase was then reassociated by adding 0.1 μg of invertase to 10–20% PEG 6000 in 40 μl, and samples were incubated 10 min at 25 °C. Unheated and heated samples were diluted to a similar concentration.

For PEG treatment of cytoplasmic invertase, wild type cells (SEY2102, transformed with pRB58) were grown at 30 °C in YP plus 5% fructose. Fructose was used as a carbon source because it represses synthesis of external invertase, but did not interfere with the invertase assay as glucose did. Cells (100 OD_{600} units) were converted to spheroplasts as described for *sec18* cells, spheroplasts were centrifuged from solution (3,000 × *g*, 5 min) and they were resuspended in 0.5 ml of water. After 10 min on ice, the lysate was centrifuged (12,000 × *g*, 10 min), and the supernatant invertase activity was measured. Invertase (0.5 units of cytoplasmic invertase, 0.5 units of purified heat-treated *sec18* invertase, or both) was suspended in 40 μl of 0.1 M KCl, 0.1 M sodium acetate, pH 5.1, plus or minus 20% PEG, and the mixture was incubated for 10 min at 25 °C.

Tunicamycin Treatment—X2180-1A cells were grown in YP containing 5% fructose at 25 °C. The culture was split and one portion was pretreated with 10 μg/ml tunicamycin for 30 min at 25 °C. Cells (30 OD_{600} units) were sedimented, resuspended in YP plus 0.1% fructose, with or without tunicamycin, and incubated at 25 °C for 3 h with shaking. Spheroplasts were prepared by incubating cells in 60 μl of Buffer A plus 0.1 M KCl for 30 min at 25 °C followed by sedimentation at 3000 × *g* for 5 min.

Gel Filtration Chromatography—PEY8-5A (*mnn9. MEL1, PHO3*) cells were grown in YP plus 2% glucose at 30 °C, cells were shifted to growth in YP plus 2% ethanol for 4 h at 30 °C, and they were then shifted to growth in YP containing 1% galactose and 0.05% glucose for 4–6 h at 25 °C. Studies with 108-3C cells showed that when cells were derepressed for α-galactosidase in this manner, only about 10% of the activity was found in the cell medium and the vast majority (80%) was found in the spheroplast supernatant (periplasmic fraction). Since α-galactosidase induction was much slower at 37 °C than at 25 °C in wild type cells, this enzyme was induced at 25 °C. Following induction, cells were washed in 0.1 M KCl, 50 mM Tris, pH 7.5, and were converted to spheroplasts in Buffer A plus 0.1 M KCl. Spheroplasts were sedimented, and the spheroplast supernatant (0.5 ml) was applied to a 1 × 60-cm Ultrogel AcA 22 gel filtration column

TABLE I

Purification of octameric invertase from sec18

Fraction	Volume	Total protein	Total activity	Specific activity	Recovery
	ml	*mg*	*units*	*units/mg*	%
Homogenate	44.0	480	68,600	140	100
Crude extract	27.0	195	45,600	230	66
QAE-cellulose chromatography	6.5	11.0	28,600	2,600	42
Sepharose 4B chromatography	27.0	5.4	22,400	4,200	33
Phenyl-Sepharose chromatography	9.4	2.9	16,100	5,600	23

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that had been equilibrated in 0.1 M KCl, 0.1 M sodium acetate, pH 5.1, 1 mM β-mercaptoethanol, 1 mM NaN_3. The elution profiles of acid phosphatase, α-galactosidase, invertase, and β-galactosidase were detected by activity assay, ferritin was detected by absorbance at 360 nm, and thyroglobulin was detected by absorbance at 280 nm.

Electrophoresis—Nondenaturing PAGE was performed in the cold according to Rodbard and Chrambach (21) using buffer system F. Samples were adjusted to 10% glycerol, and 0.1–0.5 units of invertase were loaded per lane. For invertase staining, gels were incubated in 0.1 M sucrose, 0.1 M sodium acetate, pH 5.1, at 30 °C for 10–60 min with gentle shaking. Gels were washed twice with distilled water and drained. Reducing sugars generated by invertase were localized by suspending gels in 40 ml of 0.5 M NaOH, 1 mg/ml, 1,2,5-triphenyltetrazolium chloride, followed by boiling over an open flame in a fume hood until staining was complete (22). Staining was stopped with 1 liter of water, gels were stored in 10% acetic acid, and they were dried by conventional means.

SDS-PAGE was performed according to the Laemmli technique.

Assays—Invertase (23), α-galactosidase (24), acid phosphatase (25), β-galactosidase (26), and protein (27) were determined by published procedures.

RESULTS

At a restrictive temperature, yeast *sec* mutants are secretion-defective and accumulate invertase in the ER, Golgi apparatus, or secretory vesicles (8, 9). Proteins accumulated at each stage of secretion display characteristic post-translational modifications that correlate with modifications occurring in wild type cells (9, 28). The carbohydrate moiety of invertase accumulated in the ER consists of discrete core oligosaccharide units, whereas that of invertase present in the Golgi apparatus, secretory vesicles, or periplasmic space has a heterogeneous carbohydrate "outer chain" added to most core oligosaccharide units (29, 30). When invertase is resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE) and the gel is stained for invertase activity, outer chain-containing invertase migrates as a disperse band. Under the same conditions, invertase accumulated in the ER separates into four distinct forms: I-8, I-6, I-4, and I-2 (9). When *sec18* cells (which accumulate ER) were derepressed for invertase at the restrictive temperature and lysed, and samples immediately electrophoresed through a nondenaturing gel, the majority of invertase accumulated in the ER was in the I-8 form (Fig. 1). Incubation of the cell lysate at 30 °C prior to electrophoresis caused the I-8 form to decay into the I-6, I-4 and I-2 forms without any loss of invertase activity, indicating that these forms were derived from I-8 *in vitro*.

To assess the nature of the invertase complex accumulated in the ER, the I-8 form was purified from *sec18*. The final invertase specific activity achieved was similar to that previously found by Goldstein and Lampen (23) for wild type invertase purified from the periplasmic space (Table I). SDS-PAGE of the pure complex showed several protein species with molecular masses near 80 kDa (Fig. 2). This profile was identical to that of radiolabeled invertase immunoprecipitated from *sec18* in which the species are known to differ by the number of core oligosaccharide units attached to each polypeptide chain (9). To ensure that the proteins purified from *sec18* all corresponded to invertase, a Western blot was prepared and was probed with anti-invertase antibody. All species that stained with Coomassie Blue also bound anti-invertase antibody (data not shown). We were unable to detect any proteins other than invertase that co-purified with the I-8 complex.

Velocity sedimentation centrifugation was used to estimate the molecular mass of the invertase complex. I-8 had a sedimentation coefficient of 18.5 $s^0_{20,\omega}$, corresponding to 600 kDa or seven to eight 80-kDa invertase subunits. This complex appeared to be similar to the invertase octamer secreted by wild type cells by Chu *et al.* (3).

Besides being found in the ER, I-8 invertase was present throughout the secretory pathway. Detection of I-8 was facilitated by the use of *mnn9*, a mutant strain that secretes glycoproteins with only slightly modified core oligosaccharides (31). *mnn9* cells were derepressed for invertase and converted to spheroplasts, and the material released from the cell wall was subjected to nondenaturing PAGE (Fig. 3). This strain secreted invertase that was very similar to invertase accumulated in *sec18*. Construction of *mnn9/sec18*, *mnn9/sec7* and *mnn9/sec1* double mutants that accumulate core-glycosylated invertase in the ER, Golgi apparatus or secretory vesicles, respectively, showed that invertase existed primarily in the I-8 form throughout the secretory pathway (Fig. 3). The small difference in mobility between the *mnn9* and *sec18* I-8 forms was probably due to the difference in their carbohydrate structure (8 mannose units per oligosaccharide chain for *sec18* (29) and approximately 13 mannose units per oligosaccharide chain for *mnn9* (32)). For comparison, *sec/MNN*

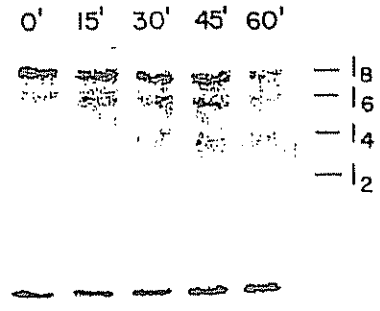


FIG. 1. **Dissociation of I-8 into I-6, I-4, and I-2.** *sec18* cells were derepressed for synthesis of invertase at the restrictive temperature. A cell lysate was prepared and samples were incubated 30 °C for 0, 15, 30, 45, or 60 min. Samples were subjected to nondenaturing PAGE on a 3.8% polyacrylamide gel and the gel was stained for invertase activity. The unidentified band represents cytoplasmic invertase. Invertase activity remained constant during this experiment. If samples were incubated up to 23 h, less than 15% loss of activity

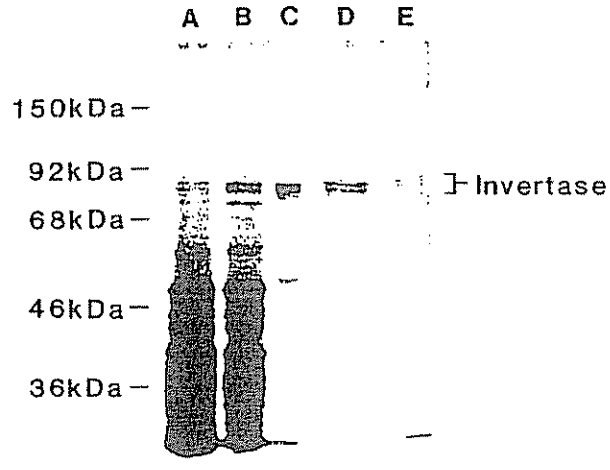


FIG. 2. **SDS-PAGE of fractions from the purification of *sec18* invertase.** Samples from each stage of the purification were diluted into SDS plus β-mercaptoethanol heated at 100 °C, and applied to a 7.5% SDS-polyacrylamide gel; the gel was stained with Coomassie Blue. *A*, homogenate; *B*, crude extract; *C*, pool from QAE-cellulose column; *D*, pool from Sepharose 4B column; *E*, peak fraction from phenyl-Sepharose column. Molecular mass standards: RNA polymerase *a*' (150 kDa), phosphorylase *b* (92 kDa), bovine serum

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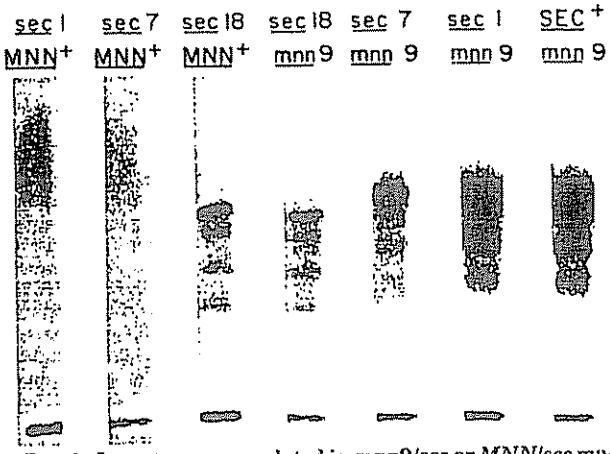


FIG. 3. **Invertase accumulated in *mnn9/sec* or *MNN/sec* mutants and secreted in *mnn9/SEC* mutants.** Cells were derepressed for invertase at the restrictive temperature, spheroplast supernatant and pellet fractions were prepared, and samples were subjected to nondenaturing PAGE through a 3-8% polyacrylamide gel; the gel was stained for invertase activity. For *mnn9/SEC* cells, spheroplast supernatants were analyzed; for all other samples, spheroplast pellets were analyzed. To visualize the *sec7* sample, it was necessary to overstain many of the other samples, so it appears as if octamer decay has occurred. In independent experiments, this was not the case.

mutants were also analyzed. The heterogeneous glycosylation of *MNN* cells caused invertase to migrate as a broad band and obscured detection of I-8 in all but *sec18*, *MNN* strains (Fig. 3).

The relationship among the four invertase species I-8, I-6, I-4, and I-2 was examined by Ferguson analysis. This method allows characterization of size and/or charge heterogeneity within a family of proteins by measuring their relative mobilities in nondenaturing gels of varying polyacrylamide concentrations (16). The log of the mobility of each species is plotted as a function of acrylamide concentration where the intercepts of the straight lines reflect the relative magnitude of the surface charge density possessed by each species, whereas the slopes of the lines are a function of the species' molecular mass. A Ferguson plot of I-8, I-6, I-4, and I-2 is shown in Fig. 4*A*; these species had similar surface charge densities (as reflected by the common intercept of each line), but differed in their molecular masses (as reflected by the slopes of the lines). When the slope of each line was compared to slopes generated by proteins of known molecular mass (10, 21), it was possible to calculate that I-2 corresponded to the invertase dimer (approximately 130 kDa), but it was impossible to accurately determine the molecular mass of I-4, I-6, or I-8 due to the limited range of the molecular mass standards (Fig. 4*B*). Extrapolation of the standard curve suggested that I-8 was approximately 600 kDa and corresponded to the invertase octamer, not a breakdown product of it.

Several different forms of pure invertase were examined by transmission electron microscopy. A nondenaturing gel of the samples chosen for inspection is shown in Fig. 5. These samples include purified I-8 (Fig. 5*A*), a fraction from the final step in I-8 purification which was enriched in I-6 and I-4 (Fig. 5*B*), and a sample similar to that in Fig. 5*B*, except that it was heated to promote formation of I-2 (Fig. 5*C*). Transmission electron micrographs of these samples are shown in Fig. 6. Sample containing mostly I-8 appeared as four associated spheres (Fig. 6*A*), sample containing all forms was composed of two, three, or four spheres associated together (Fig. 6*B*), and single spheres predominated in sample containing mostly I-2 (Fig. 6*C*). There was a clear correlation

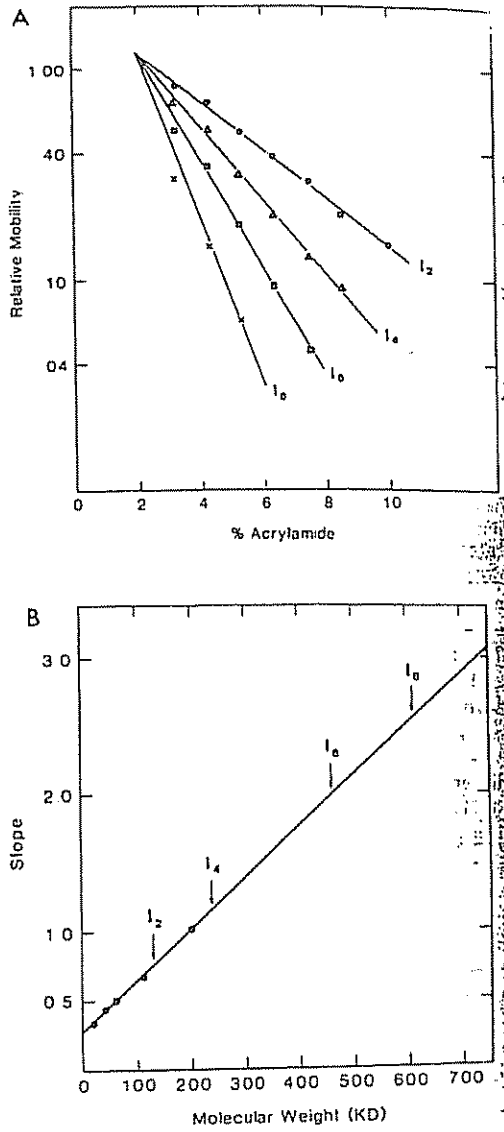


FIG. 4. **Ferguson analysis of I-8, I-6, I-4, and I-2.** *sec18* cells were derepressed for invertase at 37 °C, and a cell lysate was prepared and subjected to nondenaturing PAGE on gels of different polyacrylamide concentrations. In *A*, the gels were stained for invertase activity, and the log of the relative mobility of each invertase species was plotted *versus* the acrylamide concentration. ×, I-8; ■, I-6; ▲, I-4; ●, I-2. For *B*, to generate a molecular mass standard curve, proteins of known molecular mass were analyzed as in *A*. The negative slopes of the lines obtained in this manner were plotted *versus* the molecular mass. The slopes generated by I-2, I-4, I-6, and I-8 are indicated. Molecular mass standards: trypsin inhibitor, 21 kDa; ovalbumin, 43.5 kDa; bovine serum albumin, 67 kDa; cytoplasmic invertase, 112 kDa; bovine serum albumin trimer, 201 kDa.

between forms seen by nondenaturing PAGE and the state of sphere association in the electron microscope: I-8 corresponded to four associated spheres and I-2 corresponded to one sphere. Commercially obtained invertase was also examined in the electron microscope. It appeared as one to four associated spheres (Fig. 6*D*), confirming that these molecular associations did not result from a mutant phenotype.

Certain treatments were found to affect invertase association. Heat (50 °C for 30 min or 30 °C for 12 h) caused conversion of pure I-8 to the smaller forms, eventually forming I-2 (Fig. 7, *A* and *B*); addition of salt (0.1 M) stabilized I-8, making

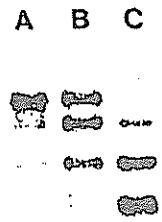


FIG. 5. **Invertase samples chosen for electron microscopy.** ire *sec18* invertase prepared as described under "Experimental ocedures" was subjected to nondenaturing PAGE through a 3.8% ilyacrylamide gel, and the gel was stained for invertase activity. *A*, ire I-8 invertase; *B*, pure invertase enriched in I-6 and I-4; *C*, sample heated at 30 °C for 12 h to form I-2.

possible to manipulate this complex *in vitro* (data not iown). Other agents such as β-mercaptoethanol, *N*-ethyl aleimide, magnesium chloride, calcium chloride, EDTA, otease inhibitors, and non-ionic detergents had no effect on 8 stability. Passage through a French pressure cell or a raun homogenizer accelerated conversion of I-8 to I-2 (data ot shown). Heat-dissociated invertase could be reassociated the I-8 form by addition of 10–20% polyethylene glycol ig. 7, *C* and *D*).

The glycosylation state of invertase affected I-8 formation stability. Cells derepressed for invertase in the presence of inicamycin, a drug which prevents asparagine-linked glycoylation (33), secreted low amounts of unglycosylated invertase dimer, not unglycosylated invertase octamer (Fig. 7, *E* nd *F*). Since this phenomenon required derepression of seeted invertase, it did not result from release of cytoplasmic ivertase during spheroplast formation (data not shown). /hen the invertase concentration in the secretory pathway as increased an order of magnitude by providing the *SUC2* ene on a multicopy plasmid (5), tunicamycin treatment still iused secretion of unglycosylated invertase dimer (data not iown). The effect of glycosylation on invertase association as independently confirmed by adding PEG to cytoplasmic inglycosylated) invertase in an attempt to make it assemble. lthough cytoplasmic invertase did not associate (Fig. 8, *A–*), purified core-glycosylated dimeric invertase, added as an iternal control, associated as usual (Fig. 8, *C–F*). This exeriment confirmed the idea that core oligosaccharide units ther stabilize I-8 or promote invertase association.

Since only secreted invertase forms octamers, this structure iay function during secretion or it may help retain invertase ithin the periplasmic space. Following treatment of *mnn9* ells with buffer (Fig. 9*A*) or β-mercaptoethanol (Fig. 9*B*), ivertase found in the cell supernatant was predominantly in ie I-2 form. When the cells were converted to spheroplasts nd the contents of the cell wall were examined, invertase as present mostly in the I-8 form (Fig. 9*C*). If the total ellular invertase was examined by removal of the cell wall in ie presence of detergent, cytoplasmic invertase was released nd secreted invertase was present mainly in the I-8 form Fig. 9*D*). These results showed that removal of the cell wall as required for I-8 to be released, but I-2 could be released ith the cell wall essentially intact.

If I-8 functions to help retain invertase in the cell wall, ther secreted yeast proteins may also form multimeric comlexes to achieve the same location. Two secreted proteins, -galactosidase (*MEL1* gene) and constitutive acid phosphaase (*PHO3* gene) were examined to see whether they were resent in the periplasmic space as high molecular mass omplexes. For this experiment, *mnn9* cells were derepressed

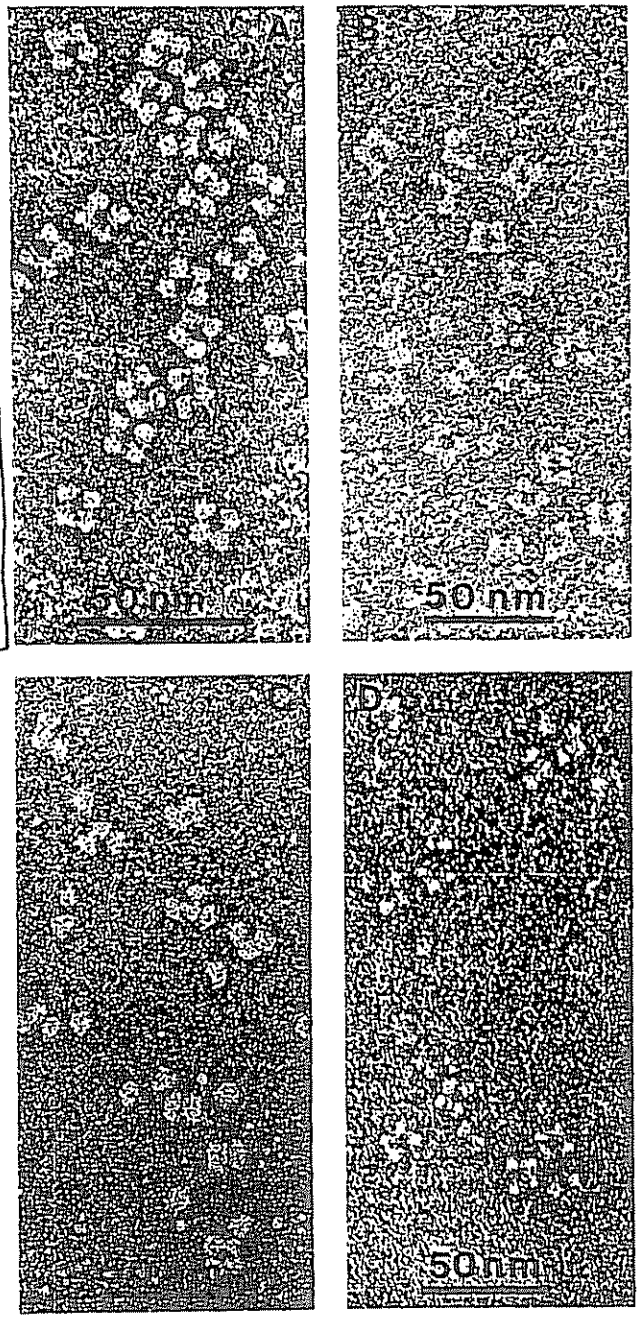


FIG. 6. **Electron microscopy of pure invertase.** Pure *sec18* invertase samples, shown in Fig. 5, were visualized in the electron microscope by negative staining (*A*, *B*, and *C*). Commercially obtained invertase was also examined (*D*); the further glycosylation of this species may cause it to bind stain differently than *sec18* invertase. *Bar* represents 50 nm.

for α-galactosidase, cells were converted to spheroplasts, and the spheroplast supernatant fraction was subjected to gel filtration chromatography. α-Galactosidase and acid phosphatase eluted with apparent molecular mass between 550 and 600 kDa (Fig. 10). Previous workers found that α-galactosidase, purified from culture medium, has a molecular mass of 300 kDa (34). Others have reported that derepressible acid phosphatase (*PHO5*), secreted by protoplasts, has a molecular mass of 560 kDa (35) or, if protein was isolated from cells

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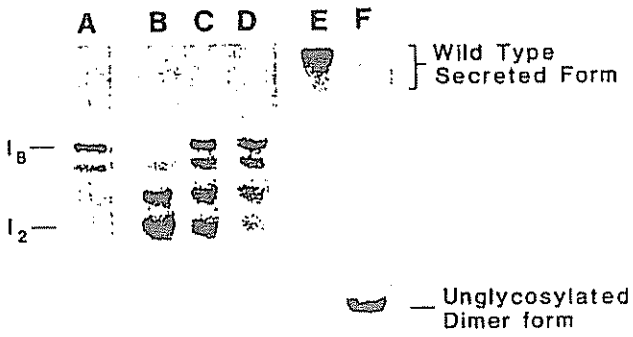


FIG. 7. **Alterations in invertase association by heat, PEG, and tunicamycin.** Pure *sec18* invertase was either kept at 4 °C (*A*) or heated at 30 °C for 12 h (*B*). Heat-treated sample was reassociated by the addition of 10% PEG (*C*) or 20% PEG (*D*). Following treatment, samples were subjected to nondenaturing PAGE through a 3.8% polyacrylamide gel, and the gel was stained for invertase activity. Wild type cells were derepressed for invertase in the absence (*E*) or the presence (*F*) of 10 μg/ml tunicamycin. Spheroplast supernatants were prepared and analyzed as in *A*—*D* except that a 6.5% polyacrylamide gel was used and the gel was incubated at 25 °C for the invertase activity stain. Invertase seen in *F* co-migrated with authentic unglycosylated invertase dimer (cytoplasmic invertase).

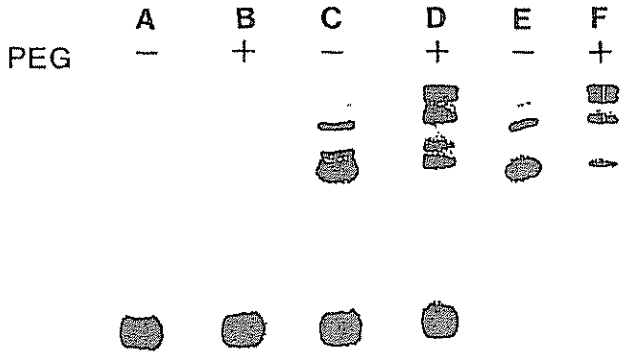
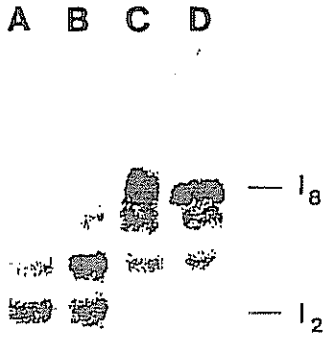


FIG. 8. **Treatment of unglycosylated invertase with PEG.** PEG was added to the indicated samples which contained cytoplasmic (unglycosylated) invertase (*A*, *B*), heat-treated invertase purified from *sec18* (*E*, *F*), or a mixture of the two (*C*, *D*). All samples were subjected to nondenaturing PAGE on a 6.5% polyacrylamide gel, and the gel was stained for invertase activity. To stain all of the *sec18* invertase bands, it was necessary to overstain the cytoplasmic invertase bands, causing them to be broader than usual.

lysed with a Braun homogenizer, the molecular mass was 252 kDa (36). These discrepancies in molecular mass may be due either to multimer dissociation during isolation (34, 36), or to aberrant protein chromatography due to unusual protein shape or extensive glycosylation. Our data are consistent with the notion that the large size of invertase, acid phosphatase, and α-galactosidase helps retain these proteins within the periplasmic space.

DISCUSSION

The results presented here indicate that invertase octamer is formed in the ER and that invertase glycosylation facilitates oligomerization. We have shown that invertase is present as the I-8 species throughout the secretory pathway and that this complex appears to play a role in retention of invertase within the periplasmic space.



— Cytoplasmic

FIG. 9. **Invertase species released from *mnn9* cells by various treatments.** *mnn9* cells were derepressed 1 h at 30 °C for synthesis of invertase, the culture was split into four portions, and each was treated for 45 min in 50-μl aliquots as described below. Treated cells were sedimented, and equal amounts of invertase activity from each supernatant fraction were analyzed by nondenaturing PAGE on a 3.8% polyacrylamide gel; the gel was stained for invertase activity. Invertase activity released by each treatment was as follows: *A*, 0.02 units; *B*, 0.12 units; *C*, 1.38 units; *D*, 1.76 units. Treatments: *A*, 0.1 M KCl, 50 mM Tris, pH 7.5, at 4 °C; *B*, 40 mM β-mercaptoethanol, 0.1 M KCl, 50 mM Tris, pH 7.5, at 4 °C; *C*, Buffer A plus 0.1 M KCl at 30 °C; *D*, Buffer A plus 0.1 M KCl, 0.1% Triton X-100 at 30 °C. Dilution of purified invertase to 0.5 unit/ml did not cause octamer dissociation.

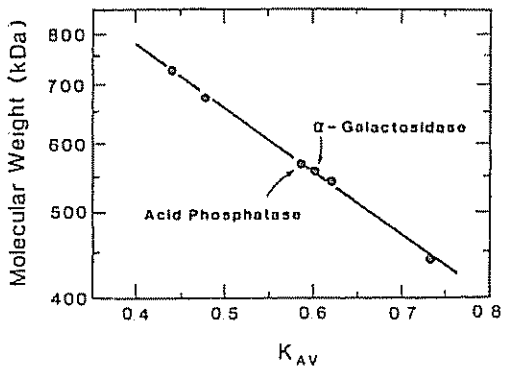


FIG. 10. **Gel filtration chromatography of acid phosphatase and α-galactosidase.** Spheroplast supernatant was prepared from *mnn9* cells which had been induced for α-galactosidase and it was applied to an Ultrogel AcA22 gel filtration column. The column was calibrated with *mnn9* invertase (720 kDa), thyroglobulin (669 kDa), β-galactosidase (540 kDa), and ferritin (440 kDa).

Velocity sedimentation, Ferguson analysis (Fig. 3), and electron microscopy (Fig. 6) demonstrate that the octamer is a complex of four invertase dimers. Octamer is dissociated by several treatments which are often used in protein isolation; shear force, heat, and low ionic strength buffer all promote conversion of octamer to dimer. Octamer instability made its detection difficult and led to the apparently erroneous conclusion that this protein is present within the periplasmic space as a dimer (2). Treatments which cause octamer dissociation do not change the kinetic parameters of the enzyme; the K_m for sucrose of invertase octamer purified from *sec18* is the same as that previously reported for dimeric invertase purified from the periplasmic space (37). In addition, dissociation of

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I-8 to I-2 does not alter the K_m or the V_{max} of the enzyme.[2]

Octamer is reconstituted from invertase dimer by the addition of PEG, a polymer that binds water and excludes protein (38), suggesting that invertase associates into octamer at high protein concentration. Many proteins self-aggregate at physiological protein concentrations, even if there are other proteins present (39–41). From this data, and from the fact that *sec18* accumulates invertase octamer, it seems likely that, in the cell, octamer forms spontaneously in the lumen of the ER. This notion is supported by preliminary observations that octamer may be visualized in wild type cell lysates using native gel electrophoresis.[3] Invertase association in octamers appears to involve specific interactions. Unlike many other protein aggregates, invertase does not form stable complexes larger than the octamer when its concentration is increased either by over production *in vivo* (Fig. 5*A*), or by addition of PEG *in vitro* (Fig. 7, *C* and *D*).

Accumulation of octamer in the lumen of the ER in *sec18* cells shows that invertase association occurs very early in the secretory pathway. We have reported previously that all of the *sec* mutants that accumulate proteins in the ER contain invertase oligomers as seen by native gel electrophoresis (9). Thus we are unable to detect stages of octamer assembly by blocking transport of glycoproteins to the Golgi body in different mutants. Furthermore, although octamer formation may be facilitated by an ER protein for which no mutation has been obtained, we detected no additional proteins associated with invertase that was purified from the ER lumen using procedures that preserved the octamer (Fig. 2). In this regard, invertase differs from the form of mouse oligomeric β-glucuronidase that is maintained in the ER by association with the protein egasyn (42). Instead, invertase oligomerization resembles that described for formation of trimeric influenza hemagglutinin which appears to assemble early in transport, probably in the ER (43).

We do not observe the formation of unglycosylated octamers either *in vivo* when invertase is synthesized in the presence of tunicamycin (Fig. 7, *E* and *F*), or *in vitro* when PEG is added to a crude fraction that contains cytoplasmic invertase (Fig. 8). We did not test octamer stability using deglycosylated secreted enzyme because the conditions required for endoglycosidase H activity (37 °C, 24 h) promote dissociation and because complete carbohydrate removal by endoglycosidase H requires that invertase be denatured (44). Our data suggest that, in addition to functions in enzyme stability (45), invertase oligosaccharide units promote octamer formation. Studies by Tammi *et al.* (46) using invertase purified from glycosylation-defective yeast mutants support this view. In contrast, Williams *et al.* (47) have found conditions (0.1 mg of protein/ml, pH 4.9) in which purified cytoplasmic invertase forms an octamer. Thus, it appears that, using pure enzyme at high protein concentration (50–300-fold higher invertase concentration than we used), the unglycosylated species can form the octamer. Similar octameric structures have been seen in concentrated solutions of cytoplasmic invertase visualized by transmission electron microscopy (46). The unglycosylated octameric form may be so unstable that it dissociates to a dimer during cell rupture.

Glycosylation plays an important, but not essential role in invertase secretion. At 25 °C, unglycosylated invertase formed in the presence of tunicamycin is secreted with a $t_{1/2}$ of about 20 min, compared to the normal $t_{1/2}$ of about 3–4 min (48, 49). This delay in secretion correlates with the failure of unglycosylated secreted invertase to form octamers. Although it is tempting to suggest a direct connection between octamer formation and rapid secretion, other proteins that are essential for invertase transport may require glycosylation for activity.

Following invertase transit through the secretory pathway, the octamer form is retained within the periplasmic space. This notion is supported by analysis of *mnn9*-secreted invertase using nondenaturing PAGE (Fig. 3), electron microscopy of *sec18* and wild type purified invertases (Fig. 6), and previous results by Chu *et al.* (3). Although very little invertase is normally released from yeast cells, the small amount that is released by washing intact cells with buffer is composed of invertase dimer (Fig. 9*A*). Dimer release is not accompanied by release of cytoplasmic invertase, showing that cell lysis had not occurred. Since invertase octamer is not released from the cell, octamer must help retain invertase within the periplasmic space. Treatment of intact cells with β-mercaptoethanol causes additional release of invertase dimer (Fig. 9*B*), suggesting that a sulfhydryl-containing cell wall component promotes invertase dimer retention. Even when intact cells are treated with β-mercaptoethanol, invertase octamer is preferentially retained in the periplasmic space; release of this invertase requires cell wall disruption (Fig. 9*C*). Previous studies with *Neurospora crassa* showed that a tetrameric form of invertase is retained within the periplasmic space while the dimer is secreted (50, 51). In addition, two other secreted yeast enzymes, acid phosphatase and α-galactosidase are present as high molecular mass species following gentle release from the periplasmic space (Fig. 10). These results support the view that retention of secreted yeast proteins in the periplasmic space is facilitated by the formation of high molecular mass complexes.

Acknowledgments—We would like to thank Ying Yang for her aid with the Model E centrifuge and Bill Kimmerly for generously providing β-galactosidase. We also thank Clinton Ballou, Lun Ballou, and John Reed for many useful discussions and suggestions.

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PROTOCOL FOR INV. PURIF →

Chu '83 Arch. Biochem Biophys 223 543–555

Invertase Purification → TRIMBLE '77 JBC 252 4407-4412

At 4°C:

Cells ↑ 50mM TRIS-HCl 7.5, 50mM EDTA, 5μM pepstatin A, 1mM PMSF + glass beads

(purification of internal invertase, buffer incl. 10mM βME, 2mM benzamidine, 5mM O-phenathrolene, & 2μg/ml leupeptin)

Sep. out supe.

↓ Supe 5k 10' (Retain supe)

Cell debris rebrkn w/ glass beads

EXTERNAL Invertase

Amm sulfate added to crude extract to 70% satn

Stir 1hr 4°C

↓ 5k 10'

Supe dialyzed against 10mM NaPO4 buffer 7.5 = BUFFER B

[Supe] Add to DE52 cellulose column

0-0.2M NaCl in buffer B = gradient run over column

[Frxns]

To obtain homogenous ~~extracts~~ enzyme load on Sephacryl S-300 (3.8 x 85cm) equil in 10mM TR 7.5 50mM NaCl

Collect Frxns

A_{280} + invertase assay

EXHIBIT 6

REDACTED IN ITS ENTIRETY

EXHIBIT 7

REDACTED IN ITS ENTIRETY

EXHIBIT 8

REDACTED IN ITS ENTIRETY

EXHIBIT 9

REDACTED IN ITS ENTIRETY