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.	)	C.A. No. 06-500-SLR **PUBLIC VERSION**

DECLARATION OF HANNAH LEE IN SUPPORT OF ZYMOGENETICS, INC.'S REPLY BRIEF IN SUPPORT OF ITS MOTION FOR SUMMARY JUDGMENT OF VALIDITY OF PATENTS-IN-SUIT WITH RESPECT TO THE CAPON PATENTS AND SEED PATENT

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Dated: August 25, 2008
Public Version: September 2, 2008

I, HANNAH LEE, declare:

1. I am an attorney with the law firm of King & Spalding LLP, counsel of record for Plaintiff ZymoGenetics, Inc. I have personal knowledge of the facts set forth in this declaration and can testify competently to those facts.

2. Attached hereto as Exhibit 1 is a true and correct copy of pages 191 and 198 from the transcript of the deposition of Andrzej Sledziewski, taken on August 21, 2007.

3. Attached hereto as Exhibit 2 is a true and correct copy of pages 15, 18 and 29 from the transcript of the deposition of Wayne Kindsvogel, taken on August 8, 2007.

4. Attached hereto as Exhibit 3 is a true and correct copy of pages from the laboratory notebook of Andrzej Sledziewski, bearing bates numbers ZG033202 and ZG033206-09.

5. Attached hereto as Exhibit 4 is a true and correct copy of pages 183-85 from the transcript of the deposition of Paul Edward Purdue, Ph.D., taken on May 5, 2008.

6. Attached hereto as Exhibit 5 is a true and correct copy of an article entitled "Stability, quarternary structure, and folding of internal, external, and core-glycosylated invertase from yeast," by Gunther Kern, et al., appearing in *Protein Science* at pp. 120-131 (Cambridge Univ. Press 1992).

7. Attached hereto as Exhibit 6 is a true and correct copy of pages 74-75 from the transcript of the deposition of Steven Levin, taken on November 8, 2007.

I declare under penalty of perjury under the laws of the State of California and the United States that each of the above statements is true and correct.

Executed on August 28, 2008 in Redwood Shores, California.



Hannah Lee

Exhibit 1

THIS EXHIBIT HAS BEEN REDACTED IN ITS ENTIRETY

Exhibit 2

THIS EXHIBIT HAS BEEN REDACTED IN ITS ENTIRETY

Exhibit 3

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Exhibit 4

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Exhibit 5

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

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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

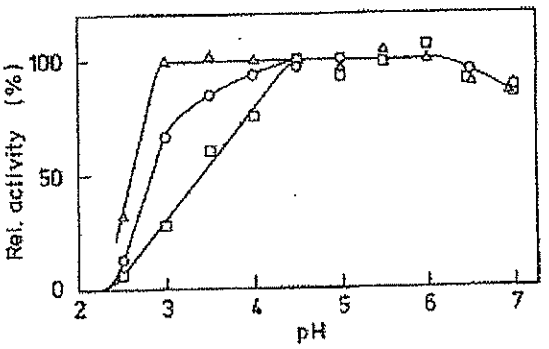

Fig. 1. Effect of pH on the long-term stability of internal (□), core-glycosylated (○), and external (△) invertase at 30 °C after 42 h. Samples of 10 μg/mL of invertase were incubated in 0.05 M citrate/0.05 M phosphate buffer plus 0.4 mg/mL NaN_3 at pH values ranging from 2.5 to 7.0. Activity was determined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

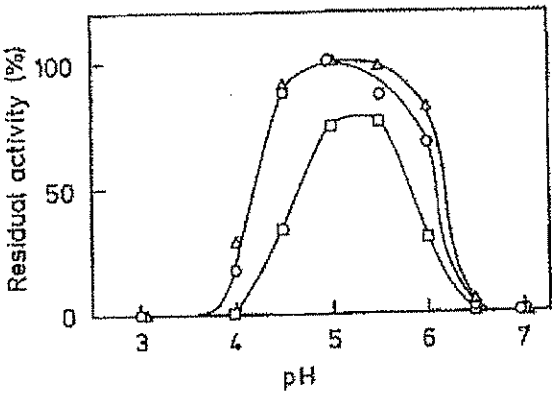

Fig. 2. Effect of pH on the stability against thermal inactivation of SUC2-encoded internal (□), core-glycosylated (○), and external (▲) invertase. Aliquots of 5 μg/mL invertase were incubated in 0.05 M citrate/0.05 M phosphate buffer at pH values ranging from 3.0 to 7.0 at 55 °C for 90 min. Fifty microliter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

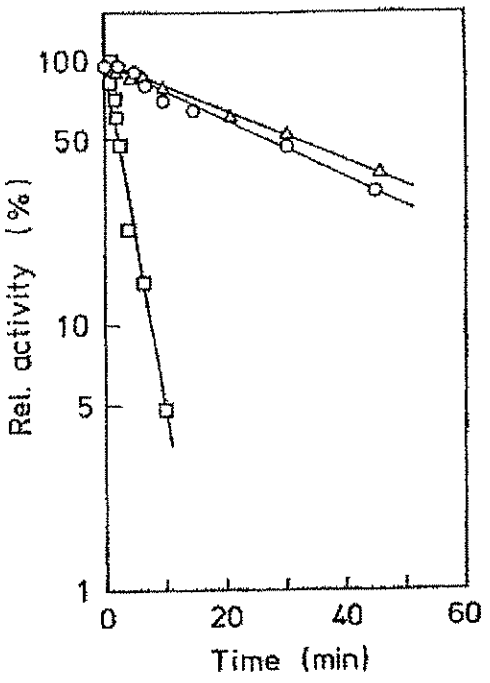

Fig. 3. Kinetics of thermal inactivation of SUC2-encoded internal (□), core-glycosylated (○), and external (▲) invertase at 65 °C and pH 5.0. Inactivation was started by adding 10 μL of invertase solution (1 mg/mL) to 990 μL 0.05 M sodium acetate, pH 5.0 (65 °C). At times t, residual activity was measured 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 half-times 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.

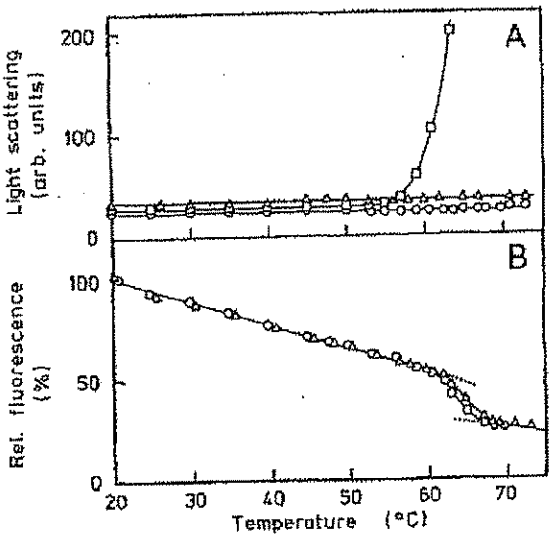

Fig. 4. Thermally induced unfolding of SUC2-encoded internal (□), core-glycosylated (○), and external (▲) invertase; 10 μg/mL of invertase in 0.05 M sodium acetate buffer, pH 5.0, were heated from 20 to 75 °C at a heating rate of 0.25 °C/min using a temperature-controlled cuvette. A: Light scattering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

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.

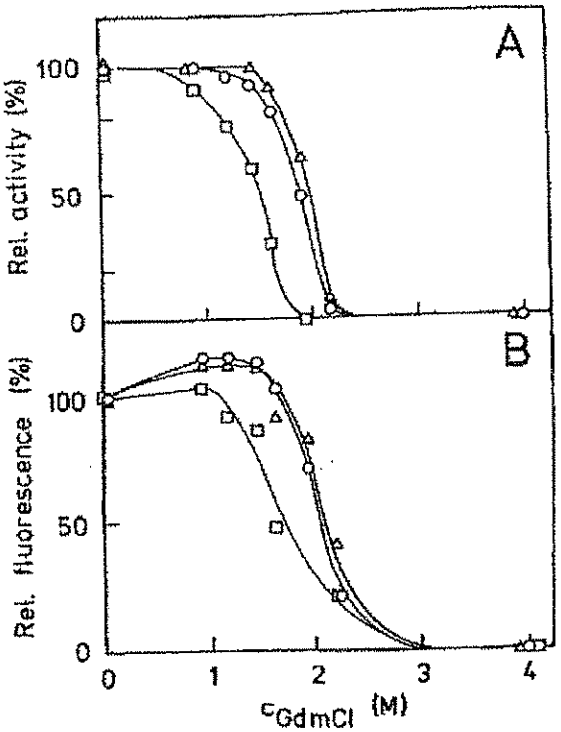

Fig. 5. GdmCl-induced inactivation (A) and unfolding (B) of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase. Aliquots of 30 μg/mL of protein were incubated in 0.05 M sodium acetate, pH 5.0, in the presence of 0–4 M GdmCl at 20 °C for 24 h. Activity was monitored under standard 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.

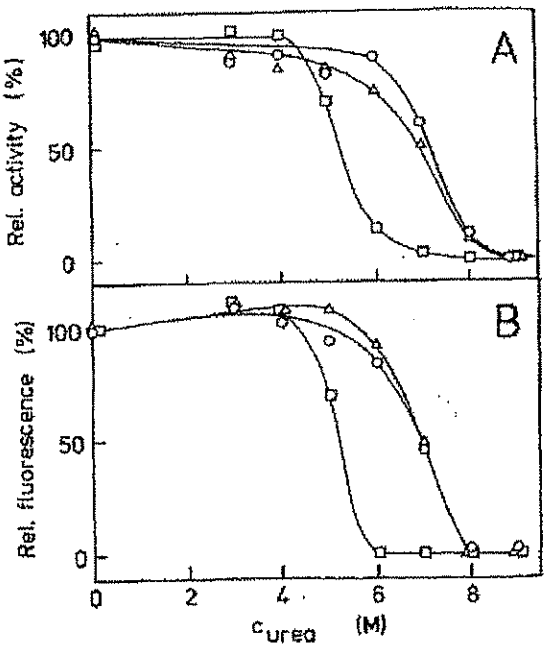

Fig. 6. Urea-induced inactivation (A) and unfolding (B) of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase. Aliquots of 5 μg/mL of protein were incubated in 0.05 M sodium acetate buffer, pH 5.0, in 0–9 M urea at 20 °C for 40 h. Activity was monitored under standard conditions; 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.

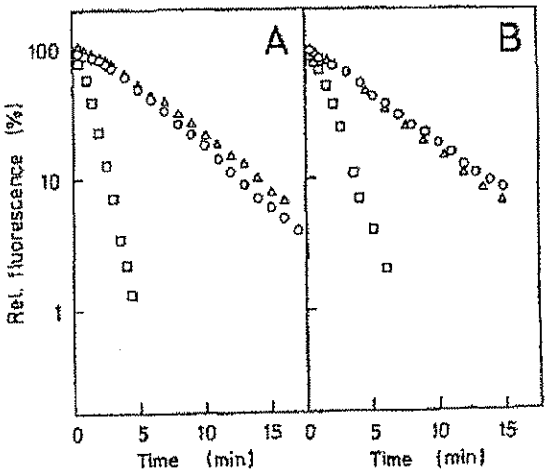

Fig. 7. Kinetics of GdmCl- (A) and urea-induced (B) unfolding of SUC2-encoded internal (□), core-glycosylated (○), and external (△) invertase. Aliquots of 10 μL of invertase (1 mg/mL) were added at a final volume of 1 mL of 4 M GdmCl in 0.05 M sodium acetate buffer, pH 4.8, at 20 °C or 9 M urea in 0.05 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).

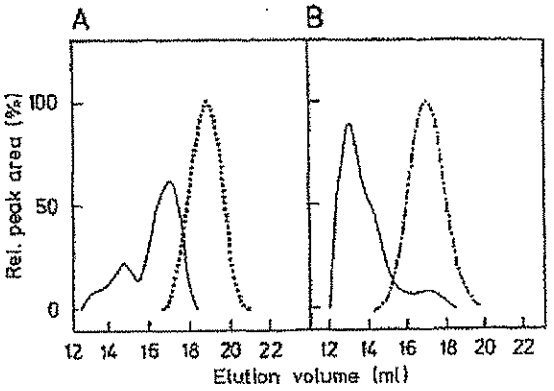

Fig. 8. Quaternary structure of internal and core-glycosylated invertase as determined by gel-permeation chromatography (TSK-3000). Aliquots of 20 μL of a 20-μg/mL protein solution in 0.05 M sodium acetate buffer, pH 5.0, 20 °C. Flow-rate was 0.5 mL/min, pressure was 6 bar, and running buffer was 0.2 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-

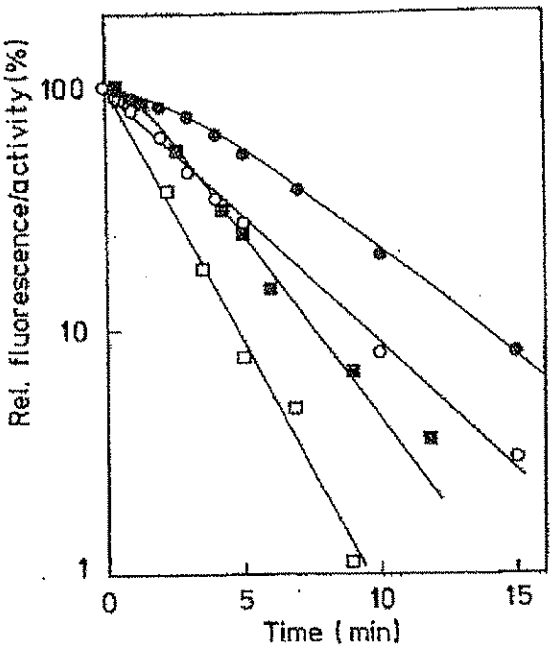

Fig. 9. Unfolding (○, ●) and inactivation (□, ■) kinetics of core-glycosylated invertase in 4 M GdmCl at 20 °C after 24 h preincubation in 4 M urea (○, □) or 1 M GdmCl (●, ■) in 0.05 M sodium acetate buffer, pH 5.0; protein concentration during preincubation was 0.5 mg/mL. Unfolding and inactivation 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.

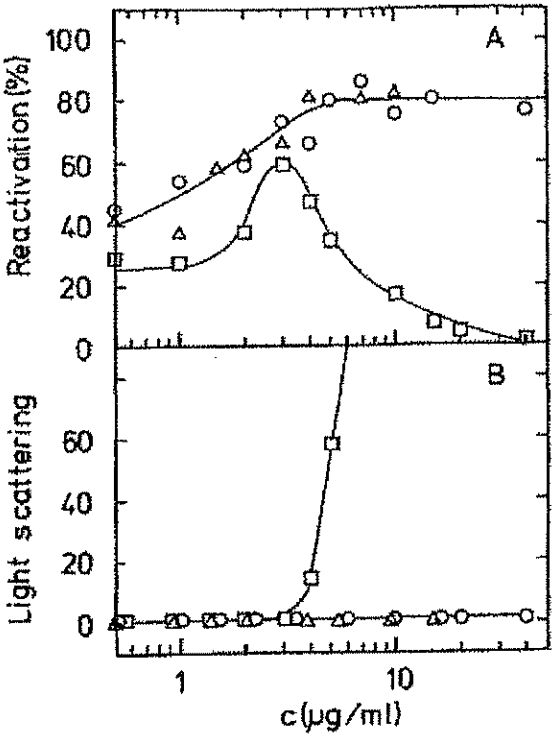

Fig. 10. Influence of protein concentration on the reactivation yield and aggregation of internal (□), core-glycosylated (○), and external (△) invertase. A: Reactivation after 1 h denaturation in 0.05 M sodium phosphate/citrate buffer, pH 7.0, in the presence of 6 M GdmCl at 20 °C. Protein concentration 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.

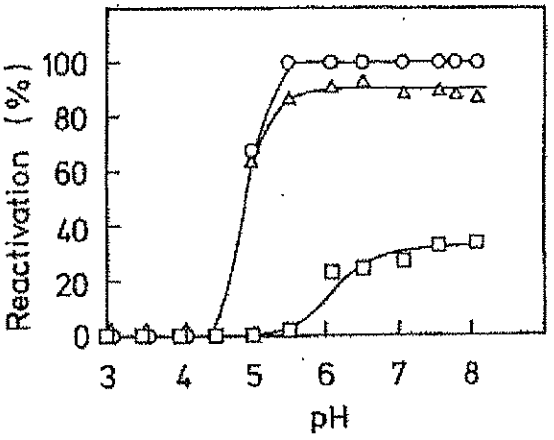

Fig. 11. Effect of pH on the yield of reactivation of internal (□), core-glycosylated (○), and external (△) invertase after 1 h denaturation with 6 M GdmCl in 0.05 M sodium phosphate/citrate buffer, pH 6.5, at 20 °C. Final protein concentration was 5 μg/mL. Reactivation was initiated by a 1:80 dilution 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-

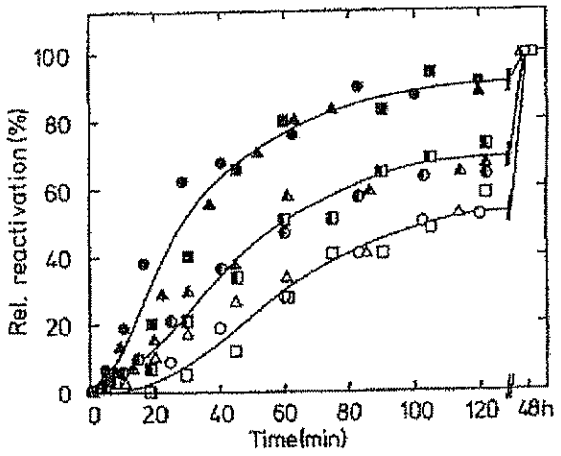

Fig. 12. Effect of protein concentration on the reactivation of internal (□, ■, ▣), core-glycosylated (○, ●, ◉), and external (△, ▲, ▲) invertase. Protein concentrations were 0.5, 2.0, and 4.0 μg/mL. Unfolding was accomplished by 1 h incubation in denaturation buffer, pH 7.0, at 20 °C. Reactivation was 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.

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References

Carlson, M., Osmond, B.C., & Botstein, D. (1980). SUC genes of yeast: A dispersed gene family. *Cold Spring Harbor Symp. Quant. Biol. 45*, 799–803.

Chu, F.K., Trimble, R.B., & Maley, F. (1978). The effect of carbohydrate depletion on the properties of yeast external invertase. *J. Biol. Chem. 253*, 8691–8693.

Chu, F.K., Watorek, W., & Maley, F. (1983). Factors affecting the oligomeric structure of yeast external invertase. *Arch. Biochem. Biophys. 223*, 543–555.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem. 28*, 350–356.

Durchschlag, H. (1986). Specific volumes of biological macromolecules and some other molecules of biological interest. In *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., Ed.), pp. 45–128. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

Esmon, B., Novick, P., & Schekman, R. (1981). Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell 25*, 451–460.

Esmon, P.C., Esmon, B.E., Schauer, I.E., Taylor, A., & Schekman, R. (1987). Structure, assembly, and secretion of octameric invertase. *J. Biol. Chem. 262*, 4387–4394.

Fischer, G. & Schmid, F.X. (1990). The mechanism of protein folding. Implications of in vitro refolding models for de novo protein folding and translocation in the cell. *Biochemistry 29*, 2205–2212.
Gabriel, O. & Wang, S. (1969). Determination of enzymatic activity in polyacrylamide gels. *Anal. Biochem. 27*, 545–554.
Gascón, S., Neumann, N.P., & Lampen, J.O. (1968). Comparative study of the properties of the purified internal and external invertases from yeast. *J. Biol. Chem. 243*, 1573–1577.
Gibson, R., Schlesinger, S., & Kornfeld, S. (1979). The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. *J. Biol. Chem. 254*, 3600–3607.
Goldstein, A. & Lampen, J.O. (1975). β-D-fructofuranoside fructohydrolase from yeast. *Methods Enzymol. 42*, 504–511.
Jaenicke, R. (1987). Folding and association of proteins. *Prog. Biophys. Mol. Biol. 49*, 117–237.
Jaenicke, R. (1991). Protein folding: Local structures, domains, subunits, and assemblies. *Biochemistry 30*, 3147–3161.
Jaenicke, R. & Rudolph, R. (1989). Folding proteins. In *Protein Structure: A Practical Approach* (Creighton, T.E., Ed.), pp. 191–223. IRL Press, Oxford.
Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagine linked oligosaccharides. *Annu. Rev. Biochem. 54*, 631–664.
Manjunath, P. & Sairam, M.R. (1983). Enhanced thermal stability of chemically deglycosylated human choriogonadotropin. *J. Biol. Chem. 258*, 3554–3558.
Marshall. (1972). Glycoproteins. *Annu. Rev. Biochem. 41*, 673–702.
Marshall. (1974). The nature and metabolism of the carbohydrate-peptide linkages of glycoproteins. *Biochem. Soc. Symp. 40*, 17–26.
Mononen, I. & Karjalainen, E. (1984). Structural comparison of protein sequences around potential N-glycosylation sites. *Biochim. Biophys. Acta 788*, 364–367.
Mortimer, R.K. & Hawthorne, D.C. (1969). Yeast genetics. In *The Yeasts*, Vol. I (Rose, A.H. & Harrison, J.S., Eds.), pp. 385–460. Academic Press, New York, London.
Myrbäck, K. (1965). Studies on yeast β-fructofuranosidase (invertase): XVII. Inhibition by 2-amino-2-hydroxymethylpropan-1,3-diol. *Ark. Kemi 25*, 315–320.
Neumann, N.P. & Lampen, J.O. (1967). Purification and properties of yeast invertase. *Biochemistry 6*, 468–475.
Nose, M. & Wigzell, H. (1983). Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA 80*, 6632–6636.
Novick, P., Field, C., & Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell 21*, 205–215.
Nozaki, Y. & Tanford, C. (1971). The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. Establishment of a hydrophobicity scale. *J. Biol. Chem. 246*, 2211–2217.
Olden, K., Bernard, B.A., Humphries, M.J., Yeo, T.K., Yeo, K.T., White, S.L., Newton, S.A., Bauer, H.C., & Parent, J.B. (1985). Function of glycoprotein glycans. *Trends Biochem. Sci. 10*, 78–82.
Olden, K., Parent, J.B., & White, S.L. (1982). Carbohydrate moieties of glycoproteins. A re-evaluation of their function. *Biochim. Biophys. Acta 650*, 209–232.
Olden, K., Pratt, R.M., Jaworski, C., & Yamada, K.M. (1979). Evidence for the role of glycoprotein carbohydrates in membrane transport: Specific inhibition by tunicamycin. *Proc. Natl. Acad. Sci. USA 76*, 791–795.
Olden, K., Pratt, R.M., & Yamada, K.M. (1978). Role of carbohydrates in protein secretion and turnover: Effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts. *Cell 13*, 461–473.
Reddy, V.A., MacColl, R., & Maley, F. (1990). Effect of oligosaccharides and chloride on the oligomeric structures of external, internal, and deglycosylated invertase. *Biochemistry 29*, 2482–2487.
Schekman, R. (1982). The secretory pathway in yeast. *Trends Biochem. Sci. 7*, 243–246.
Schülke, N. (1988). Der Einfluß der N-Glykosylierung auf die Struktur, die Stabilität und den Faltungsmechanismus von Invertase aus *Saccharomyces cerevisiae*. Dissertation, Universität Regensburg, Regensburg, West Germany.
Schülke, N. & Schmid, F.X. (1988a). The stability of invertase is not significantly influenced by glycosylation. *J. Biol. Chem. 263*, 8827–8831.
Schülke, N. & Schmid, F.X. (1988b). Effect of glycosylation on the mechanism of renaturation of invertase from yeast. *J. Biol. Chem. 263*, 8832–8836.
Sharon, N. (1984). Surface carbohydrates and surface lectins are recognition determinants in phagocytosis. *Immunol. Today 5*, 143–147.
Sharon, N. & Lis, H. (1982). Glycoproteins. In *The Proteins*, Vol. 5, 3rd Ed. (Neurath, H., Ed.), pp. 1–144. Academic Press, New York, London.
Tarentino, A.L., Plummer, T.H., Jr., & Maley, F. (1974). The release of intact oligosaccharides from specific glycoproteins by endo-β-N-acetylglucosaminidase H. *J. Biol. Chem. 249*, 818–824.
Taussig, R. & Carlson, M. (1983). Nucleotide sequence of the yeast SUC2 gene for invertase. *Nucleic Acids Res. 11*, 1943–1954.
Trimble, R.B. & Maley, F. (1977). The use of endo β-N-acetylglucosaminidase H in characterizing the structure and function of glycoproteins. *Biochem. Biophys. Res. Commun. 78*, 935–944.
Williams, R.S., Trumbly, R.J., MacColl, R., Trimble, R.B., & Maley, F. (1985). Comparative properties of amplified external and internal invertase from the yeast SUC2 gene. *J. Biol. Chem. 260*, 13334–13341.
Yphantis, D.A. (1964). Equilibrium ultracentrifugation of dilute solutions. *Biochemistry 3*, 297–317.
Ziegler, F.D., Maley, F., & Trimble, R. (1988). Characterization of the glycosylation sites in yeast external invertase. *J. Biol. Chem. 263*, 6986–6992.

Exhibit 6

THIS EXHIBIT HAS BEEN REDACTED IN ITS ENTIRETY

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CERTIFICATE OF SERVICE

I, Philip A. Rovner, hereby certify that on September 2, 2008, the within document was filed with the Clerk of the Court using CM/ECF which will send notification of such filing(s) to the following; that the document was served on the following counsel as indicated; and that the document is available for viewing and downloading from CM/ECF.

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