Kinetics of folding and association of differently glycosylated variants of invertase from *Saccharomyces cerevisiae*

GUNTHER KERN,¹ DOROTHEE KERN,² RAINER JAENICKE,¹ AND ROBERT SECKLER¹

¹ Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-93040 Regensburg, Germany

² Max-Planck-Arbeitsgruppe Enzymologie der Peptidbindung, Weinbergweg 16A, D-06120 Halle/Saale, Germany

(RECEIVED July 14, 1993; ACCEPTED August 11, 1993)

Abstract

A core-glycosylated form of the dimeric enzyme invertase has been isolated from secretion mutants of *Saccharomyces cerevisiae* blocked in transport to the Golgi apparatus. This glycosylation variant corresponds to the form that folds and associates during biosynthesis of the protein in vivo. In the present work, its largely homogeneous subunit size and well-defined quaternary structure were utilized to characterize the folding and association pathway of this highly glycosylated protein in comparison with the nonglycosylated cytoplasmic and the high-mannose-glycosylated periplasmic forms of the same enzyme encoded by the *suc2* gene.

Renaturation of core-glycosylated invertase upon dilution from guanidinium-chloride solutions follows a unimolecular reaction scheme with consecutive first-order subunit folding and second-order association reactions. The rate constant of the rate-limiting step of subunit folding, as detected by fluorescence increase, is $k_1 = 1.6 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$ at 20 °C; it is characterized by an activation enthalpy of $\Delta H^\ddagger = 65 \text{ kJ/mol}$. The reaction is not catalyzed by peptidyl-prolyl *cis-trans* isomerase of the cyclophilin type. Reactivation of the enzyme depends on protein concentration and coincides with subunit association, as monitored by size-exclusion high-pressure liquid chromatography. The association rate constant, estimated by numerical simulation of reactivation kinetics, increases from $5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ to $7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ between 5 and 30 °C.

Although reactivation kinetics and yields of the core-glycosylated and high-mannose-glycosylated invertase are essentially identical between 5 and 40 °C, the nonglycosylated cytoplasmic form displays strongly reduced reactivation yields at the high end and significantly reduced reactivation rates at the low end of this temperature range.

Keywords: association kinetics; glycoprotein folding; invertase; refolding

One of the most frequent covalent modifications of proteins is N-glycosylation. Many biological functions have been ascribed to the sugar moiety, but so far without general agreement on its role for protein structure, stability, and folding (Sharon & Lis, 1982; Julius et al., 1984; Gross et al., 1987; Paulson, 1989; Winther et al., 1991). During biosynthesis of secreted proteins, the transfer of oligosaccharide chains onto asparagine residues (core-glycosylation) occurs cotranslationally, as soon as the polypeptide chain enters the lumen of the endoplasmic reticulum (Sharon & Lis, 1982; Kornfeld & Kornfeld, 1985). Consequently, the initial conjugation reaction may coincide with domain folding, and it is certainly expected to precede subunit association in the case of oligomeric enzymes. Upon exit

from the endoplasmic reticulum, for which the native folded structure of proteins appears to be a prerequisite (Schekman, 1985; Hurtley & Helenius, 1989; Gething & Sambrook, 1990, 1992), carbohydrate chains are trimmed and elongated extensively during subsequent transfer through the Golgi apparatus. Thus, core-glycosylation may assist efficient folding, whereas extensive glycosylation of secreted proteins may interfere with it.

Previous studies with differentially glycosylated ribonucleases did not unravel an effect of glycosylation on protein folding and stability (Grafl et al., 1987). However, ribonucleases are small monomeric proteins that fold rapidly, and their potential glycosylation sites are located at the surface of the folded proteins (Beintema, 1986). In contrast to ribonuclease, glycosylation has a stabilizing effect in the case of yeast invertase. Glycosylation protects the enzyme from irreversible aggregation during thermal

Reprint requests to: Robert Seckler, Biochemie II, Universität Regensburg, D-93040 Regensburg, Germany.

unfolding and during refolding after solvent-induced denaturation (Kern et al., 1992b). The enzyme occurs in the form of a cytosolic nonglycosylated dimer of 58.5-kDa subunits and a high-mannose-glycosylated form that is secreted into the periplasmic space of the yeast cell. In addition, a core-glycosylated form of invertase is accessible from a temperature-sensitive yeast mutant (*sec18*) transformed with a plasmid carrying the *suc2* gene coding for one of the six isoforms of yeast invertase (Roitsch & Lehle, 1989; Kern et al., 1992b). In this strain, protein transport to the Golgi is blocked at nonpermissive temperature (Novick et al., 1980; Esmon et al., 1981; Schekman, 1982), so that core-glycosylated invertase with fairly homogeneous carbohydrate chains accumulates in the endoplasmic reticulum. Because this mutant produces nonglycosylated and high-mannose-glycosylated invertase from the same plasmid-encoded *suc2* gene at permissive temperature, this approach avoids the complications of genetic inhomogeneity frustrating earlier comparisons of glycosylated and nonglycosylated invertase (Carlson et al., 1980; Schülke & Schmid, 1988a,b; Kern et al., 1992b).

Core-glycosylated invertase may be taken to resemble the glycosylated form of the enzyme that folds and assembles during biosynthesis *in vivo*. Its homogeneous subunit size has facilitated a quantitative description of the tendency of glycosylated invertase to form high-molecular-mass association products (Esmon et al., 1987; Kern et al., 1992b). For the same reason, a detailed characterization of its folding and association pathway appeared feasible. This was attempted in the present study in comparison with nonglycosylated and high-mannose-glycosylated forms expressed from the same structural gene.

Results

The reconstitution pathways of three differentially glycosylated forms of invertase containing 0, 0.5, and 1.6 g carbohydrate/g protein were characterized by a variety of techniques focusing on the different organization levels of oligomeric proteins. Two spectroscopic signals exhibit large amplitude changes during refolding of invertase. Circular dichroism in the far-UV (220 nm), mainly reflecting the secondary structure of the protein, changes from $[\theta]_{MR} = -1,000 \pm 500 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ to $[\theta]_{MR} = -7,000 \pm 500 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. The intrinsic fluorescence intensity measured at 325 nm (excitation at 280 nm) exhibits a 2.6-fold increase with a concomitant blue shift of the emission maximum by 20 nm, indicating that tryptophan residues become shielded from solvent upon tertiary structure formation or at subunit interfaces of the dimeric protein during subunit association. The spectroscopic signal changes were identical within experimental error for all three invertase glycosylation isoforms. In addition to the spectroscopic experiments, reconstitution of enzyme activity was measured, and subunit association

was followed by size-exclusion high-pressure liquid chromatography (HPLC).

Multiphasic reconstitution kinetics of invertase

Three kinetic phases were distinguishable during reconstitution of the invertase glycosylation variants upon dilution from guanidinium chloride solutions, as illustrated in Figure 1 for the core-glycosylated protein. Within the dead-time of manual mixing (20 s), the far-UV CD increased to $[\theta]_{MR,220\text{nm}} = -7,000 \pm 500 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ ($\Delta \cdots \Delta$). In the same time range, a twofold increase of fluorescence intensity was observed (----) concomitant with a blue shift of the emission maximum. Evidently, essentially all of the native secondary structure was formed and aromatic amino acid residues became buried in the interior of the protein. This fast folding reaction was followed by a much slower further increase in fluorescence intensity, which could be well described by a single-exponential rise with a time constant in the minutes range (Fig. 1, ---; Fig. 2A). At low protein concentration, reactivation exhibited a lag. Activity of the enzyme continued to increase after completion of the second kinetic phase observed in fluorescence, but no further change in spectroscopic signals was observed (Fig. 1, $\circ - \circ$). Apparently, folding and subunit association reactions determine the kinetics of invertase reconstitution, as is observed commonly for oligomeric proteins (Jaenicke, 1987). At low protein concentration, where subunit assembly became rate limiting for reactivation, single steps along the reconstitution pathway were well separated kinetically.

Fig. 1. Reconstitution kinetics of core-glycosylated invertase at 20 °C followed by far-UV CD ($\Delta \cdots \Delta$), fluorescence spectroscopy (----), and enzymatic activity ($\circ - \circ$). Renaturation was initiated by an 80-fold dilution of the denatured protein into renaturation buffer, pH 7.0. CD was recorded at 220 nm in a cell with a 1-mm pathlength (protein concentration: 50 $\mu\text{g}/\text{mL}$) and corrected for buffer background. Fluorescence emission at 325 nm was measured at a protein concentration of 5 $\mu\text{g}/\text{mL}$ and was normalized relative to the signal observed for the completely renatured enzyme, which was set to 100%. Invertase activity was measured for 0.5–1 min after a 1:50 dilution into the assay mixture, as described previously (Kern et al., 1992b). The activity observed after 48 h of reactivation was set to 100%.

Fig. 2. Subunit folding of invertase. **A:** Kinetics of refolding at 25 °C of internal ($\square$) and external (∇) invertase at 2 $\mu\text{g/mL}$ as well as core-glycosylated invertase at 8 $\mu\text{g/mL}$ ($\blacktriangle$) and 16 $\mu\text{g/mL}$ ($\bullet$), followed by fluorescence emission at 325 nm. The fluorescence signals determined by extrapolation to $t = 0$ and $t = \infty$ were set to 0 and 100%, respectively. The solid line represents a single-exponential rise with a rate constant of $k_1 = 3 \times 10^{-3} \text{ s}^{-1}$. **B:** Eyring plot for the determination of the activation enthalpy $\Delta H^\ddagger$ of the subunit folding reaction. First-order rate constants were determined by nonlinear regression of fluorescence data measured at a protein concentration of 5 $\mu\text{g/mL}$. **C:** Effect of peptidyl-prolyl *cis-trans* isomerase (PPIase) on the kinetics of subunit folding of core-glycosylated invertase at 25 °C. Refolding of invertase at a protein concentration of 20 $\mu\text{g/mL}$ (0.34 μM subunits) was followed by a change in fluorescence emission at 325 nm in the absence ($\circ$) and in the presence of 2.2 μM ($\bullet$) or 10 μM ($\blacktriangle$) PPIase.

Thus, it was possible to examine the effect of differential glycosylation on individual kinetic phases of the overall reaction.

Subunit folding

As mentioned above, the $\sim 30\%$ increase in fluorescence intensity observed on the minutes time scale could be fit-

ted by a single exponential, suggesting that the fluorescence change is indicative of a first-order folding reaction. This conclusion was confirmed when the reaction was studied at varying protein concentrations (Fig. 2A): kinetics were essentially superimposable for all protein concentrations studied (2–16 $\mu\text{g/mL}$; i.e., 34–270 nM invertase subunits). As explained below, the first-order reaction observed spectroscopically precedes formation of invertase dimers. Therefore, it must correspond to a rate-limiting subunit folding reaction. The rate constant of subunit folding at 25 °C ($k = 3.5 \pm 1.0 \times 10^{-3} \text{ s}^{-1}$) was identical within experimental error for all three glycosylation variants, as apparent from the data presented in Figure 2A.

Because reconstitution yields of core-glycosylated invertase are high and largely independent of protein concentration (Kern et al., 1992b) and temperature (see below), the subunit folding reaction could be characterized in more detail. From the temperature dependence of the rate constant (Fig. 2B), an activation enthalpy of $\Delta H^\ddagger = 64 \pm 3 \text{ kJ/mol}$ was calculated according to the Eyring equation. The relaxation time at 25 °C ($\approx 285 \text{ s}$) corresponds to a free energy of activation of $\Delta G^\ddagger \approx 85 \text{ kJ/mol}$. These values are close to those observed commonly for the *cis-trans* isomerization of peptide bonds preceding proline residues (Brandts & Lin, 1986). Isomerization of X-Pro peptide bonds has been shown to be a rate-limiting reaction during the folding of a number of proteins. The reaction is catalyzed by the enzyme peptidyl-prolyl *cis-trans* isomerase (PPIase), if the X-Pro peptide bond in the folding intermediate is accessible for this enzyme (Schönbrunner et al., 1991; Schmid, 1993; Schmid et al., 1993). As the rate-limiting reaction of invertase subunit folding might mirror a *cis-trans* isomerization at one of its 26 proline residues, different concentrations of PPIase were added to the renaturation buffer prior to refolding. No acceleration of this folding reaction was observed in the presence of the isomerase at up to 30-fold molar excess over invertase subunits (Fig. 2C).

Reactivation of invertase follows a consecutive uni-bimolecular reaction scheme

Because most oligomeric proteins become active only after subunit association, their reactivation often follows second-order kinetics at low protein concentration. The time course of reactivation of core-glycosylated and high-mannose-glycosylated invertase at 20 °C was determined for protein concentrations varied between 0.5 and 4 $\mu\text{g/mL}$ (corresponding to subunit concentrations of 8.5–68 nM). The nonglycosylated form of the enzyme was not included in the experiment, because folding intermediates of the nonglycosylated enzyme begin to form inactive aggregates at the upper end of this concentration range, resulting in decreased reactivation yields (Kern et al., 1992b). For both glycosylated forms, reactivation kinetics were sigmoidal and indistinguishable (Fig. 3). The simplest model

Fig. 3. Reactivation kinetics of core-glycosylated (open symbols) and high-mannose-glycosylated external (filled symbols) invertase at 20 °C. The enzymatic activity determined after 48 h of reactivation was taken as 100%. The subunit concentrations during refolding were 8.5 nM (circles), 17 nM (triangles), and 68 nM (diamonds). The solid lines are numerical simulations of consecutive uni-bimolecular kinetics using the first-order rate constant $k_1 = 1.6 \times 10^{-3} \text{ s}^{-1}$ determined by fluorescence and a bimolecular rate constant of $k_2 = 1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$.

to explain the observed sigmoidicity and the protein concentration dependence of the reactivation rate is a uni-bimolecular folding-association mechanism, where the unfolded chain (U) first folds to structured but inactive monomers (M), which subsequently associate to active dimers (D):

To test this model, the formation of active dimers was calculated for the experimental range of protein concentrations (0.5, 1, and 4 $\mu\text{g}/\text{mL}$) according to a uni-bimolecular folding mechanism by numerical simulation. Reasonable agreement of the simulation with the experimental data was observed when the rate constant of the unimolecular step ($\text{U} \rightarrow \text{M}$) was fixed at the value determined by fluorescence spectroscopy ($k_1 = 1.6 \times 10^{-3} \text{ s}^{-1}$) and an association rate constant of $k_2 = 1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ was used (Fig. 3, solid lines). Thus, reactivation kinetics of the glycosylated invertase variants are well described by a consecutive uni-bimolecular reaction mechanism.

Temperature dependence of invertase reactivation

Glycosylation of yeast invertase increases the resistance of the enzyme against thermal inactivation and its stability against denaturants and also suppresses aggregation during refolding at elevated protein concentration (Kern et al., 1992b). For a number of proteins, aggregation as the primary off-pathway reaction competing with folding is not only observed at high protein concentration but is favored strongly at elevated temperature (Mitraki & King, 1989; Seckler & Jaenicke, 1992). As shown in Figure 4D, a strong decrease of the refolding yield at increasing tem-

Fig. 4. Temperature dependence of invertase reactivation kinetics and yields. **A–C:** Reactivation kinetics at 5 °C (○), 10 °C (●), 20 °C (▽), and 30 °C (▼) of core-glycosylated (A), external (B), and internal invertase (C). The protein concentration was 3 $\mu\text{g}/\text{mL}$, and the final yields after 48 h were set to 100%. The solid lines represent numerical simulations according to a sequential uni-bimolecular folding mechanism using the first-order rate constants determined from the kinetics of fluorescence change at the respective temperature, and the following bimolecular rate constants selected to fit the data for the glycosylated enzymes: 5 °C ($k_1 = 3.4 \times 10^{-4} \text{ s}^{-1}$; $k_2 = 5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$), 10 °C ($k_1 = 6.0 \times 10^{-4} \text{ s}^{-1}$; $k_2 = 8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$), 20 °C ($k_1 = 1.6 \times 10^{-3} \text{ s}^{-1}$; $k_2 = 2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$), and 30 °C ($k_1 = 3.8 \times 10^{-3} \text{ s}^{-1}$; $k_2 = 7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). **D:** Reactivation yields of internal (□), core-glycosylated (○), and external (▽) invertase under the conditions used in A–C.

perature was indeed observed for the nonglycosylated invertase (□) but not for the core-glycosylated (○) and the high-mannose-glycosylated (▽) forms of the enzyme. Although reconstitution yields of all variants at 5–10 °C were identical within experimental error ($60 \pm 10\%$), reconstitution of the nonglycosylated enzyme was progressively less efficient above 20 °C. At 40 °C, no reconstitution of invertase activity was observed for the nonglycosylated form at a protein concentration of 3 $\mu\text{g}/\text{mL}$ (51 nM invertase subunits), whereas reconstitution yields for the glycosylated forms were 65–80%. Obviously, glycosyla-

tion protects invertase from premature aggregation during refolding at high protein concentration and high temperature. This protection may result from (1) increased solubility of the glycosylated folding intermediates, and (2) alterations in the folding pathway. To determine whether altered rates of folding or association contribute to the dramatic difference between reconstitution yields of glycosylated and nonglycosylated invertase at elevated temperature, reconstitution kinetics were measured at varied temperature (5–30 °C) for the three invertase glycosylation variants. For the core-glycosylated (Fig. 4A) and for the high-mannose-glycosylated enzyme (Fig. 4B), the observed reconstitution kinetics at all temperatures coincided within experimental error. The data for the glycosylated forms coincided fairly well with simulations according to the above uni-bimolecular scheme when the first-order rate constant of subunit folding at the respective temperature was fixed at the value determined spectroscopically (cf. Fig. 2B). The association rate constants used in the simulations (solid lines in Fig. 4) mark an activation enthalpy of $\Delta H^\ddagger \approx 70$ kJ/mol. At all temperatures, reactivation kinetics of the nonglycosylated invertase were slightly slower than those of the glycosylated invertase forms. Significant deviations from the simulations were observed at low temperature (Fig. 4C). Because side reactions of folding intermediates forming inactive aggregates or adsorbing to vessel walls complicate reactivation kinetics of the nonglycosylated enzyme at elevated temperature (Fig. 4D), no further quantitative analysis was attempted.

No evidence for slow reshuffling at the dimer level

Slow folding reactions have often been observed to follow subunit association during reconstitution of oligomeric proteins (Jaenicke, 1987). Such reshuffling reactions may exhibit very high activation enthalpies and thus become rate limiting during refolding at low temperature (Goldenberg & King, 1982; Fuchs et al., 1991; Rehder & Jaenicke, 1993). To test for the occurrence of a dimeric low-temperature intermediate, reconstitution of core-glycosylated invertase was initiated at 5 °C and the reappearance of enzymatic activity was followed (Fig. 5). After 2 h, when reactivation was about 40% completed, the sample was shifted to 30 °C. The kinetics of reactivation after the temperature shift coincided with the trace observed after initiation of reconstitution at 30 °C, suggesting that reactivation coincides with subunit association, and no partially folded dimer is accumulated at low temperature. The uniform subunit size of core-glycosylated invertase allowed confirmation of this conclusion by size-exclusion HPLC, measuring subunit association directly (Fig. 5B). Upon initiation of refolding at 5 °C, the formation of dimers closely matched the reappearance of enzymatic activity during the first 2 h of the reactivation experiment (Fig. 5, cf. ● in A and B). A similar analysis attempted

Fig. 5. Subunit association and test for slow reshuffling at the dimer level during refolding of core-glycosylated invertase. **A:** Reactivation kinetics at 30 °C (○) and 5 °C with a temperature shift to 30 °C after 120 min (●). The protein concentration was 10 µg/mL. **B:** Reassociation kinetics of core-glycosylated invertase at 5 °C under the conditions preceding the temperature shift in A. After the times indicated, samples of 20 µL were injected onto a size-exclusion HPLC column in order to follow changes in monomer (○), dimer (●), and tetramer (▽) concentrations.

for internal invertase failed because of the tendency of the nonglycosylated subunits to adsorb to the column material.

Discussion

Reconstitution of glycosylated invertase after solvent denaturation follows a simple uni-bimolecular mechanism of consecutive subunit folding and association reactions. As is common for large proteins (Jaenicke, 1987; Seckler & Jaenicke, 1992), multiphasic subunit folding was observed. Transfer of tryptophans into a hydrophobic environment and the bulk of secondary structure formation occurred within the mixing time. This fast reaction was followed on the minutes time scale by a rate-limiting subunit folding reaction indicated by an increase in fluorescence quantum yield and connected with a high activation enthalpy ($\Delta H^\ddagger = 64$ kJ/mol). Although subunit folding was not accelerated by PPLase, this does not exclude *cis-trans* isomerization of proline residues from being involved in the slow-folding reaction. This is because PPLase efficiently catalyzes the isomerization only of peptide bonds readily accessible in folding intermediates (Schmid, 1993; Schmid et al., 1993). Reactivation of the dimeric enzyme is protein-concentration dependent and coincides with subunit association. Because of the uni-

form size of the core-glycosylated subunits, this could be directly demonstrated by size-exclusion HPLC.

The present comparison of refolding and association kinetics extends previous results on the stability and refolding of yeast invertase glycosylation variants, which showed that the core-glycosylation occurring in the endoplasmic reticulum is sufficient to protect the enzyme against irreversible aggregation during unfolding or refolding and to stabilize the protein against thermal denaturation (Kern et al., 1992b). Reactivation, as well as spectroscopically observed changes, occurred with identical kinetics for the core-glycosylated secretion intermediate, and the high-mannose-glycosylated external invertase with carbohydrate contents of $\approx 35\%$ and $\approx 65\%$, respectively. Thus, in contrast to earlier suggestions (Schülke & Schmid, 1988b), extensive glycosylation of the external enzyme does not retard folding or association compared to the form that folds and associates in the endoplasmic reticulum *in vivo*.

The lack of glycosylation in the significantly less stable cytoplasmic form of invertase (Kern et al., 1992b) also does not alter subunit folding kinetics. However, significantly reduced overall rates of reactivation were observed for the nonglycosylated enzyme. This effect was most prominent at low temperature, where reactivation yields of all invertase forms are high. Thus, the differences in reactivation kinetics do not appear to be the consequence of side reactions, but rather of altered association rates or of a (spectroscopically undetected) folding reaction that becomes rate determining in the case of the nonglycosylated enzyme. Further analysis by size-exclusion HPLC was hampered by the tendency of the nonglycosylated subunits to adsorb to the column material.

Although all invertase forms refolded with similar yields at low temperature, the yields differed progressively with increasing temperature. For the glycosylated invertase forms, yields of refolding were essentially independent of temperature between 5 and 40 °C. Folding yields of the nonglycosylated enzyme, however, strongly decreased with increasing temperature until, at 40 °C, no significant reactivation was observed. As shown previously, low folding yields for the internal enzyme are due to competing irreversible aggregation; they can be increased by the GroEL/S chaperone proteins (Kern et al., 1992a).

An earlier characterization of the glycoprotein by protein-chemical methods revealed that, on the average, 2 of the 9–10 carbohydrate chains in glycosylated invertase are inaccessible to endoglycosidases in the native enzyme (Trimble et al., 1983; Ziegler et al., 1988). These two chains are sufficient for the stabilizing effect of glycosylation (G. Kern & R. Jaenicke, unpubl.). As shown in the present study, subunit folding kinetics are unaffected, whereas reactivation of invertase dimers is accelerated by glycosylation. This suggests that the inaccessible chains become buried during association of dimers at the subunit interfaces rather than during subunit folding.

Materials and methods

Enzymes

Nonglycosylated, core-glycosylated, and high-mannose-glycosylated forms of invertase were obtained from *Saccharomyces cerevisiae* strain SEY 5188 carrying the *suc2* gene on plasmid pRT51.83 (Roitsch & Lehle, 1989). Purification and determination of enzymatic activity were performed as described by Kern et al. (1992b). PPIase from pig kidney was purified as described (Fischer et al., 1984). Protein concentrations refer to the polypeptide parts of the enzymes and were determined spectroscopically using specific absorbances of $A_{280\text{nm}}^{1\text{mg/mL}} = 2.25$ for invertase and $A_{280\text{nm}}^{1\text{mg/mL}} = 0.457$ for PPIase.

Unfolding and refolding

Denaturation was performed by incubation in 0.05 M sodium acetate, 5.3 M ultrapure guanidinium chloride (ICN-Schwarz/Mann), pH 5.0, at 20 °C for ≥ 1 h. Prior to refolding, the sample of denatured protein and the dilution buffer were equilibrated at the refolding temperature. Renaturation was initiated by a 1:80 dilution of the denatured protein into 0.05 M sodium citrate, 0.05 M sodium phosphate, pH 7.0.

Spectroscopy

Fluorescence was measured in stirred quartz cells employing a Perkin-Elmer LS-5B luminescence spectrometer with thermostatted cell holder. The temperature was monitored inside the cell using a telethermometer. For digital data registration, the spectrometer was interfaced to a personal computer through an AN-2400-2-0-D digital voltmeter (Analogic, Peabody, Massachusetts). An excitation wavelength of 280 nm was used, and the spectral bandpass was 5 nm for both excitation and emission.

CD was measured using thermostatted cylindrical cells with a 1-mm optical pathlength in a Jasco J-500 A spectropolarimeter. A mean residue molar mass of 114.2 g/mol was used to calculate residue ellipticities.

All spectroscopic signals were corrected for buffer background using mock samples lacking the protein.

HPLC

Size-exclusion chromatography made use of a TSK G3000SW HPLC column (7.5 $\times$ 300 mm) and a Kontron model 420 inert pump at 5 °C. The flow rate was 0.5 mL/min, and eluting protein was detected by its fluorescence at 325 nm with excitation at 280 nm. The fluorescence detector (E. Merck-Hitachi F1000) was connected to a Spectra-Physics 4270 Integrator for evaluation of peak areas.

Regression and numerical simulations

To determine subunit folding rate constants, single-exponential functions were fitted to the fluorescence data. Nonlinear regression made use of the Marquardt-Levenberg algorithm built into the commercial graphics program SigmaPlot (Jandel Scientific, San Rafael, California) with default settings. To estimate second-order rate constants of the association reaction, the measured first-order rate constants were used in numerical simulations of invertase reactivation kinetics according to a scheme of consecutive first- and second-order reactions ($2A \rightarrow 2B \rightarrow C$), where only C is an active species. The step size used in the simulations was 0.24 min.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 43-A10, Se 517/5-1) and the Fonds der Chemischen Industrie.

References

- Beintema, J.J. (1986). Do asparagine-linked carbohydrate chains in glycoproteins have a preference for β -bends? *Biosci. Rep.* 6, 709–714.
- Brandts, J.F. & Lin, L.-N. (1986). Proline isomerization studied with proteolytic enzymes. *Methods Enzymol.* 131, 107–126.
- 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.
- 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., Bang, H., & Mech, C. (1984). Nachweis einer Enzymkatalyse für die *cis-trans*-Isomerisierung der Peptidbindung in Prolin-haltigen Peptiden. *Biomed. Biochim. Acta* 43, 1101–1111.
- Fuchs, A., Seiderer, C., & Seckler, R. (1991). In vitro folding pathway of phage P22 tailspike protein. *Biochemistry* 30, 6598–6604.
- Gething, M.-J. & Sambrook, J. (1990). Transport and assembly processes in the endoplasmic reticulum. *Semin. Cell Biol.* 1, 65–72.
- Gething, M.-J. & Sambrook, J. (1992). Protein folding in the cell. *Nature* 355, 33–45.
- Goldenberg, D. & King J. (1982). Trimeric intermediate in the in vivo folding and subunit assembly of the tail spike endorhamnosidase of bacteriophage P22. *Proc. Natl. Acad. Sci. USA* 79, 3403–3407.
- Grafl, R., Lang, K., Vogl, H., & Schmid, F.X. (1987). The mechanism of folding of pancreatic ribonucleases is independent of the presence of covalently linked carbohydrate. *J. Biol. Chem.* 262, 10624–10629.
- Gross, V., Steube, K., Tran-Thi, T.-A., Häussinger, D., Legler, G., Decker, K., Heinrich, P.C., & Gerok, W. (1987). The role of N-glycosylation for the plasma clearance of rat liver secretory glycoproteins. *Eur. J. Biochem.* 162, 83–88.
- Hurtley, S.M. & Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5, 277–307.
- Jaenicke, R. (1987). Folding and association of proteins. *Prog. Biophys. Mol. Biol.* 49, 117–237.
- Julius, D., Schekman, R., & Thorner, J. (1984). Glycosylation and processing of prepro- α -factor through the yeast secretory pathway. *Cell* 36, 309–318.
- Kern, G., Schmidt, M., Buchner, J., & Jaenicke, R. (1992a). Glycosylation inhibits the interaction of invertase with the chaperone GroEL. *FEBS Lett.* 305, 203–205.
- Kern, G., Schülke, N., Schmid, F.X., & Jaenicke, R. (1992b). Stability, quaternary structure, and folding of internal, external, and core-glycosylated invertase from yeast. *Protein Sci.* 1, 120–131.
- Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54, 631–664.
- Mitraki, A. & King, J. (1989). Protein folding intermediates and inclusion body formation. *Bio/Technology* 7, 690–697.
- 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.
- Paulson, J.C. (1989). Glycoproteins: What are the sugar chains for? *Trends Biochem. Sci.* 14, 272–276.
- Rehaber, V. & Jaenicke, R. (1993). The low-temperature folding intermediate of hyperthermophilic D-glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* shows a native-like cooperative unfolding transition. *FEBS Lett.* 317, 163–166.
- Roitsch, T. & Lehle, L. (1989). Structural requirements for protein N-glycosylation. Influence of acceptor peptides on cotranslational glycosylation of yeast invertase and site-directed mutagenesis around a sequon sequence. *Eur. J. Biochem.* 181, 525–529.
- Schekman, R. (1982). The secretory pathway in yeast. *Trends Biochem. Sci.* 7, 243–246.
- Schekman, R. (1985). Protein localization and membrane traffic in yeast. *Annu. Rev. Cell Biol.* 1, 115–143.
- Schmid, F.X. (1993). Prolyl isomerase: Enzymatic catalysis of slow protein-folding reactions. *Annu. Rev. Biophys. Biomol. Struct.* 22, 123–143.
- Schmid, F.X., Mayr, L., Mücke, M., & Schönbrunner, E.R. (1993). Prolyl isomerases: Their role in protein folding. *Adv. Protein Chem.* 44, 25–66.
- Schönbrunner, E.R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N., & Schmid, F.X. (1991). Catalysis of protein folding by cyclophilins from different species. *J. Biol. Chem.* 266, 3630–3635.
- 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, 8832–8837.
- Seckler, R. & Jaenicke, R. (1992). Protein folding and protein refolding. *FASEB J.* 6, 2545–2552.
- Sharon, N. & Lis, H. (1982). Glycoproteins. In *The Proteins*, 3rd Ed., Vol. V (Neurath, H. & Hill, R.L., Eds.), pp. 1–144. Academic Press, New York.
- Trimble, R.B., Maley, F., & Chu, F.K. (1983). Glycoprotein biosynthesis in yeast. Protein conformation affects processing of high mannose oligosaccharides on carboxypeptidase Y and invertase. *J. Biol. Chem.* 258, 2562–2567.
- Winther, J.R., Stevens, T.H., & Kielland-Brandt, M.C. (1991). Yeast carboxypeptidase Y requires glycosylation for efficient intracellular transport, but not for vacuolar sorting, in vivo stability, or activity. *Eur. J. Biochem.* 197, 681–689.
- Ziegler, F.D., Maley, F., & Trimble, R. (1988). Characterization of the glycosylation sites in yeast external invertase. II. Location of the endo- β -N-acetylglucosaminidase H-resistant sequons. *J. Biol. Chem.* 263, 6986–6992.