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Reconstitution of a Heat Shock Effect in Vitro: Influence of GroE on the Thermal Aggregation of α-Glucosidase from Yeast

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ABSTRACT: α-Glucosidase from yeast is inactivated rapidly at temperatures above 42 °C. The thermal inactivation is accompanied by aggregation. The molecular chaperone GroEL suppresses the formation of aggregates by binding the thermally inactivated α-glucosidase. Spectroscopic studies suggest that GroEL binds α-glucosidase in an intermediately folded state. The complex between α-glucosidase and GroEL can be dissolved by MgATP. GroES accelerates the MgATP-dependent dissociation of the α-glucosidase–GroEL complex. At elevated temperatures this release leads to the formation of aggregates, while at lower temperatures native, enzymatically active molecules are formed.

All organisms respond to an increase of the growth temperature above the physiological level by inducing the synthesis of a number of highly conserved proteins (Lindquist & Craig, 1988). It has been proposed that these heat shock proteins are involved in maintaining cellular proteins in their native form or dissolve protein aggregates formed at elevated temperatures (Pelham, 1986). To describe the protective role of some members of the heat shock protein family in protein structure formation (without becoming part of the final structure), the term molecular chaperone has been coined [cf. Ellis (1987) and Ellis and van der Vies (1991)].

The *Escherichia coli* heat shock proteins GroEL[1] and GroES are most thoroughly characterized in terms of both structure and function. GroEL in its native form is a homotetradecamer. The subunits (M_r = 57 000) are arranged in a double ring with 7-fold symmetry (Hendrix, 1979). GroES (M_r = 10 000) is a homoheptamer which also exhibits a ring-like quaternary structure (Chandrasekhar et al., 1986; Tilly & Georgopoulos, 1982). The GroEL and GroES genes, constituting the GroE operon, are members of the heat shock regulon of *E. coli* (Georgopoulos & Eisen, 1974; Fayet et al., 1986). Synthesis of the GroE protein, which accounts for approximately 1% of the cellular protein at 37 °C, increases to about 10% of the total protein content upon shifting the growth temperature to 46 °C (Herendeen et al., 1979; Hemmingsen et al., 1988). The GroE gene products must therefore play an essential role in the cellular response to thermal stress. However, both GroEL and GroES are also necessary for bacterial growth and viability at low temperatures (Fayet et al., 1989).

Recent reports demonstrate that the GroE system participates in protein folding (and association) both in vivo and in vitro (Goloubinoff et al., 1989a,b; Laminet et al., 1990; Viitanen et al., 1990; Buchner et al., 1991; Martin et al., 1991; Mendoza et al., 1991). Taken together, these studies have shown that GroE facilitates renaturation of various monomeric or oligomeric proteins in vitro in a MgATP-dependent fashion. Nonnative proteins form a stable complex with GroEL, which inhibits both their folding to the native state and their ac-

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; DTE, dithioerythritol; GroE, complex of GroEL and GroES; GroEL, tetradecamer of the 57-kDa *Escherichia coli* heat shock protein, which is a member of the chaperonin 60 (cpn 60) protein family; GroES, heptamer of the 10-kDa *E. coli* heat shock protein, which is a member of the chaperonin 10 (cpn 10) protein family; *p*-NPG, *p*-nitrophenyl α-D-glucopyranoside; Rubisco, ribulose-1,5-bisphosphate carboxylase.

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companying unproductive aggregation reactions (Buchner et al., 1991). Most probably, GroEL binds folding intermediates which exhibit structural features of a "molten globule" state (Martin et al., 1991). Release of the protein from GroEL is achieved in the presence of MgATP, K^+ ions, and, depending on the protein, GroES (Viitanen et al., 1990). Participation of the GroE gene products in protein-folding processes may be the vital function of these heat shock proteins under physiological temperature conditions.

In the present report, we describe the interaction of GroE with a thermally inactivated protein. For this functional in vitro analysis of heat shock proteins at elevated temperatures, we use α-glucosidase as a substrate. Monomeric α-glucosidase from yeast (M_r = 68 000) is rapidly inactivated at elevated temperatures (i.e., ≥42 °C). The inactive molecules show a strong tendency to precipitate. GroEL has no effect on the time course of the inactivation process. However, it completely suppresses aggregation of thermally inactivated α-glucosidase at equimolar concentrations. Addition of MgATP at elevated temperatures leads to the release and subsequent aggregation of α-glucosidase, while at lower temperatures (i.e., 25 °C) the released protein is able to regain its enzymatic activity. GroES accelerates the MgATP-dependent dissociation of the α-glucosidase–GroEL complex. Fluorescence measurements indicate that the thermally inactivated α-glucosidase molecules interacting with GroEL are not completely unfolded but rather are in a partially folded conformation.

Experimental Procedures

Materials

Purified α-glucosidase (maltase, EC 3.2.1.20) from yeast with a specific activity of >130 units/mg was a gift from Dr. A. Grossmann (Boehringer Mannheim GmbH). Yeast α-glucosidase is a cytoplasmic enzyme, which is necessary for growth on maltose as the sole carbon source. *Saccharomyces cerevisiae* strain BY 85 contains two isoforms of the enzyme, PI and PII, which can be distinguished by their isoelectric points (Kopetzki et al., 1989a). Here we used α-glucosidase PI, which consists of 583 amino acid residues (M_r = 68 000). The native monomeric protein contains four cysteine residues which are not engaged in disulfide bonds (Kopetzki et al., 1989b).

GroEL and GroES were purified as described (Buchner et al., 1991). Molar concentrations are calculated for the GroEL tetradecamer (M_r = 14 × 57 000) and for the GroES heptamer (M_r = 7 × 10 000).

ATP and *p*-NPG were from Boehringer Mannheim. Nile Red was obtained from Eastman Kodak. All reagents used were of analytical grade.

Methods

Protein Concentration. The protein concentration of GroEL, GroES, and α-glucosidase was determined according to Bradford (1976) using bovine serum albumin as standard.

Activity Assay. The enzymatic activity of α-glucosidase was determined as described (Kopetzki et al., 1989b). In short, the change in absorption observed upon release of *p*-nitrophenol from *p*-NPG at 30 °C was monitored at 405 nm using an Eppendorf photometer, Model 1101.

Denaturation/Renaturation. α-Glucosidase was denatured in 8 M urea, 10 mM potassium phosphate, 1 mM EDTA, and 2 mM DTE, pH 7.0, for 1 h at room temperature. Renaturation was achieved by diluting denatured α-glucosidase 100-fold into 0.1 M Tris-HCl, pH 7.6 at 25 °C. To analyze the kinetics of reactivation, aliquots were taken at defined times.

Light Scattering. Light scattering was followed using a Hitachi F-4000 fluorescence spectrophotometer equipped with a temperature-controlled cell holder and a magnetic stirrer. The excitation and emission wavelengths were set at 360 nm. The excitation and emission slits were set at 5 nm. Aggregation kinetics were recorded under stirring. The reaction was started by adding α-glucosidase to a solution preequilibrated at the given temperature.

Intrinsic Protein Fluorescence. Fluorescence spectra were recorded in a Hitachi F-4000 spectrophotometer. The excitation wavelength was 280 nm. Excitation and emission slits were set at 5 nm.

Results

Thermal Inactivation of α-Glucosidase. Previous studies have shown that the α-glucosidases from yeast are temperature-sensitive enzymes (Halvorson & Ellias, 1958; Khan & Eaton, 1967; Tabata et al., 1984). Incubation at elevated temperatures leads to inactivation of α-glucosidase PI from bakers' yeast (Figure 1A). The time course of inactivation, which follows first-order kinetics, is strongly temperature-dependent. At 52 °C, α-glucosidase loses its activity with an estimated half-time of 1 min, while at 46 °C the half-time of inactivation is about 15 min. Light scattering measurements show that inactivation of the enzyme leads to aggregation (Figure 1B). Inactivation and aggregation occur in the same time range. Since thermal inactivation and aggregation are consecutive processes, the increase in light scattering exhibits a sigmoidal time course.

Thermal Inactivation of α-Glucosidase in the Presence of GroEL. The kinetics of inactivation of α-glucosidase are not affected by the presence of GroEL (Figure 2A). While the chaperone protein does not protect α-glucosidase from inactivation, it does however prevent the aggregation of the thermally inactivated enzyme (Figure 2B). To determine the stoichiometry of GroEL and α-glucosidase necessary for complete inhibition of aggregation, light scattering measurements were performed in the presence of different concentrations of GroEL. As shown in Figure 2C, a small molar excess of GroEL over α-glucosidase is sufficient for total suppression of aggregation. At a molar ratio of GroEL to α-glucosidase of 1.5:1 to 2:1, no increase in light scattering is observed. At equimolar concentrations of GroEL and α-glucosidase only a slight increase in light scattering is detected. Substoichiometric amounts of GroEL (i.e., 0.5:1, 0.25:1) slow down the aggregation of α-glucosidase, but aggregation is not suppressed (Figure 2C). These data imply the formation of a complex between thermally inactivated α-glucosidase and GroEL. While associated with GroEL, α-gluosidase cannot undergo unspecific aggregation reactions.

In the presence of K^+ ions and MgATP, however, aggregation processes occur comparable to those observed in the absence of GroEL. Therefore, one may conclude that no stable complex between GroEL and α-glucosidase can be formed under these conditions. The results show that the suppression of aggregation by GroEL is a specific effect of the chaperone protein GroEL. Control experiments with bovine serum albumin at high molar excess do not show a comparable suppression of the aggregation of α-glucosidase at elevated temperatures (data not shown).

Release of Thermally Inactivated α-Glucosidase Bound to GroEL. Light scattering measurements show that GroEL is sufficient to prevent aggregation of α-glucosidase during thermal stress. The complex of thermally inactivated α-glucosidase and GroEL can be dissociated by addition of GroES, MgATP, and K^+ ions. Release of α-glucosidase from GroEL

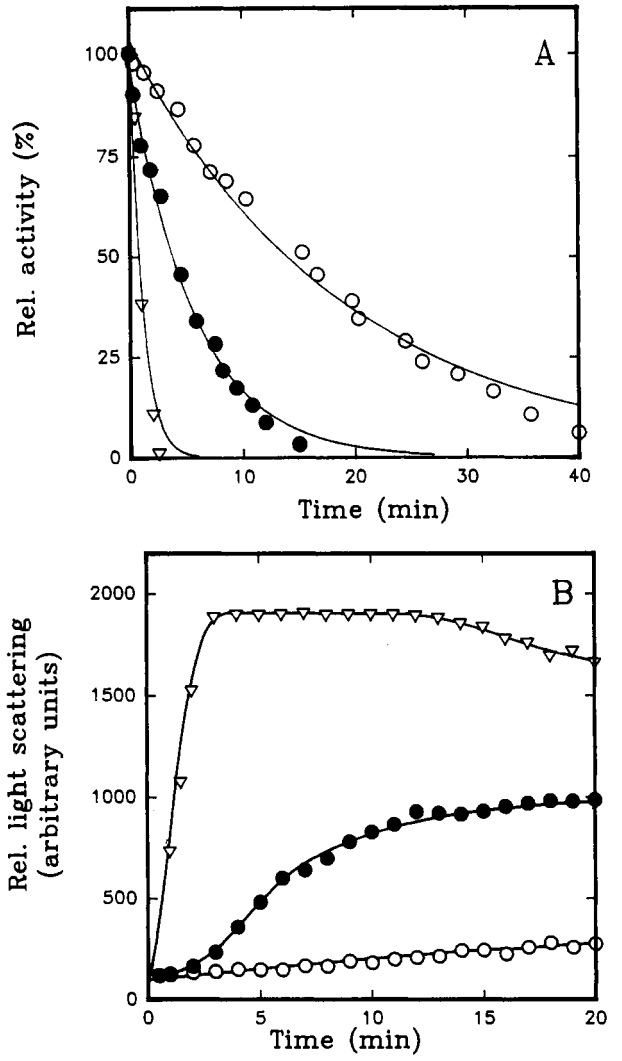

FIGURE 1: Thermal inactivation and aggregation of α-glucosidase. (A) Time course of thermal inactivation of α-glucosidase. α-Glucosidase was diluted to a final concentration of 147 nM in 0.1 M Tris-HCl, pH 7.6. Activity assays were performed after various times of incubation at 46.3 °C (○), 48.7 °C (●), and 52.5 °C (▽). Full lines are calculated for first-order reactions using the following rate constants: 8.7×10^{-4} s^{-1} at 46.5 °C, 3.0×10^{-3} s^{-1} at 48.7 °C, and 1.6×10^{-2} s^{-1} at 52.5 °C. (B) Kinetics of aggregation of α-glucosidase as determined by light scattering. α-Glucosidase was diluted to a final concentration of 147 nM in 0.1 M Tris-HCl, pH 7.6, equilibrated at 41.2 °C (○), 46.7 °C (●), and 49.2 °C (▽).

at elevated temperatures leads to precipitation, as shown by the increase in light scattering (Figure 3A). After addition of both GroES and MgATP, a rapid dissociation of the α-glucosidase–GroEL complex is observed. Addition of MgATP in the absence of GroES, however, leads to a slow release of α-glucosidase, as shown by the slow increase in light scattering. Subsequent addition of GroES accelerates the dissociation reaction. In the absence of MgATP, GroES does not induce the dissociation of the α-glucosidase–GroEL complex (data not shown). All these experiments were carried out in the presence of K^+ ions. Like the chaperone-mediated refolding of Rubisco (Viitanen et al., 1990) the MgATP-driven dissociation of the complex is dependent on K^+ ions (data not shown).

To determine the amount of GroES which is necessary for rapid dissociation of the α-glucosidase–GroEL complex, various amounts of GroES were added to the preformed complex together with a constant amount of MgATP (Figure 3B). At a molar ratio of 1:0.15 (GroEL:GroES) the release reaction is already significantly accelerated as compared to

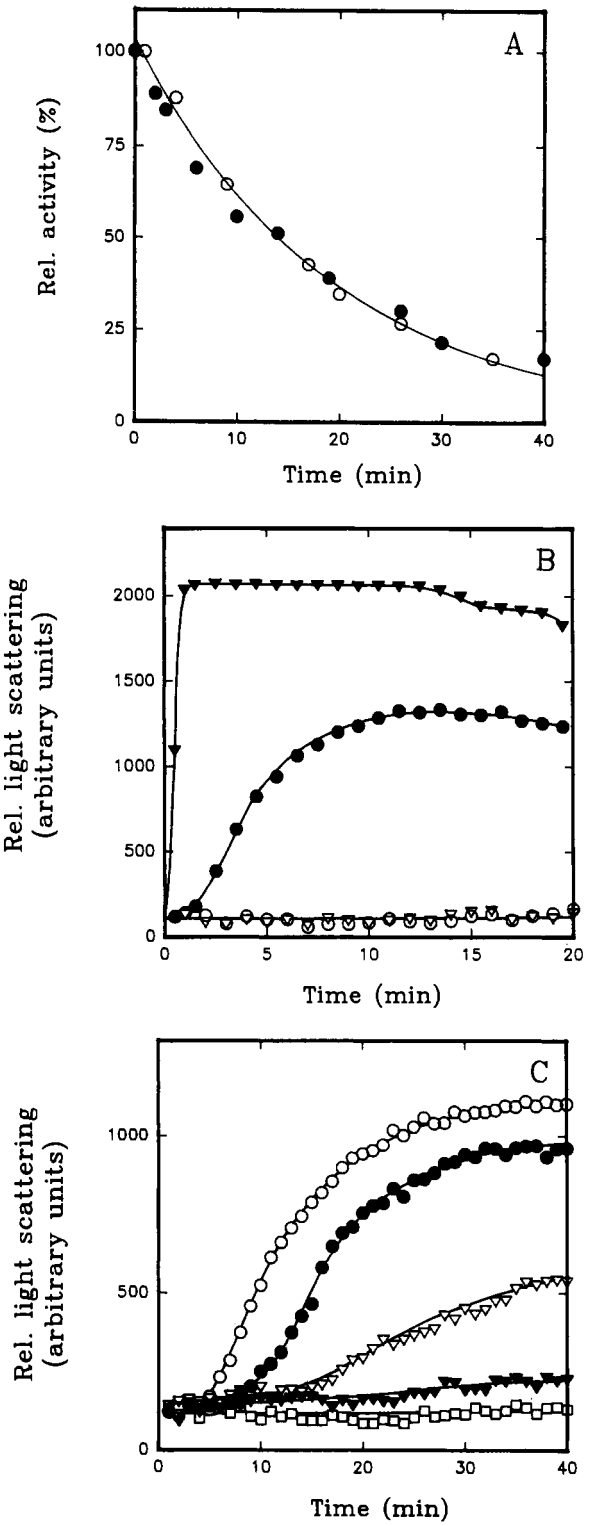

FIGURE 2: Thermal inactivation and aggregation of α-glucosidase in the presence of GroEL. (A) Thermal inactivation of α-glucosidase in the presence of GroEL. α-Glucosidase was diluted to a final concentration of 147 nM in 0.1 M Tris-HCl, pH 7.6, in the absence (●) and presence (○) of 220 nM GroEL. Activity measurements were performed after various times of incubation at 46.5 °C. The full line is calculated for a first-order reaction with a rate constant of 8.7×10^{-4} s^{-1}. (B) Suppression of the thermal aggregation of α-glucosidase by GroEL. α-Glucosidase was diluted to a final concentration of 147 nM in 0.1 M Tris-HCl, pH 7.6, equilibrated at 47.5 °C (○, ●) or 52.5 °C (▽, ▼), in the absence (closed symbols) or presence (open symbols) of 220 nM GroEL. (C) Suppression of the thermal aggregation of α-glucosidase at various ratios of GroEL:α-glucosidase. α-Glucosidase was diluted to a final concentration of 147 nM in 0.1 M Tris-HCl and 10 mM KCl, pH 7.6, equilibrated at 47 °C, in the presence of 37 nM (○), 74 nM (●), 147 nM (▽), 220 nM (▼), or 290 nM (□) GroEL.

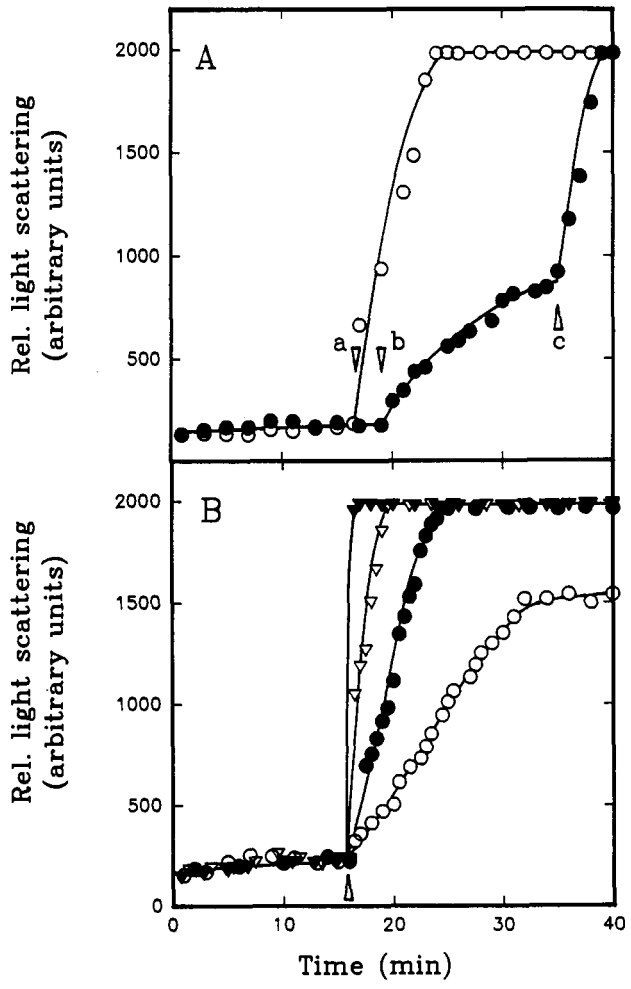

FIGURE 3: Dissociation of the α-glucosidase–GroEL complex. (A) Dissociation of the α-glucosidase–GroEL complex by MgATP and GroES. α-Glucosidase (147 nM) and GroEL (220 nM) were incubated in 0.1 M Tris-HCl and 10 mM KCl, pH 7.6 at 47 °C. At the times indicated by arrowheads, the following components were added: (a) 2 mM ATP, 10 mM $MgCl_2$, *and* 147 nM GroES; (b) 2 mM ATP and 10 mM $MgCl_2$; (c) 147 nM GroES. (B) Acceleration of the dissociation of the α-glucosidase–GroEL complex by increasing concentrations of GroES. α-Glucosidase (147 nM) and GroEL (220 nM) were incubated in 0.1 M Tris-HCl and 10 mM KCl, pH 7.6 at 47 °C. After 17-min incubation 2 mM ATP, 10 mM $MgCl_2$, and the following concentrations of GroES were added: 22 nM (○), 54 nM (●), 109 nM (▽), and 438 nM (▼).

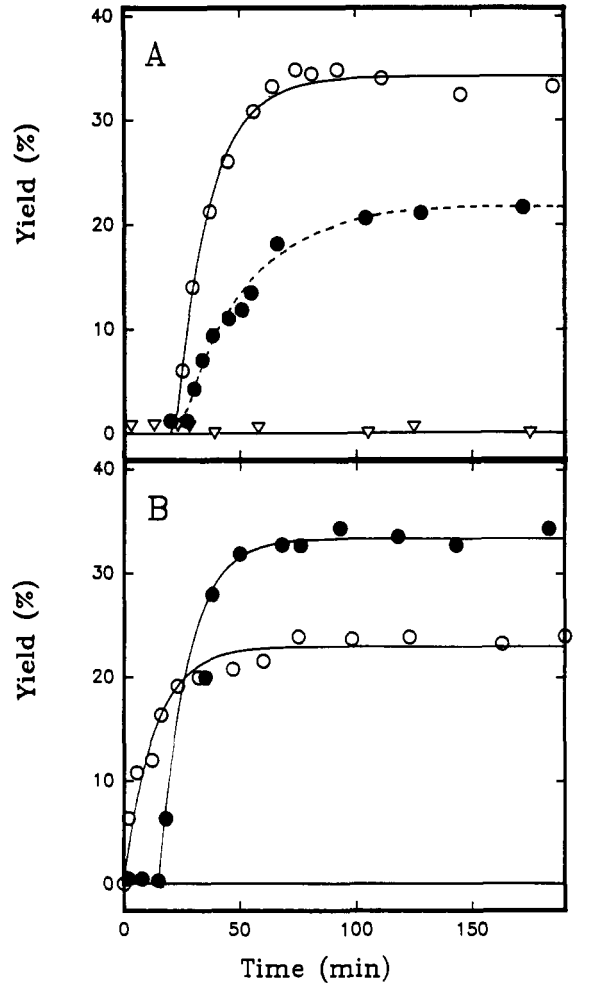

FIGURE 4: Reactivation of thermally inactivated and of chemically denatured α-glucosidase. (A) Reactivation of thermally inactivated α-glucosidase. α-Glucosidase (147 nM) and GroEL (147 nM) were incubated in 0.1 M Tris-HCl and 10 mM KCl, pH 7.6 at 47 °C, for 60 min. After being cooled to 25 °C, reactivation was determined in the absence of further additives (▽) and after addition of 2 mM ATP and 10 mM $MgCl_2$ (●) or 2 mM ATP, 10 mM $MgCl_2$, *and* 147 nM GroES (○). The full line is calculated for a first-order reaction determined by a rate constant of 1.1×10^{-3} s^{-1}. (B) Reactivation of chemically denatured α-glucosidase. α-Glucosidase was denatured in 8 M urea and renatured at a concentration of 147 nM as described under Experimental Procedures. Reactivation determined in the absence of chaperone proteins (○) is characterized by a first-order rate constant of 1.3×10^{-3} s^{-1}. Reactivation in the presence of 147 nM GroEL was initiated by addition of 2 mM ATP, 10 mM $MgCl_2$, 10 mM KCl, and 147 nM GroES (●). In this case the full line is calculated for a first-order reaction determined by a rate constant of 1.4×10^{-3} s^{-1}.

the dissociation induced by MgATP in the absence of GroES (cf. Figure 3A). Higher amounts of GroES cause a further increase of the rate of dissociation of the α-glucosidase–GroEL complex.

Reactivation of Thermally Inactivated α-Glucosidase. Incubation of α-glucosidase at elevated temperatures leads to irreversible precipitation, while binding to GroEL suppresses aggregation completely. The α-glucosidase–GroEL complex can be dissolved in a MgATP-dependent reaction. To investigate the possibility of functional reactivation of α-glucosidase bound to GroEL, complex formation was carried out at 47 °C for 60 min. Subsequent incubation of the complex at 25 °C (for up to 4 h) does not result in spontaneous reactivation of α-glucosidase (Figure 4A). However, release and reactivation of α-glucosidase can be achieved by addition of either MgATP or MgATP and GroES. In the presence of both MgATP and GroES reactivation follows first-order kinetics determined by a rate constant of 1.1×10^{-3} s^{-1}. In the absence of GroES, however, distinct differences are observed in the kinetics of reactivation: Both the rate and the yield of reactivation are significantly reduced (Figure 4A). Dissociation of the α-glucosidase–GroEL complex becomes rate-determining in the absence of GroES (cf. light scattering measurements, Figure 3). Therefore, both the decrease in rate and the slight sigmoidicity of the reactivation curve may derive from the consecutive mechanism of dissociation followed by refolding.

Reactivation of α-glucosidase after chemical denaturation in 8 M urea exhibits first-order kinetics with a rate constant of 1.3×10^{-3} s^{-1} (Figure 4B). By dilution of urea-denatured α-glucosidase in buffer containing GroEL, reactivation is completely inhibited. However, normal reactivation with a rate constant of 1.4×10^{-3} s^{-1} is observed after subsequent addition of MgATP and GroES (Figure 4B). In this case, the yield of reactivation is increased as compared to reactivation in the absence of the chaperone proteins. Within the limits of experimental error normally observed for folding kinetics,

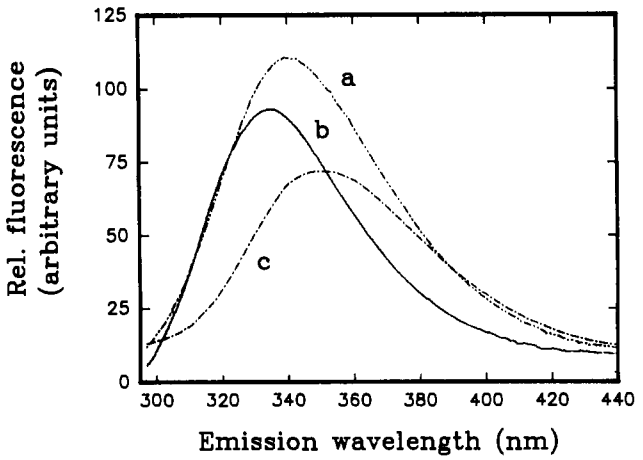

FIGURE 5: Fluorescence of thermally inactivated α-glucosidase bound to GroEL. The fluorescence of thermally inactivated α-glucosidase bound to GroEL (a) was determined after 60-min incubation of α-glucosidase (147 nM) and GroEL (220 nM) in 0.1 M Tris-HCl, pH 7.6 at 47 °C, and subsequent cooling to 25 °C. In order to obtain the fluorescence spectrum of bound α-glucosidase without contributions from GroEL (which does not contain tryptophan residues), the spectrum of GroEL was subtracted from the fluorescence spectrum of the complex. Fluorescence spectra of native (b) and denatured (c) α-glucosidase were recorded at 25 °C in the absence or presence of 8 M urea, respectively.

identical rate constants are obtained for reactivation of thermally and of chemically denatured α-glucosidase.

Conformation of Thermally Inactivated α-Glucosidase Bound to GroEL. In order to investigate the conformational state of α-glucosidase bound to GroEL, fluorescence spectra of the native and denatured enzyme are compared to the fluorescence of thermally inactivated α-glucosidase bound to GroEL (Figure 5). Due to its strong tendency to aggregate, spectra of the thermally inactivated protein cannot be recorded in the absence of GroEL. Native α-glucosidase shows a maximum of fluorescence emission at 334 nm. In 8 M urea, the emission maximum is shifted to 350 nm, which corresponds to the fluorescence maximum of tryptophan in aqueous solution. The spectrum of the complexed form of α-glucosidase exhibits a maximum of fluorescence emission at 340 nm. The fluorescence intensity of the complexed enzyme is higher than that of the native protein. This result implies that the protein bound to GroEL is neither completely unfolded nor nativelike. The increase in fluorescence intensity may be due to the changes in the environment of the chromophores of α-glucosidase induced upon thermal unfolding or it may reflect interactions of the partialy unfolded protein with GroEL.

Discussion

The enzymatic activity of α-glucosidase from yeast decreases rapidly during incubation at elevated temperatures (i.e., ≥42 °C). The reaction is irreversible since no activity is regained by downshifting the temperature after previous thermal inactivation of the enzyme. As shown by light scattering, inactivation is accompanied by aggregation processes. The kinetics of both inactivation and aggregation are strongly dependent on the incubation temperature employed. The temperature range applied here corresponds to the range where the heat shock response is observed in vivo in mesophilic organisms (Lindquist & Craig, 1988). Since a number of proteins are denatured under these conditions, it has been proposed that heat shock proteins help to protect cellular proteins from irreversible damage (Pelham, 1986; Ellis & van der Vies, 1991). The ability of GroE to prevent aggregation of nonnative proteins during renaturation has been shown for citrate synthase (Buchner et al., 1991). Interestingly, aggregates formed in the absence of GroE cannot be disintegrated by GroE. We therefore assume that GroEL has to be present during stress conditions in order to protect nonnative proteins from interacting unspecifically. To test this hypothesis, we use heat inactivation of monomeric α-glucosidase as a model system. While α-glucosidase is rapidly inactivated at temperatures above 42 °C, GroEL as well as GroES is stable up to 60 °C (Lissin et al., 1990; Schmidt, 1991). GroEL present during the inactivation process of α-glucosidase does not change the kinetics of inactivation nor does it influence the temperature dependence of the inactivation reaction. Therefore, GroEL has no stabilizing effect on α-glucosidase per se. However, GroEL completely suppresses the aggregation of thermally inactivated α-glucosidase. The complex between α-glucosidase and GroEL formed at elevated temperatures is very stable. Release of α-glucosidase is only possible in the presence of MgATP. GroES, as in the case of pre-β-lactamase (Laminet et al., 1990) and dihydrofolate reductase (Martin et al., 1991), is not necessarily required for this reaction, but it accelerates the process. In contrast, both Rubisco (Goloubinoff et al., 1988b) and citrate synthase (Buchner et al., 1991; Schmidt, 1991) require the presence of both MgATP and GroES for release from GroEL. We assume that this reflects differences in the binding of these proteins to GroEL.

α-Glucosidase released from GroEL at elevated temperatures aggregates rapidly. The time course and the extent of aggregation are comparable to the aggregation process observed in the absence of GroEL. If the release reaction is carried out at lower temperatures, α-glucosidase folds to its native, enzymatically active conformation. The time course and the yield of reactivation are the same for the renaturation of urea-denatured α-glucosidase and of the thermally inactivated, GroEL-bound protein after relase by MgATP and GroES. These results imply that rate-limiting folding steps, which determine the kinetics of reactivation, occur after the release of α-glucosidase. Reactivation in the presence of MgATP alone is slower, and the yield of active protein is significantly lower. Similarly, light scattering measurements show that the release of α-glucosidase at elevated temperatures is slow in the presence of MgATP as compared to the release in the presence of both MgATP and GroES. We therefore conclude that GroES increases the efficiency of the release reaction. In the presence of MgATP incomplete release and rebinding may interfere with reactivation similar to results obtained for the GroE-mediated folding of rhodanese (Martin et al., 1991).

At elevated temperatures, α-glucosidase is trapped by GroEL in an intermediately folded conformation, as indicated by the maximum emission wavelength and the intensity of the intrinsic fluorescence. As in the case of dihydrofolate reductase and rhodanese (Martin et al., 1991), the fluorescence properties of the thermally inactivated α-glucosidase bound to GroEL suggest that the protein may process a molten globule like conformation.

Previous reports on the function of the GroE complex were focused primarily on its interaction with proteins during in vitro folding. However, it is not known whether the refolding processes observed after chemical denaturation are equivalent to in vivo folding. Since heat shock proteins help organisms to survive under unfavorable environmental conditions, the function of chaperones should also be determined under stress conditions. In the present report, we show that GroE protects thermally inactivated protein from precipitation. α-Gluco-

sidase bound to GroEL is characterized by an intermediately folded conformation, differing from both the native and the denatured states. A more detailed description of the conformational properties is not possible on the basis of fluorescence measurements. At present, only high-resolution techniques such as nuclear magnetic resonance spectroscopy will allow an unambiguous structural analysis of proteins or peptides bound to GroEL (Landry & Gierasch, 1991).

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Registry No. Mg-ATP, 1476-84-2; K, 7440-09-7; α-glucosidase, 9001-42-7.

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