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Glycosylation inhibits the interaction of invertase with the chaperone GroEL

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During refolding and reassociation of chemically denatured non-glycosylated invertase from *Saccharomyces cerevisiae*, aggregation competes with correct folding, leading to low yields of reactivation (Kern et al. (1992) Protein Sci. 1, 120–131). In the presence of the chaperone GroEL, refolding is completely arrested. This suggests the formation of a stable complex between GroEL and non-native non-glycosylated invertase. Addition of MgATP results in a slow release of active invertase from the chaperone complex. When GroEL/ES and MgATP are present during refolding, the final reactivation yield increases from 14% to 36%. In contrast, refolding of the core-glycosylated and the high-mannose glycosylated forms of invertase is not arrested by GroEL. Only a short lag phase at the beginning of reactivation and a slightly increased reactivation yield (64% to 86% for core-glycosylated and 62% to 76% for external invertase) indicate a weak interaction of the glycosylated forms with the chaperone.

Chaperone; GroE; Folding; Aggregation; Glycosylation; Invertase

1. INTRODUCTION

Protein folding is assumed to depend exclusively on the primary structure of the polypeptide chain and the solvent conditions [1,2]. However, successful in vitro refolding of proteins is often limited by competition with aggregation [2,3]. Recently, 'helper proteins' have been identified which recognize non-native proteins supporting their correct structure formation [4–6]. The chaperone GroE from *Escherichia coli* is one of the best-characterized helper proteins. It effects protein folding both in vivo and in vitro [4,5,7,8]. The GroE system consists of two proteins, GroEL and GroES. The homotetradecamer GroEL binds chemically or thermally denatured proteins [5,6,9,10,11]. In most cases, their release is dependent on the heptameric GroES protein and MgATP. The mechanism of binding and release is still unclear. For a few proteins, GroE has been shown to suppress aggregation of non-native folding intermediates [6,9,12].

In order to investigate the influence of protein glycosylation on the interaction of non-native proteins with GroE, we used three different forms of yeast invertase for in vitro refolding experiments in the presence and absence of the GroE-system: non-glycosylated 'internal' invertase, the core-glycosylated form, and the fully glycosylated 'external' enzyme. All three forms of invertase have the same amino acid sequence. The glycosylated forms carry 10 *N*-linked sugars. The core-glycosylated enzyme (with 34% sugar) contains short oligosaccharide side chains of the structure $(GlcNAc_2)_2$-$(Man)_{8-9}$ [13]. The external invertase differs from the core-glycosylated form only by the additional outer mannose chains [14]. The internal form exhibits a strong tendency to form aggregates at concentrations above 4 μg/ml and temperatures above 10°C during refolding [15]. Glycosylation increases the solubility of the protein [12,13,15], presumably by covering hydrophobic patches of the polypeptide chain. Thus the carbohydrate moiety favors correct folding by suppressing aggregation. The fact that the refolding of glycosylated forms of invertase, in contrast to the non-glycosylated form, is not arrested by GroEL suggests that the sugar moieties sterically interfere with the interaction of the chaperone with potential intermolecular binding sites on the surface of the non-native protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

Non-glycosylated internal-, core-glycosylated- and high-mannose-glycosylated external invertase were purified as described [15]. Protein concentrations were calculated for the monomer (M_r = 58,500). Guanidinium chloride (GdmCl), ultrapure reagent grade, was purchased from Schwarz/Mann (Orangeburg, New York), the GOD-assay kit for the determination of glucose from Boehringer Mannheim.

GroEL and GroES were purified, and molar concentrations of the monomers were determined as described [9]. All chemicals were of analytical grade. Quartz-bidistilled water was used throughout.

2.2. *Denaturation/Renaturation*

The different forms of invertase were denatured in 5.3 M GdmCl, 50 mM NaAc, 10 mM EDTA, pH 5.0 at 20°C. Incubation time was 1 h for non-glycosylated and 2 h for the glycosylated forms of inver-

Figure 1

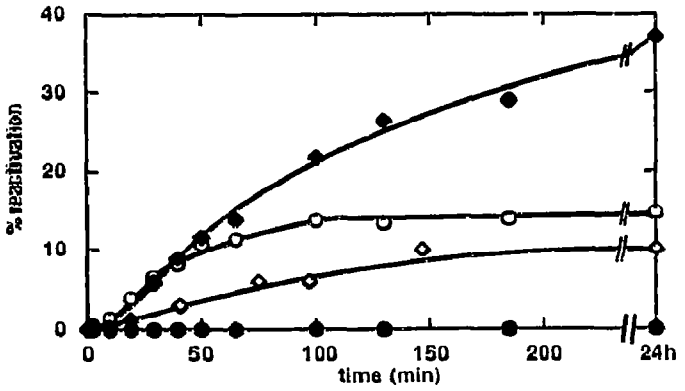

Fig. 1. GroE-dependent reactivation of chemically denatured non-glycosylated internal invertase. Invertase was denatured in 5.3 M GdmCl, 50 mM NaAc, 10 mM EDTA, pH 5.0 for 1 h at 20°C. Reactivation was started by a 1:80 dilution in different renaturation buffers. Invertase activity was determined under standard conditions [15] after various times of incubation. Final concentration of internal invertase was 0.34 μM. Standard renaturation buffer: 50 mM HEPES, 10 mM KCl, pH 7.0. Standard buffer without additives (○); in standard buffer, in the presence of 0.67 μM GroEL(14-mer), as well as 0.67 μM GroEL(14-mer) plus 0.67 μM GroES (7-mer) no reactivation is observed (●); standard buffer plus 0.68 μM GroEL(14-mer), 10 mM $MgCl_2$ and 2 mM ATP (◇); standard buffer plus 0.67 μM GroEL(14-mer), 0.67 μM GroES (7-mer), 10 mM $MgCl_2$ and 2 mM ATP (♦).

tase. Renaturation was initiated by a 1:80 dilution into 50 mM HEPES buffer, 10 mM KCl, pH 7.0 at 20°C to a final invertase concentration of 0.34 μM. The influence of GroE on refolding was tested by adding either GroEL (0.67 μM), or GroEL (0.67 μM) plus 10 mM $MgCl_2$/2 mM ATP, or GroEL (0.67 μM) plus GroES (0.67 μM)/10 mM $MgCl_2$/2 mM ATP to the same buffer, prior to renaturation. Activity was determined as described [15].

3. RESULTS AND DISCUSSION

In previous studies invertase was employed as a model system to investigate the influence of glycosylation on protein folding [15]. For the non-glycosylated enzyme, formation of inactive aggregates was found to be kinetically favored compared to correct folding at protein concentrations > 4 μg/ml and temperatures beyond 10°C. Glycosylation was found to suppress aggregation of non-native invertase [15].

GroEL was shown to prevent aggregation of a number of proteins during refolding [6,9,10]. We examined whether chaperone proteins and protein glycosylation may serve a similar function, i.e. to prevent aggregation of non-native proteins and keep them in a soluble state. To test this hypothesis, we refolded non-glycosylated, core-glycosylated and high-mannose glycosylated invertase in the absence and in the presence of GroE.

3.1. *In vitro refolding of non-glycosylated invertase is affected by GroE*

Fig. 1 shows the reactivation of internal invertase in the absence and presence of GroE. Internal invertase, at a concentration of 20 μg/ml and 20°C, reactivates spontaneously to 14%. In the presence of GroEL or GroEL plus GroES, reactivation is completely suppressed. Obviously, intermediates on the folding pathway form a stable complex with GroEL, as observed for a number of other proteins [5,9,11,16]. Addition of 2 mM ATP and 10 mM $MgCl_2$ to the GroEL/invertase complex results in a slow release of active invertase with a yield (10%) below the one observed for spontaneous reactivation. This effect was invariant, whether Mg/ATP was added prior, simultaneously, or after binding of denatured invertase to GroEL. The complete GroE system, however, increases the final reactivation yield of internal invertase from 14% to 36%, proving that the GroE-Mg/ATP complex favors correct folding of the non-glycosylated enzyme. To test for non-specific effects of GroEL/GroES, we refolded internal invertase under the same buffer conditions, but substituted the chaperone by 0.5 mg/ml mitochondrial malate dehydrogenase which corresponds to the concentration of GroE used in the previous experiments. In this control, both the kinetics and the final reactivation yield were the same as observed for the spontaneous reactivation in the absence of the chaperone (14 ± 2%) (data not shown).

3.2. *Glycosylation suppresses the formation of a stable complex between GroEL and invertase*

In order to determine the influence of glycosylation on the interaction with GroE, we used core-glycosylated invertase (short sugar side chains), and external invertase (long sugar side chains) for refolding experiments in the absence and in the presence of GroE (Fig. 2). In contrast to the non-glycosylated form, none of the glycosylated proteins formed a stable complex with GroEL during reactivation. The lag-phase in the presence of GroEL alone indicates that a transient complex with GroEL may be formed. This lag-phase disappeared when GroEL, GroES, and MgATP were present during refolding. The yield of reactivated protein increased in the presence of both GroEL and GroEL/GroES from 64% to 86% for core-glycosylated invertase (Fig. 2A), and from 62% and 76% for the external enzyme (Fig. 2B). This suggests, that glycosylation inhibits tight binding of folding intermediates of invertase to GroEL, but still allows some weak interactions with the chaperone, thus assisting correct folding. This effect was less pronounced for the high mannose glycosylated external invertase. Increased steric hindrance of the interaction between GroEL and hydrophobic sites on the surface of non-native glycoproteins with the extended carbohydrate moiety could be the reason. We suggest a model, where GroEL has a high affinity to parts of the non-native polypeptide chain which are buried in the native state. In the case of glycosylated proteins, such sites may be protected by glycosylation. This implies that both

Figure 2

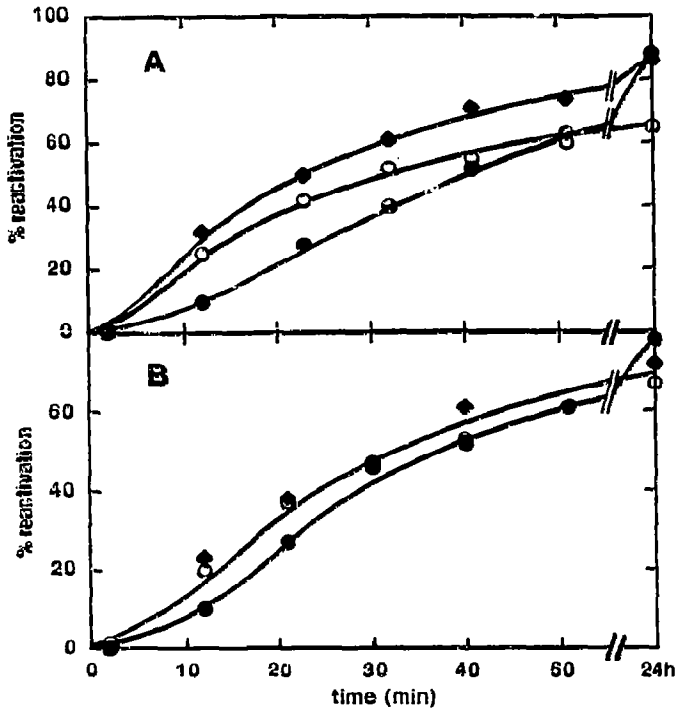

Fig. 2. GroE dependent reactivation of chemically denatured coreglycosylated (A) and external high-mannose glycosylated invertase (B). Denaturation/renaturation conditions and symbols as in Fig. 1.

chaperone action and glycosylation function by avoiding 'wrong' intermolecular interactions of non-native proteins, this way keeping them in solution and preventing non-specific aggregation.

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