Chaperonin GroE-facilitated refolding of disulfide-bonded and reduced Taka-α-amylase A from Aspergillus oryzae

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The refolding characteristics of Taka-α-amylase A (TAA) from Aspergillus oryzae in the presence of the chaperonin GroE were studied in terms of activity and fluorescence. Disulfide-bonded (intact) TAA and non-disulfide-bonded (reduced) TAA were unfolded in guanidine hydrochloride and refolded by dilution into buffer containing GroE. The intermediates of both intact and reduced enzymes were trapped by GroEL in the absence of nucleotide. Upon addition of nucleotides such as ATP, ADP, CTP or UTP, the intermediates were released from GroEL and recovery of activity was detected. In both cases, the refolding yields in the presence of GroEL and ATP were higher than spontaneous recoveries. Fluorescence studies of intrinsic tryptophan and a hydrophobic probe, 8-anilinonapthalene-1-sulfonate, suggested that the intermediates trapped by GroEL assumed conformations with different hydrophobic properties. The presence of protein disulfide isomerase or reduced and oxidized forms of glutathione in addition to GroE greatly enhanced the refolding reaction of reduced TAA. These findings suggest that GroE has an ability to recognize folding intermediates of TAA protein and facilitate refolding, regardless of the existence or absence of disulfide bonds in the protein.

Keywords: disulfide bond formation/folding intermediate/ GroE/protein folding/Taka-α-amylase A

Introduction

Protein folding is one of the critical issues in both fundamental and applied research fields, especially in protein engineering. In these fields, it is sometimes necessary to refold an over-expressed or engineered protein in vitro when the protein aggregates as an inclusion body in a cell because an effective way to overcome inclusion body formation in vivo is not available. Recently, it has been demonstrated clearly that the folding of many proteins in vivo is not an isolated process but is facilitated by proteins called chaperonins (Gething and Sambrook, 1992; Hartl, 1996; Fenton and Horwich, 1997). Utilization of the chaperonin as an in vitro refolding system, therefore, may be a good way to overcome this problem.

The chaperonin GroE (GroEL/ES) from Escherichia coli is one of the most extensively studied chaperonins. GroE is composed of two types of subunits; GroEL (14-mer), a 57 kDa polypeptide which forms two toroidal heptameric rings, and GroES (7-mer), a 10 kDa polypeptide which forms a heptameric ring (Henrich, 1979; Chandrasekhar et al., 1986; Hemmingsen et al., 1988). The X-ray crystal structures of these GroEL and GroES proteins have been elucidated recently (Braig et al., 1994; Hunt et al., 1996; Xu et al., 1997a) and have provided a wealth of information from which more detailed studies on structure and function relationships are possible (Hartl, 1996; Fenton and Horwich, 1997). The functional mechanism of GroE can be divided broadly into two parts: binding of protein folding intermediates prone to aggregation and the controlled release of these intermediates to allow maximum refolding yields. In the former step, GroEL recognizes a folding intermediate and binds it specifically by hydrophobic and electrostatic interactions (Martin et al., 1991; Hoshino et al., 1996; Katsumata et al., 1996b). By forming this complex, the folding intermediate is stabilized and irreversible aggregation is suppressed. In the latter step, the intermediate is released and the native protein is formed efficiently when ATP is added. In this step, the GroES protein plays an important role in assisting the function of GroEL (Kubo et al., 1993; Hayer-Hartl et al., 1996; Fenton and Horwich, 1997).

The GroE protein has been shown to facilitate the in vitro folding of many proteins regardless of various differences in structural characteristics (Kawata et al., 1994; Hartl, 1996; Fenton and Horwich, 1997). Among the target proteins studied are proteins possessing disulfide bonds, such as β-lactamase (Laminet et al., 1990; Zahn et al., 1994a; Gervasoni and Plückthun, 1997), α-lactalbumin (Hayer-Hartl et al., 1994; Okazaki et al., 1994, 1997), immunoglobulin Fab fragment (Schmidt et al., 1994), apo-α-lactalbumin (Katsumata et al., 1996a,b), ribonuclease T1 (Walter et al., 1996) and pro-urolase (Xu et al., 1997b). Although studies have been reported in which the disulfide bonds of some of these enzymes had been fully or partially reduced, a comparative study on the chaperonin-facilitated refolding of the disulfide-bonded and reduced forms of the same enzyme has been performed only for β-lactamase (Gervasoni and Plückthun, 1997; Gervasoni et al., 1998).

In this work, we studied the effects of GroE on the refolding of Taka-α-amylase A (TAA) as a model of an extra-cellular protein having disulfide bonds in its tertiary structure. TAA from Aspergillus oryzae (EC 3.2.1.1; α-1,4-glucan 4-glucohydrolase) is a monomeric enzyme of M, 55 000 containing post-translationally modified sugars, four disulfide bonds and one free sulfhydryl group in its tertiary structure (Matsuura et al., 1980; Toda et al., 1982). Both refolding intermediates of disulfide-bonded (intact) TAA and the non-disulfide-bonded (reduced) TAA from a guanidine hydrochloride (Gdn.HCl)-unfolded state were trapped by GroEL in the absence of nucleotide and formed stable complexes of GroEL-refolding intermediates. Upon addition of various nucleotides, both intermediates were released and refolded efficiently under the conditions used. The finding that GroE facilitated refolding of the enzyme regardless of the existence or absence of disulfide bonds in its tertiary structure suggests that the functional ability of the chaperonin is very adaptable, and useful in various applications in the protein engineering research field.
Materials and methods

Nomenclature

We use the term ‘SS-TAA’ to denote the protein in which native intramolecular disulfide bonds are present and the term ‘SH-TAA’ to denote the protein in which all intramolecular disulfide bonds had been reduced. ‘TAA’ used singly denotes both forms of the enzyme.

Materials

Gdn.HCl (finest grade) was obtained from Nacalai Tesque (Kyoto, Japan). Nucleotide preparations, bovine serum albumin and reduced and oxidized forms of glutathione were obtained from Sigma, St Louis, MO, USA). All other reagents were obtained from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque. GroEL and GroES proteins were purified from a GroE-overproducing strain, Escherichia coli DH1/pKY206, according to the method of Kubo et al. (1993). TAA from Aspergillus oryzae was purified from a commercial product, Takadiastase Sankyo, and crystallized according to the method of Akabori et al. (1995). The concentration of GroEL was determined spectrophotometrically on a Hitachi U-2000 spectrophotometer, using an absorption coefficient $A_{195}^\text{cm} = 2.36$ at 277 nm (Mizobata et al., 1992). The concentrations of TAA and GroES were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Unfolding and reduction of disulfide bonds

SS-TAA was unfolded in 6 M Gdn.HCl for 30 min at 30°C. SH-TAA was prepared by incubating SS-TAA in 2 mM DTT in 25 mM Tris–HCl buffer, pH 8.0, containing 6 M Gdn.HCl and 1 mM EDTA for 30 min at 30°C. This mixture was then passed through a PD-10 (Sephadex G-25) desalting column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM MOPS–KOH buffer, pH 7.0, containing 4 M Gdn.HCl. The sample was checked for extent of disulfide reduction using Ellman’s reagent (Ellman, 1959) and immediately thereafter used for the refolding experiments with GroEL/ES.

Refolding assay

Appropriate aliquots of SS-TAA and SH-TAA proteins unfolded in 6 and 4 M Gdn.HCl, respectively, were subsequently diluted into refolding buffer. The buffer used for SS-TAA refolding was 50 mM MOPS–KOH, pH 7.0, containing 10 mM KCl, 10 mM Mg(CH$_3$COO)$_2$ and 1 mM CaCl$_2$, and that for SH-TAA was 50 mM TAPS–KOH, pH 8.0, containing 10 mM KCl, 10 mM Mg(CH$_3$COO)$_2$, 1 mM CaCl$_2$, 2 mM reduced glutathione (GSH) and 0.2 mM oxidized glutathione (GSSG). The 2 mM GSH–0.2 mM GSSG mixture was always present for the refolding of SH-TAA unless stated otherwise. The concentrations of enzyme and Gdn.HCl during the refolding reaction were 2 µg/ml and 50 mM, respectively, and the refolding temperature was 37°C. A 15-fold molar excess of GroEL and GroES oligomer relative to refolding enzyme and 2 mM nucleotide were selectively added to the refolding mixture. The refolding yield was determined as the percentage ratio of the activity of the refolding enzyme relative to that of native enzyme.

Enzyme assay

The enzyme activity was assayed according to the method of Bernfeld (1951) at 30°C. Briefly, 100 µl of the refolding mixture solution were added to 33 mM acetate buffer, pH 5.3, containing 2 mM Ca(CH$_3$COO)$_2$ and 0.5% starch, which had been previously heat treated at 85°C for 30 min. After incubation for 10 min at 30°C, the enzyme reaction was stopped by adding 1 ml of stop solution (2 M NaOH containing 1% 3,5-dinitrosalicylate and 30% tartrate) and was boiled in a water-bath for 5 min. The absorbance of the colored solution at 540 nm was measured after cooling.

Complex formation of GroEL and refolding intermediates of TAA protein

The GroEL-trapped TAA intermediates were prepared as follows: a 5-fold molar excess of unfolded TAA in 4 or 6 M Gdn.HCl relative to GroEL 14-mer was diluted into buffer containing GroEL as described in the section Refolding assay, and immediately loaded on to a Sephacryl S-300 gel-filtration column (250×12 mm i.d.) which had been equilibrated with the same buffer. The GroEL–intermediate complex thus isolated was used for the fluorescence measurements. The isolated complex was also confirmed by SDS–PAGE of isolated samples.

Measurements of fluorescence spectra

Tryptophyl fluorescence and 8-anilinonaphthalene-1-sulfonate (ANS) fluorescence were measured on a Hitachi F-4010 spectrofluorimeter. The temperature was maintained at 25°C with a thermostatically controlled cell holder. The buffer used was 50 mM MOPS–KOH buffer, pH 7.0, containing 10 mM KCl, 10 mM Mg(CH$_3$COO)$_2$ and 1 mM CaCl$_2$. The excitation wavelength was set to 295 nm for tryptophyl fluorescence and 370 nm for ANS fluorescence. For GroEL–TAA complex experiments, a control fluorescence spectrum of GroEL in sample buffer was subtracted from the observed spectrum.

Results

Complex formation with the intermediates of SS-TAA and SH-TAA

In order to examine whether GroEL forms a stable complex with a refolding intermediate of SS-TAA in the absence of nucleotide, the ability of the enzyme to refold in the presence of various concentrations of chaperonin was measured. The net refolding yield in the presence of GroEL was used to estimate the ratio of SS-TAA molecules which escaped complex formation with GroEL. As shown in Figure 1, the refolding yield
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Fig. 2. Effects of oxidative reagents on the refolding of SH-TAA in the presence of GroE and 2 mM ATP. The refolding reaction was performed at 37°C. The concentration of SH-TAA was 2 µg/ml (36 nM) and a 15-fold molar excess of GroE 21-mer was used. Open squares, in the absence of reagent (air oxidation with stirring); closed squares, in the presence of 2 µM Cu²⁺; open circles, 50 mM oxidized DTT; closed triangles, 2 mM GSH–0.2 mM GSSG; closed circles, 8.3 µg/ml (150 nM) PDI. To prevent air oxidation, all experiments except the air oxidation experiment were performed under nitrogen.

Fig. 3. GroE-facilitated refolding reaction of SS-TAA (a) and SH-TAA (b). Open and closed circles represent refolding in the presence of buffer only (spontaneous refolding) and in the presence of a 15-fold molar excess of GroE 21-mer and 2 mM ATP, respectively. Open squares represent refolding in the presence of a 15-fold molar excess of GroE 21-mer without nucleotide. Closed squares indicate the changes in the activity when ATP was added (at the time indicated by the arrow) to the refolding mixture containing GroE and TAA (open squares) to a concentration of 2 mM. The concentration of TAA was 2 µg/ml and the refolding temperature was 37°C.

Effects of oxidative reagents on the refolding of SH-TAA in the presence of GroE

In order to monitor the refolding of SH-TAA by enzyme activity, correct formation of disulfide bonds is required during the refolding process. In order to optimize the refolding conditions of SH-TAA in the presence of GroE and 2 mM ATP, we examined several oxidative reagents and conditions for the formation of correct disulfide bonds, as shown in Figure 2. Air oxidation and air oxidation in the presence of 2 µM Cu²⁺ were more effective than the reaction with 50 mM oxidized DTT. The highest refolding yields were achieved, however, by using 8.3 µg/ml (0.15 µM) protein disulfide isomerase (PDI) and 2 mM GSH–0.2 mM GSSG. Interestingly, the refolding in the presence of PDI was faster than the spontaneous rate whereas the refolding in the presence of GSH–GSSG was slower. An acceleration of refolding rate in the presence of PDI has also been reported for the renaturation kinetics of reduced lysozyme (Puig and Gilbert, 1994). Surprisingly, the final refolding yield of the enzyme using PDI or GSH–GSSG was about 120% of the initial activity. This observation could be explained if we consider that the one free sulfhydryl group of the cysteine residue at position 227 near the active site (Matsuura et al., 1980) might be modified or oxidized during storage of the purified enzyme but is restored to the active conformation during refolding. In the present study, we added GSH–GSSG in all refolding reactions of SH-TAA.

Chaperonin-facilitated refolding of SS-TAA and SH-TAA

The refolding reactions facilitated by GroE were characterized for both SS-TAA and SH-TAA, as shown in Figure 3. As SH-TAA requires the formation of correct disulfide bonds for expression of activity, it is reasonable that the refolding time of SH-TAA was longer than that of SS-TAA. Both refolding reactions were suppressed almost completely (20 min for SS-TAA and 100 min for SH-TAA) when a 15-fold molar excess of GroE protein was present in the refolding buffer in the absence of nucleotide. When 2 mM ATP was added to the refolding mixture at the indicated time, a rapid increase in the enzyme activity was observed; the correct refolding of both SS-TAA and SH-TAA was facilitated by the chaperonin. In both cases, it should be noted that the refolding yield in the presence of GroE was higher than that for spontaneous refolding, which indicated that GroE protein facilitated the refolding reaction of the enzyme. These results show clearly that GroE has an ability to recognize the refolding intermediates of TAA and to facilitate the correct refolding reaction regardless of the existence of disulfide bonds.

Chaperonin-facilitated refolding of SS-TAA and SH-TAA

In the GroE-facilitated refolding reaction of yeast enolase (Kubo et al., 1993), we have shown that not only ATP but...
also ADP, CTP and UTP are effective in the presence of GroES, which could be explained by a conformational change upon binding of nucleotide to GroEL protein, which facilitates a favorable release of intermediates (Kawata et al., 1994; Hayer-Hartl et al., 1996). In order to determine whether the refolding of TAA is also facilitated by nucleotides other than ATP, we examined the effects of various nucleotides for GroEL-mediated refolding in the presence or absence of GroES. As expected, Figure 4 shows that ADP and CTP were also good effectors for the refolding of the enzyme in the presence of GroES. UTP was, however, effective only for the refolding of SS-TAA. The effectiveness of CTP in the presence of GroES and of ATP in the absence of GroES for the GroEL-facilitated refolding of SS-TAA was higher than that of SH-TAA. In contrast, the effect of ADP addition in the presence of GroES is stronger for the refolding of SH-TAA than that of SS-TAA. It should be noted that for all the nucleotides tested, the refolding yields of both types of TAA in the presence of GroES were higher than those in the absence of GroES. These differences in the effectiveness of nucleotides for SS-TAA and SH-TAA may be due to the differences in the characteristics of the intermediates.

Characteristics of the intermediates bound to GroEL

In order to elucidate the structural characteristics of the intermediate that was bound to GroEL, we measured the intrinsic tryptophyl fluorescence (Figure 5) and ANS binding characteristics of the refolding intermediates (Figure 6). The intrinsic tryptophyl fluorescence spectrum of native SS-TAA had a peak maximum at about 330 nm upon excitation at 295 nm, whereas that of unfolded SS-TAA in 6 M Gdn.HCl had a peak maximum at about 350 nm and a decreased intensity, as shown in Figure 5a. The decrease in intensity of the unfolded state was larger for SS-TAA than SH-TAA (Figure 5b). The characteristics of the tryptophyl fluorescence spectra of the folding intermediates of SS-TAA and SH-TAA trapped by GroEL were seen to be mid-way between those of the native and unfolded states with regard to both fluorescence intensity and peak wavelengths.

Figure 6 shows the fluorescence spectra of a hydrophobic probe, ANS, co-existing in protein solutions of various states. Whereas no differences were observed in the spectra of SS-TAA in the native state and of SS-TAA and SH-TAA in the unfolded state, an increased ANS fluorescence for both TAA proteins trapped by GroEL was observed. These increases in fluorescence spectra were much greater than the increase observed for GroEL in buffer, as shown in the inset in Figure 6, indicating that the trapped intermediates of TAA have a strong hydrophobic character. In contrast to the result obtained from tryptophyl fluorescence, a difference in the ANS fluorescence intensities of the refolding intermediates of SS-TAA and SH-TAA was observed; the SH-TAA intermediate showed an intensity which was higher than that of SS-TAA (Figure 6). This finding suggests that the intermediate of SH-TAA bound to GroEL was more hydrophobic than that of SS-TAA.
and trapped it to form a stable GroEL–intermediate complex in the absence of nucleotide (Figures 1 and 3). This specific interaction was observed for the refolding reaction of both SS-TAA and SH-TAA and, in both cases, a net increase in refolding yield compared with that of spontaneous refolding was mediated by the addition of ATP (Figure 3). This result indicates that GroE has an ability to facilitate the refolding reaction of the protein regardless of the existence of intramolecular disulfide bonds. This finding is in sharp contrast to the case of Gdn.HCl unfolded β-lactamase where it was found that the refolding intermediate of non-disulfide-bonded β-lactamase interacted with GroEL but that of disulfide-bonded β-lactamase did not (Gervasoni and Plückthun, 1997).

Conformation of intermediate trapped by GroEL and the nucleotide specificity of the chaperonin-assisted reaction

One of the conspicuous characteristics of chaperonin function is the broad specificities for the recognition and the interaction of GroE toward refolding intermediates. Although it has been reported that GroEL interacts with the folding intermediates of various proteins by hydrophobic and electrostatic interactions (Martin et al., 1991; Hoshino et al., 1996; Katsumata et al., 1996b), the identity of the intermediate trapped by GroEL in various cases is not fully understood and a variety of approaches have been utilized to address this issue (Robinson et al., 1994; Zahn et al., 1994b; Hlodan et al., 1995; Lin et al., 1995; Murai et al., 1995; Aoki et al., 1997; Goldberg, et al., 1997; Torella et al., 1998). In the present work, we tried to detect structural differences of the GroEL-trapped intermediates between SS-TAA and SH-TAA. That the two forms were different from a mechanistic point of view was demonstrated by the results in Figure 4, which show a difference in the nucleotide specificity for the release of TAA intermediates. Although the tryptophyl fluorescence spectra of the two intermediates were indistinguishable (Figure 5), the differences in ANS-fluorescence reflected a minute difference in structure. The ANS-binding fluorescence experiments (Figure 6) suggested that the intermediate of SH-TAA bound to GroEL was more hydrophobic than that of SS-TAA; in other words, the conformations of the trapped intermediates were different. This difference may also be responsible for the different efficiencies of individual nucleotides for the release of folding intermediates from GroEL (Figure 4).

A novel finding in the present study was that GroEL can interact stably with different folding intermediate states (disulfide-bonded and the reduced form) of the same protein (Figures 1 and 6). Very recently, it was also reported that GroEL trapped two different sets of conformations of heat-denatured β-lactamase (Gervasoni et al., 1998). These results are rare but may not be surprising from the point of view of the iterative mechanism of chaperonin function (Hartl, 1996; Fenton and Horwich, 1997). We did not see whether GroEL could bind to a refolding intermediate from partially disulfide-bond(s) reduced TAA, but this interaction would be likely to occur because both refolding reactions from fully disulfide-bonded TAA and reduced TAA were completely trapped by GroEL in the absence of nucleotide (Figures 1 and 3). It has also been reported that the partially folded α-lactalbumin, in which one or two of four disulfide bonds had been selectively reduced, was stably trapped by GroEL (Hayer-Hartl et al., 1994). The ability of GroEL to interact with different intermediates of the same protein indicates that the recognition mechanism of GroEL is based on some common characteristic
of the protein refolding intermediate such as hydrophobic characteristics (Martin et al., 1991; Mendoza et al., 1992; van der Vies et al., 1992; Hayer-Hartl et al., 1994; Hoshino et al., 1996), but not the presence or absence of intramolecular disulfide bonds.

Conclusion

The chaperonin GroE from E. coli was found to facilitate the in vitro refolding of TAA protein in the presence of various nucleotides regardless of intramolecular disulfide bonds. The intermediates trapped by GroEL differed slightly in hydrophobicity between the disulfide-bonded and the reduced TAA proteins. This finding suggests that GroEL can interact stably with different conformations of the same protein. The present study utilizing the chaperonin in refolding of proteins having intramolecular disulfide bonds may provide a way of attaining a good yield in in vitro refolding of protein which would otherwise be aggregated and unusable.

Acknowledgement

This research was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References