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INTERACTION OF β-LACTOGLOBULIN WITH CHAPERONIN GROEL

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The roles of electrostatic and hydrophobic interactions in the substrate recognition of GroEL were studied with bovine β-lactoglobulin as a substrate. We previously reported that, although the native state of β-lactoglobulin does not interact with GroEL, the modified protein in which the two disulfide bonds were reduced formed a stable complex[22]. To clarify the extent of denaturation necessary for the interaction, we examined the interaction of GroEL with the intermediate state of β-lactoglobulin in which the free thiol group was specifically modified with 5,5'-dithiobis(2-nitrobenzoic acid). The thiol-modified β-lactoglobulin formed a complex with GroEL as measured by size-exclusion chromatography, indicating that moderate denaturation is enough for the interaction. We then measured the interaction between GroEL and the refolding intermediate of the disulfide intact β-lactoglobulin. Although the interaction was marginal under low salt conditions, notable interaction was observed by the addition of KCl. Since pI values of GroEL and β-lactoglobulin are 4.7 and 4.5, respectively, the addition of salt was considered to shield the unfavourable charge repulsion. Tryptophyl fluorescence of the kinetic intermediate indicated that tryptophan residues were buried upon binding. These results confirmed the previous view that suppression of the unfavourable electrostatic repulsion in addition to exertion of favourable hydrophobic interaction is important for the binding of substrate proteins to GroEL.

Key Words: β-Lactoglobulin; GroEL; Protein Folding; Substrate Recognition; Salt Effects; Hydrophobic Interaction

Introduction

Chaperonin GroEL from *Escherichia coli*, known to assist the correct and efficient folding of denatured proteins in the presence of GroES and ATP, is one of the most extensively studied molecular chaperones[1-5]. X-ray crystallographic analyses have shown that GroEL is composed of 14 identical subunits of 57 kDa, which form a cylinder containing a central cavity[6-8]. Each subunit is composed of three domains: an apical domain interacting with substrate proteins and GroES, an equatorial domain with an ATP binding site, and an intermediate domain connecting the other two domains.

GroEL recognizes various conformations of substrate proteins such as folding intermediates, random coil-like state and different conformations of the same protein[9-13]. Braig *et al.*[6,7] proposed that helix H8 and H9, and the loop between helix H10 and strand S11 constitute the substrate binding site. There are many hydrophobic residues in the binding site, suggesting that the interaction of GroEL with substrate protein is dominated by hydrophobic interactions. Several reports supported the importance of hydrophobic interaction[1-4,12-15]. On the other hand, the importance of electrostatic interactions was also indicated in several cases. By the addition of salts, while the interactions of negatively charged GroEL (pI = 4.7) with positively charged substrate proteins (e.g. cytochrome *c* with pI = 10[12,16]), staphylococcal nuclease with pI = 9.8[17] or barnase with pI = 8.8[18] were weakened, the interactions of GroEL with negatively charged substrates (e.g. α-lactalbumin with pI = 4.7[19-21]), β-lactoglobulin with pI = 4.5[22] were strengthened. As salts shield the electrostatic interactions, these results indicated the role of electrostatic interactions in modulating the GroEL-substrate interactions: while the attraction between the negatively charged GroEL and positively charged substrate proteins promotes binding, the repulsion between GroEL and negatively charged substrate proteins weakens the binding. In fact, there are many

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acidic residues in the substrate binding site, consistent with the above mechanism. Thus, both hydrophobic and hydrophilic interactions have been considered to be important for the interaction of GroEL with substrate proteins. Nevertheless, the exact mechanism of the recognition of substrate proteins is still unknown. Since GroEL interacts with a variety of protein, it will be important to examine the interactions with various proteins to establish the general mechanism of interaction.

We have been studying the mechanism of substrate recognition of GroEL with bovine β-lactoglobulin along with the folding reaction of β-lactoglobulin[23-27]. β-Lactoglobulin consists of 162 amino acid residues (18.4 kDa) and contains two disulfide bonds (Cys66-Cys160, Cys106-Cys119) and a free thiol (Cys121)[28-30] (Fig. 1). Although it is a predominantly β-sheet protein, it has a high helical propensity and, intriguingly, it formed a kinetic intermediate with non-native helical structure during its refolding reaction. Thus, β-lactoglobulin has been considered to be an intriguing model protein to understand the interplay between the local and nonlocal interactions during protein folding. Moreover, since α-to-β transition has been suggested for several important biological phenomena, the folding mechanism of β-lactoglobulin might provide a clue to understand those phenomena. On the other hand, we showed that reduction of the disulfide bonds produced a collapsed state which can bind to GroEL, monitored by size-exclusion gel chromatography and single molecule detection of the complex[22].

In the course of studying the conformation and stability of β-lactoglobulin, we noted that the free thiol group of β-lactoglobulin can be specifically modified with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)[25]. The conformation of the 2-nitro-5-thiobenzoic acid-modified β-lactoglobulin (TNB-β-lactoglobulin) was less stable than that of the native state, but evidently more ordered than that of the disulfide bond-reduced state. To address the extent of denaturation required for the interaction with GroEL, we first examined the interaction of TNB-β-lactoglobulin with GroEL by size-exclusion chromatography. Previously, we could not obtain the stable complex formation between the refolding kinetic intermediate of intact β-lactoglobulin and GroEL. By carefully measuring the interaction in the presence of high salt, we demonstrated the complex formation. The spectrum of the trapped intermediate indicated the burial of tryptophan residues, consistent with the importance of hydrophobic interactions.

Materials and Methods

Chaperonin GroEL. GroEL was prepared from a GroE-overproducting strain, *Escherichia coli* DH1/pKY206, as described[31]. Purification of GroEL was performed according to the method of Buchner *et al.*,[32] with some modification[12]. Obtained GroEL was further purified by the method of Clark *et al.*[33].

Chemically Modified Substrate Protein

Bovine β-lactoglobulin was purchased from Sigma. We first prepared cy5-labelled β-lactoglobulin by incubating β-lactoglobulin at 5 mg ml^{-1} with 200 μg of cy5 (Amersham Life Science) in 10 mM Tris-HCl buffer (pH 7.5) for 1 hr at room temperature. After removing the excess reagent by gel filtration with PD10 column (Pharmacia Biotech), the molar ratio of β-lactoglobulin: cy5 was determined from the absorption spectrum. About one mole of amino group per mole of β-lactoglobulin was modified with cy5 and the absorption at 640 nm was used for detection. TNB-β-lactoglobulin was prepared by titrating the thiol group of cy5-labelled-β-lactoglobulin at 0.4 mg ml^{-1} with 0.4 mM DTNB in 1.0 M Gdn-HCl and 10 mM Tris-HCl buffer at pH 7.5[25]. The titration reaction was followed by measuring the absorption increase at 412 nm and it was confirmed that one mole of thiol group reacted with DTNB. The excess reagents were removed by gel filtration.

HPLC Measurements

The binding of substrate proteins to GroEL was monitored by size-exclusion chromatography experiments using a Gilson high performance liquid chromatography (HPLC) system as described before in ref. [12]. Typically, 2 μg of substrate protein was added to a 1 equimolar amount of GroEL (total volume was 60 μl), and solution was incubated for 30 minutes at room temperature. The protein solution was applied to a Bio-Rad SEC 40XL column (300 mm x 7.8 mm) equilibrated with 10 mM Tris-HCl (pH 7.5) at a flow-rate of 0.5 mg ml^{-1}. The experiments were carried out in the presence of various concentration of KCl in the sample and buffer solutions.

Fluorescence Measurements

Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, F4500, at 20°C. Tryptophan fluorescence was measured with an excitation at 295 nm at a protein concentration of 0.2 mg ml^{-1}.

Conformation of TNB-β-lactoglobulin

We first summarize the conformation of TNB-β-lactoglobulin. The circular dichroism (CD) spectra and fluorescence spectra of TNB-β-lactoglobulin (Fig. 2) were reproduced from our previous paper[25], since they represent the overall conformation well. The far-UV CD spectrum of TNB-β-lactoglobulin at pH 7.5 with a minimum at around 205 nm is distinct from that of the native state, although it is different from the spectrum in 4 M guanidine hydrochloride (Gdn-HCl) (Fig. 2A). The absence of sharp peaks in the aromatic region confirmed the disorder of the rigid native structure. On the other hand, the near-UV CD showed a broad positive maximum at around 300 nm, probably arising from the loosely fixed TNB group. These results are distinct from the modification of β-lactoglobulin by mercaptoethnol or mercaptopropionic acid[34], in which only slight changes in the far- and near-UV CD spectra have been reported. This difference probably arises from the difference in the size of the reagent molecules: mercaptoethanol and mercaptopropionic acid are smaller than TNB.

β-Lactoglobulin has two Trp residues at positions 19 on the βA strand and 61 at the end of βB strand. While Trp19 is fully buried, Trp61 is relatively exposed to the solvent[28,29]. The fluorescence spectrum of the native state showed a maximum at 335 nm at pH 7.5 (Fig. 2B). Upon denaturation by 7.0 M Gdn-HCl, the maximal wavelength shifted to 350 nm and the maximal fluorescence intensity increased upon unfolding.

The reaction with DTNB resulted in a dramatic decrease of fluorescence intensity, which was accompanied by a red shift in maximal emission wavelength to 345 nm (Fig. 2B). The absorption of TNB group with a maximum at 325 nm exactly overlaps with the tryptophan fluorescence. Therefore the quenching of tryptophan fluorescence is probably caused by a transfer of the fluorescence energy to the TNB group. Quenching of the fluorescence by the newly created mixed disulfide at Cys121 is also likely to be involved because the disulfide bond is one of the most important quenchers of tryptophan fluorescence, as observed for the immunoglobulin domains[35,36]. The red shift of the emission maximum suggests that the buried tryptophan (Trp19) is preferentially quenched, consistent with the proximity of Cys121 and Trp19 (Fig. 1).

Upon unfolding by 7.0 M Gdn-HCl, the fluorescence intensity increased. This observation also indicated that TNB-β-lactoglobulin in the absence of denaturant assumes a compactly folded structure in which the tryptophan residues and quenchers are adjacent. Nevertheless, the fluorescence intensities in the unfolded state were less than those of the intact β-lactoglobulin in the unfolded state, indicating that the TNB group still quenches the tryptophan fluorescence.

It has been shown that intact β-lactoglobulin assumes a dimeric structure at pH 7, whereas the monomer predominates at pH below 3[34,37-39]. Sakai *et al.*[25] examined the monomer-dimer equilibrium of TNB-β-lactoglobulin by sedimentation equilibrium. The dimerization constant of TNB-β-lactoglobulin ($2.8 \times 10^3\ M^{-1}$) was one-twentieth of the value of the intact β-lactoglobulin and the dimer fraction was calculated to be 19% at 1 mg ml^{-1}, much less than that of the intact β-lactoglobulin under the same conditions. The 1D NMR spectra of the TNB-β-lactoglobulin at pH 7.5 was typical for a denatured protein or a molten globule state, indicating that the side chains are largely fluctuating. In comparison, the NMR spectrum of the intact β-lactoglobulin at pH 7.5 was dispersed although the peaks were broad because of the dimer formation[25].

Unfolding transitions of the intact β-lactoglobulin and TNB-β-lactoglobulin induced by Gdn-HCl at pH 7.5 were measured using tryptophan fluorescence[25]. Apparently, the unfolding transition of TNB-β-

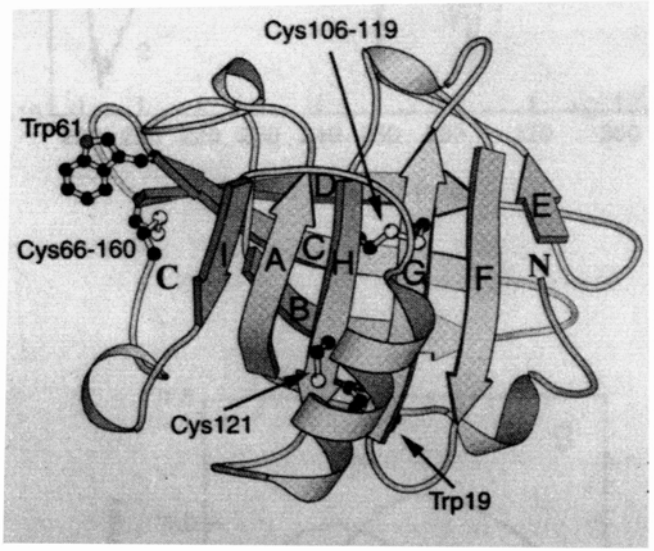

Fig. 1 X-ray crystal structure of β-lactoglobulin drawn by Molscript with Protein Data Bank file 3blg[29]

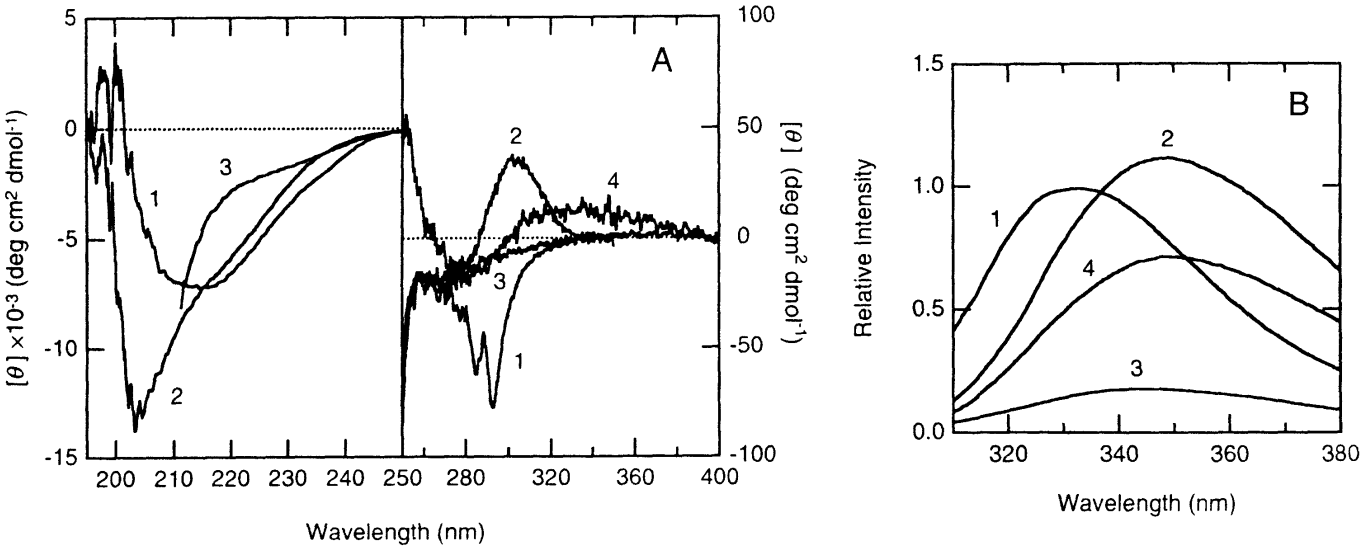

Fig. 2 CD and fluorescence spectra of TNB-β-lactoglobulin. (A) Far- and near-UV CD spectra of intact- and TNB-β-lactoglobulin at pH 7.5. (1) Intact β-lactoglobulin, (2) TNB-β-lactoglobulin, (3) TNB-β-lactoglobulin in 4 M Gdn-HCl. (B) Fluorescence spectra of intact and TNB β-lactoglobulin at pH 7.5. (1) Intact β-lactoglobulin, (2) TNB-β-lactoglobulin, (3) intact β-lactoglobulin in 4 M Gdn-HCl, (4) TNB-β-lactoglobulin in 4 M Gdn-HCl. (Taken from ref. [25] with permission.)

lactoglobulin occurred at Gdn-HCl concentrations similar to that of the intact β-lactoglobulin, although the transition of TNB-β-lactoglobulin was less cooperative than that of intact β-lactoglobulin. Taken together, TNB-β-lactoglobulin adopts a unique compact denatured structure with fluctuating side chains, like the molten globule state observed for several proteins[40-42].

Interaction of GroEL with TNB-β-lactoglobulin

The interaction of TNB-β-lactoglobulin with GroEL was monitored by size exclusion chromatography with cy5 labelled β-lactoglobulin (Fig. 3). Elution peak at 16 min indicated the complex formation of TNB-β-lactoglobulin with GroEL, consistent with the interaction between GroEL and the disulfide bond reduced β-lactoglobulin[22]. When the complex was incubated in the presence of 2 mM MgATP, the dissociation of the complex was observed, indicating that the complex is formed specifically.

The CD and fluorescence spectra of the disulfide bond reduced β-lactoglobulin showed that the structure is substantially disordered, although more structured than the highly denatured state in high concentrations of Gdn-HCl. In contrast, TNB-β-lactoglobulin prepared in the present study was more structured than the disulfide bond-reduced protein and the extent of exposure of the hydrophobic residues are less than that of the disulfide reduced protein (Fig. 2). These results indicate that, for the interaction of β-lactoglobulin with GroEL, partial exposure of the hydrophobic residues will be enough.

Interaction of GroEL with Refolding Intermediate

We previously thought that the refolding intermediate of β-lactoglobulin does not form a stable complex with GroEL. The complex formation of TNB-β-lactoglobulin or the disulfide bond reduced β-lactoglobulin with GroEL under high salt conditions suggested that the refolding intermediate of β-lactoglobulin might form the complex in the presence of salt. In this regards, we examined the complex formation under different concentrations of KCl monitored by size exclusion chromatography. Cy5-labelled β-lactoglobulin denatured in 4 M Gdn-HCl was diluted into the refolding buffer containing GroEL and then applied to the column. We confirmed that cy5 label did not affect the refolding kinetics of β-lactoglobulin. Under low salt conditions, marginal complex formation was observed (Fig. 4). In contrast, substantial amount of complex was formed in the presence of high concentrations of KCl. In the presence of 300 mM KCl, the fraction of complex was about 70% judging from the peak areas, although we cannot estimate the equilibrium amount from the gel filtration experiment.

Tryptophan Fluorescence of Bound Substrate

Since GroEL has no tryptophan residues, the fluorescence spectrum of the complex with the intact β-lactoglobulin excited at 295 nm directly represents

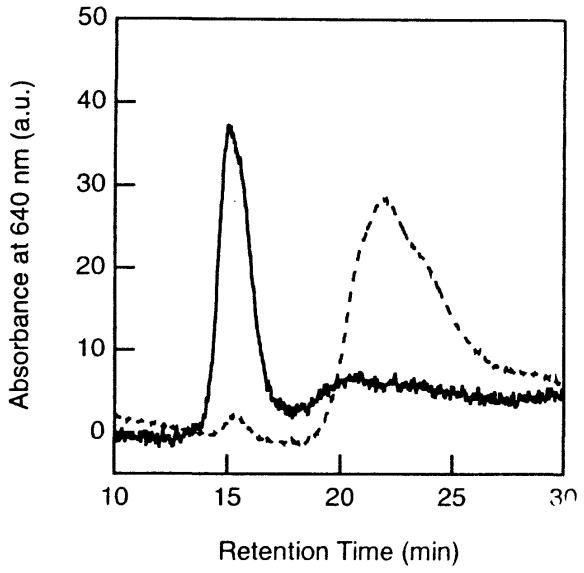

Fig. 3 Interaction of GroEL and TNB-β-lactoglobulin measured by size-exclusion chromatography. Elution profiles of a mixture of GroEL and cy5-labelled TNB-β-lactoglobulin in the absence (solid line) or presence (dashed line) of 2 mM Mg-ATP, were monitored by the absorption of cy5 at 640 nm. Buffers used were 10 mM Tris-HCl (pH 7.5) and 300 mM KCl for the elution in the absence of ATP and 10 mM Tris-HCl (pH 7.5), 50 mM KCl 10 mM $MgCl_2$ and 2 mM ATP for the elution in the presence of ATP

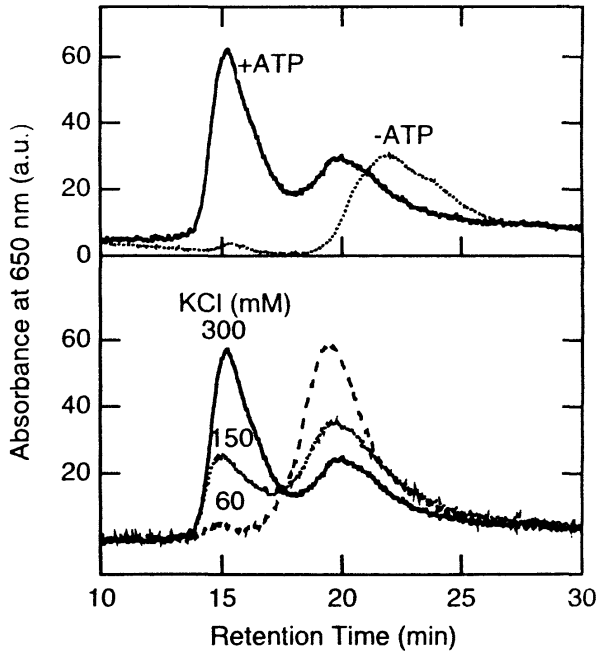

Fig. 4 Interaction of GroEL and the kinetic intermediate of β-lactoglobulin measured by size-exclusion chromatography. Elution of cy5-labelled β-lactoglobulin was monitored by the absorption of cy5 at 640 nm. (A) Elution profiles of a mixture of GroEL and refolding intermediate of cy5-labelled β-lactoglobulin in the presence of different concentrations of KCl. (B) The effect of 2 mM Mg-ATP in the presence of 300 mM KCl

the fluorescence spectrum of the bound substrate. We examined the fluorescence spectra of intact β-lactoglobulin under different conditions (Fig. 5). As described above, the fluorescence spectrum of the native state showed a maximum at 331 nm at pH 7.5 and the maximum shifted to 348 nm upon denaturation by 4 M Gdn-HCl. When kinetic intermediate of β-lactoglobulin formed a complex with GroEL, the maximum wavelength shifted to 333 nm and the intensity was higher by 1.5 fold than that of the denatured state. This showed that the tryptophan residues were buried in the hydrophobic environment by the complex formation. When ATP was added to this complex, the native-like spectrum was reproduced, showing that β-lactoglobulin was dissociated and refolded to the native state.

Combined Role of Hydrophobic and Electrostatic Interactions

We previously proposed with apocytochrome *c* (pI = 10) that, while the hydrophobic interaction plays major role in the interaction between GroEL and substrate proteins, electrostatic interaction modifies this interaction[12]. The pI value of GroEL is 4.7. When the substrate protein has an overall positive charge like

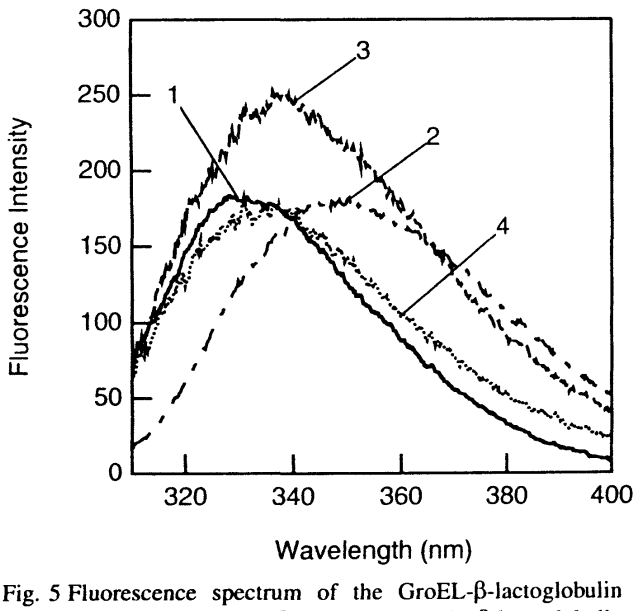

Fig. 5 Fluorescence spectrum of the GroEL-β-lactoglobulin complex. (1) Native β-lactoglobulin, (2) β-lactoglobulin denatured in 4.0 M Gdn-HCl, (3) the complex of GroEL-β-lactoglobulin complex, (4) β-lactoglobulin refolded by adding 2 mM ATP to the solution of (3). Buffers used were 10 mM Tris-HCl (pH 7.5), 50 mM KCl and 10 mM $MgCl_2$. Excitation wavelength was 295 nm. Concentration of β-lactoglobulin was 0.2 mg ml^{-1}. The concentration of GroEL was one equimolar amount of β-lactoglobulin and solution was incubated for 30 minutes at room temperature

cytochrome *c*[12,16], staphylococcal nuclease[17] or barnase[18], electrostatic attractions stabilize the interaction and the addition of salt destabilizes the interaction. On the other hand, when the substrate protein has negative charge like β-lactoglobulin[22] or α-lactalbumin[19-21], electrostatic repulsive forces work and the addition of salts tends to stabilize the interaction. Present results show another clear example that the interaction is determined by the combination of hydrophobic and electrostatic interactions.

The interaction of kinetic intermediate of intact β-lactoglobulin with GroEL was not so strong under low salt conditions. On the other hand, TNB-β-lactoglobulin was bound to GroEL tightly as detected by size exclusion chromatography under relatively low salt conditions. Thus, the kinetic intermediate accumulated after dilution may be more structured than that of TNB-β-lactoglobulin, with less exposed hydrophobic area. Consistent with this, analysis of the refolding kinetics monitored by H/D exchange indicated that the hydrophobic core as well as the non-native helical structure at the N-terminal of the molecule is formed rapidly in several m sec after refolding[26,27]. However, by the addition of salt, which shields the charge repulsion and thus increases the net interaction, even such structured intermediate can be bound tightly to GroEL as detected by gel-filtration chromatography. The present results also indicated the substantial burial of tryptophan residues of β-lactoglobulin upon interaction with GroEL. Formation of the collapsed state is the most likely reason of the red shift. In addition, it is probable that the binding to GroEL buries the tryptophan residues located at the interface between GroEL and substrate.

In the present paper, we did not examine the effects of GroES since we want to close up the interaction of substrate protein and GroEL. However, GroES has been proposed to share the same binding site on GroEL as the substrate proteins. Moreover, the binding of GroEL produces a ternary complex in which the substrate protein is encapsulated in the GroEL/GroES complex. The detailed effects of GroES and various nucleotides on the interaction of GroEL and substrate proteins will be important to relate the present results with the functional cycle of GroEL.

In conclusion, we showed that refolding intermediate of β-lactoglobulin, which previously could not form the stable complex with GroEL, does form a complex under high salt conditions. The results added another example that the interaction is determined by the combined effects of hydrophobic and electrostatic interactions. While the hydrophobic interaction is determined by the structure of the substrate protein itself, electrostatic interactions are modified depending on the solvent conditions.

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