The interaction of β_2 -glycoprotein I domain V with chaperonin GroEL: The similarity with the domain V and membrane interaction

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Abstract

To clarify the mechanism of interaction between chaperonin GroEL and substrate proteins, we studied the conformational changes of the fifth domain of human β_2 -glycoprotein I upon binding to GroEL. The fifth domain has a large flexible loop, containing several hydrophobic residues surrounded by positively charged residues, which has been proposed to be responsible for the binding of β_2 -glycoprotein I to negatively charged phospholipid membranes. The reduction by dithiothreitol of the three intramolecular disulfide bonds of the fifth domain was accelerated in the presence of stoichiometric amounts of GroEL, indicating that the fifth domain was destabilized upon interaction with GroEL. To clarify the GroEL-induced destabilization at the atomic level, we performed H^2H exchange of amide protons using heteronuclear NMR spectroscopy. The presence of GroEL promoted the H^2H exchange of most of the protected amide protons, suggesting that, although the flexible loop of the fifth domain is likely to be responsible for the initiation of binding to GroEL, the interaction with GroEL destabilizes the overall conformation of the fifth domain.

Keywords: β_2 -Glycoprotein I; chaperonin GroEL; disulfide bond reduction; heteronuclear NMR; H^2H exchange

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 (Coyle et al. 1997, 1999; Fenton and Horwich 1997; Rye et al. 1997; Bukau and Horwich 1998; Kawata et al. 1999; Taguchi et al. 2001; Hartl and Hayer-Hartl 2002). X-ray crystallographic analyses have shown that GroEL is composed of 14 identical sub-

units of 57 kD, which form a cylinder containing a central cavity (Braig et al. 1994, 1995; Xu et al. 1997). 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. Although our understanding of the GroEL reaction cycle in terms of the cooperative function of these domains has advanced, one of the remaining questions is the conformation of the bound substrate, which is the most intriguing subject with respect to the conformation of the non-native state of proteins.

GroEL recognizes various conformations of substrate proteins such as folding intermediates, random coil-like state, and different conformations of the same protein (Goldberg et al. 1997; Kawata et al. 1998; Wang et al. 2000). Studies using isolated apical domain fragment "mini-chaperones" confirmed that the apical domain is responsible for the binding of substrate proteins (Buckle et al. 1997;

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Abbreviations: β_2 GPI, β_2 -glycoprotein I; Domain V, recombinant fifth domain of β_2 GPI; DTT, dithiothreitol; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; PF, protection factor; p^2H , pH meter reading of 2H_2O solution; HPLC, high-performance liquid chromatography.

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Golbik et al. 1998; Chatellier et al. 2000a, 2000b; Wang et al. 2000; Smoot et al. 2001). Moreover, detailed structural changes of GroEL upon binding to denatured substrate proteins were reported (Ma et al. 2000; Falke et al. 2001). On the other hand, the conformation of substrate proteins bound to GroEL is still ambiguous. In this study, we attempted to characterize conformation of GroEL-bound substrate protein at the atomic level with recombinant β_2 -glycoprotein I (β_2 -GPI) fifth domain (Domain V) expressed in methylophilic yeast *Pichia pastoris*.

Human β_2 -GPI is a positively charged plasma protein consisting of 326 amino acid residues with a molecular mass of 50 kD. It has five repeated domains, each consisting of about 60 amino acid residues. β_2 -GPI has been shown to bind negatively charged substances such as DNA, heparin, dextran sulfate, and negatively charged phospholipids such as cardiolipin (CL) (Krøll et al. 1976; Polz et al. 1980; Wurm 1984; Schousboe and Rasmussen 1998). It has been identified as a cofactor in the recognition by a subset of antiphospholipid antibodies in autoimmune diseases. Antiphospholipid antibodies bind to β_2 -GPI after complex formation with CL. Aberrant fifth domain (Domain V, 86 amino acids corresponding to Thr241–Cys326 of the whole molecule, 10 kD, pI = 10), with a long and hydrophobic flexible loop, plays important roles in the binding of β_2 -GPI to negatively charged compounds (Steinkasserer et al. 1992; Hunt et al. 1993; Hagihara et al. 1997; Sanghera et al. 1997; Hoshino et al. 2000; Hong et al. 2001).

Usually, GroEL does not interact with the native proteins. However, we expected the positively charged Domain V to interact with the negatively charged GroEL (pI = 4.7) through the flexible and hydrophobic loop of Domain V, similarly to the binding of Domain V to negatively charged membranes. As the size of Domain V is suitable for NMR analysis, we may be able to obtain detailed structural information on the GroEL-bound state. First, we confirmed the interaction of GroEL and Domain V by using the fluorescence spectrum of the single tryptophan residue located in the flexible loop of Domain V. Then, we characterized the effects of interaction on the conformational stability of Domain V by following the reduction of disulfide bonds by dithiothreitol (DTT) and the H^2/H exchange reaction of amide protons monitored by NMR. The results indicated that binding through the flexible loop of Domain V results in global destabilizing of the protein structure.

It has been reported that the GroEL–GroES complex can unfold a trapped protein using the free energy of ATP binding and hydrolysis (Shtilerman et al. 1999). On the other hand, GroEL can unfold proteins in a passive mass action sense, without ATP and GroES (Zahn et al. 1994, 1996). Although the biologic significance of passive unfoldase activity is unknown, the present results imply that it plays a role for some native proteins with the flexible and hydrophobic loops.

Results

Interactions monitored by tryptophan fluorescence

GroEL contains no tryptophan residues, while Domain V has one (Trp76) in the flexible loop. As tryptophan fluorescence can reflect polarity of its environment, we examined the changes in tryptophan fluorescence spectrum upon interaction with GroEL (Fig. 1). In the absence of GroEL, the fluorescence spectrum had a maximum at 348 nm, consistent with its exposure to the solvent. When GroEL was added, the fluorescence intensity increased accompanied by a slight blue shift of the maximal wavelength to 343 nm (Figs. 1C and 1D). This indicated that the tryptophan residue was transferred to a more hydrophobic environment, reflecting its burial in a less polar environment upon interaction with GroEL.

As the interaction of GroEL with Domain V as indicated by fluorescence spectrum might be simply caused by non-specific electrostatic attraction, we also performed a similar experiment using GroES instead of GroEL. The cochaperonin GroES is an acidic protein (pI = 5.0) with no tryptophan residues, a good reference protein with which to examine the contribution of general electrostatic effects. Upon addition of GroES, a slight change was observed in the fluorescence spectrum (Fig. 1B). However, the increase in intensity was much less than that of GroEL, and no shift was observed in the maximum wavelength. The results indicated that the change in the fluorescence spectrum observed by the addition of GroEL represents specific binding in which burial of the flexible loop is involved.

From the initial slope of the titration curve with 2 μ M of Domain V (Fig. 1C), about 4 mole of Domain V was estimated to bind tightly to 1 mole of GroEL 14-mer. On the other hand, the results of the disulfide bond reduction of Domain V, as will be described below, indicated that about 2 mole of Domain V bind to 1 mole of GroEL 14-mer, corresponding to one Domain V to each heptamer. This inconsistency may be explained by the nonspecific electrostatic binding of Domain V to GroEL as observed for GroES–Domain V interaction.

We attempted to monitor the interaction of GroEL with Domain V by gel filtration chromatography as we used for other proteins (Hoshino et al. 1996; Yamasaki et al. 1999). However, no stable complex was detected (data not shown). The present results with tryptophan fluorescence suggested that, although the binding constant is large, the association and dissociation rates are too fast to retain the complex during gel chromatography. It is likely that the small size of the binding region, that is, the flexible loop of about 10 residues, prevents the persistent complex formation required for detection by gel filtration.

Fig. 1. Tryptophan fluorescence spectra of Domain V measured upon excitation at 295 nm at pH 6.0 and 25°C. The concentration of Domain V was 2 μ M. (A) Spectra in the presence of various concentrations of GroEL. GroEL concentration: 1, 0 μ M; 2, 0.5 μ M; 3, 3 μ M. (B) Spectra in the presence of various concentrations of GroES. The GroES concentration: 1, 0 μ M; 2, 1 μ M; 3, 5 μ M. (C) Relative increase in fluorescence intensity at 348 nm as a function of the concentration of GroEL (open circles) or GroES (open squares). The increase in fluorescence intensity in the presence of 3 μ M GroEL was arbitrarily assigned a value of 100. (D) Maximum emission wavelength as a function of GroEL (open circles) or GroES (open squares).

Reduction of the disulfide bonds

Domain V has three intramolecular disulfide bonds (Cys5–Cys56, Cys41–Cys66, Cys48–Cys86), where the numbering of amino acid residues in the whole β 2-GPI molecule can be obtained by adding 240: Cys5 in this article corresponds to Cys245 in the whole molecule. Reduction of disulfide bonds by a reducing reagent, such as DTT, is dependent on protein structure and stability. We monitored the reduction of disulfide bonds of Domain V by DTT at pH 8.0 using reversed-phase high-performance liquid chromatography (HPLC) (Fig. 2A). The intact Domain V (i.e., oxidized Domain V) with three disulfide bonds gave a single elution peak at 19 min. When the protein was incubated with 10 mM DTT, another peak appeared at 25 min and its intensity increased with incubation time. From the 5,5'-dithiobis (2-nitrobenzoic acid) titration of free thiol groups, the new peak was confirmed to be the reduced Domain V with all three disulfide bonds reduced. At an incubation time point of 300 min, the peak for oxidized Domain V disappeared completely. Intriguingly, no disulfide bond reduction inter-

mediate (i.e., species with one or two disulfide bonds) accumulated, indicating that reduction of disulfide bonds occurred highly cooperatively.

The relative fraction of oxidized Domain V (f_{ox}), calculated from its peak area, decreased exponentially against incubation time, t (Fig. 2B). We assumed first-order kinetics with the apparent rate constant, k_{app} : $f_{ox} = \exp(-k_{app} \times t)$ and k_{app} was obtained by least-squares curve fitting to the observed kinetics. The reaction was dependent on the concentration of DTT: The reduction was accelerated with increases in the concentration of DTT. The apparent rate constant increased linearly with increases in the concentration of DTT (Fig. 2C).

We also examined the reduction of disulfide bonds in the presence of 8 M urea and 10 mM DTT (Fig. 2D). The reaction was very rapid with $k_{app} = 2.55 \text{ min}^{-1}$, an increase of about 140-fold in comparison with the rate in the absence of urea (Fig. 2B, squares), indicating that the disulfide bonds in the native state are protected from rapid reduction even though one (Cys48–Cys86) is fairly exposed to the solvent.

Fig. 2. Reduction of disulfide bonds of Domain V by DTT at pH 8.0 and 25°C. (A) Reversed-phase HPLC elution profiles of Domain V (20 μ M) after the indicated incubation time in the presence of 10 mM DTT at pH 8.0 and 25°C. No DTT indicates the profile before addition of DTT. Peak intensity of the oxidized form of Domain V (ox) decreased with time, and that of the reduced-form (red) increased. (B) Decrease of the relative fraction of oxidized Domain V (f_{ox}) with time at various DTT concentrations: (open circles), 2 mM; (open squares), 10 mM; (open up triangles), 50 mM; (open down triangles), 100 mM; (open diamonds), 200 mM. The lines show the simulated kinetics with estimated apparent first-order rate constants. (C) Dependence of the apparent first-order rate constant on the concentration of DTT. (D) Reduction of the disulfide bonds in the presence of 8 M urea and 10 mM DTT. The line shows the first-order kinetics with $k_{app} = 2.55 \text{ sec}^{-1}$.

To examine the effects of GroEL on the reduction of disulfide bonds, we performed the same experiments in the presence of GroEL at a fixed DTT concentration of 10 mM. As GroEL has no disulfide bond, the presence of GroEL itself should not affect the reduction rate. However, we observed notable acceleration of reduction by addition of GroEL (Fig. 3A). The apparent first-order rate constant of reduction (k_{app}) increased with increasing concentration of GroEL, and was saturated at 10 μ M (Fig. 3B). As the concentration of Domain V was 20 μ M, this indicated that 2 moles of Domain V bind to 1 mole of GroEL 14-mer. As mentioned above, we considered this stoichiometry to be more reliable than that obtained from the fluorescence titration with respect to specific binding to the substrate binding site of GroEL.

We also performed the same experiment using GroES, a control protein to examine the specificity of the effects of GroEL (Fig. 3C). No acceleration of the reduction rate was detected by addition of 10 μ M GroES. Thus, nonspecific binding as indicated for GroES did not promote disulfide bond reduction.

H²H exchange monitored by NMR

The acceleration of disulfide bond reduction upon interaction with GroEL suggested the conformational destabilization of Domain V. To clarify the GroEL-induced destabilization at the atomic levels, we examined H^2H exchange of Domain V in the presence and absence of GroEL monitored by heteronuclear NMR.

Fig. 3. Reduction of disulfide bonds of Domain V (20 μM) in the presence and absence of GroEL by 10 mM DTT in 50 mM Tris-HCl (pH 8.0) and 25°C. (A) Decrease with time of the fraction of oxidized Domain V in the absence (open circles) and presence of 3 μM (open up triangles), 5 μM (open squares), 10 μM (open down triangles), and 15 μM (open diamonds) GroEL. The lines indicate the exponential curves with the estimated apparent first-order rate constants. (B) Dependence of the apparent first-order rate constant on the concentration of GroEL. (C) Reduction of Domain V in the absence (open circles) or presence of 10 μM GroEL (open up triangles) or 10 μM GroES (open squares). The lines are the exponential curves with the estimated apparent first-order rate constants.

First, we carried out H^2H exchange of ^{15}N -labeled Domain V in the absence of GroEL at 5°C and p^2H_r 6.0. We employed p^2H_r 6.0 instead of p^2H_r 8.0 to slow down the exchange reaction. The ^{15}N - 1H HSQC spectrum of Domain V showed the backbone 83 amide protons (NHs) among 88 NHs (Hoshino et al. 2000). H^2H exchange reaction was started by dissolving the lyophilized Domain V in 20 mM deuterated Na phosphate buffer (p^2H_r 6.0) at a protein concentration of 1 mM. By recording a series of HSQC spectra, we monitored the exchange reaction of a total of 39 NHs, which were protected from exchange within the sample preparation (a few minutes) and/or recording period of the HSQC spectrum (20 min) (Fig. 4). It was clear without further analysis that NHs highly protected from exchange were clustered on the β -strands C (Ser38, Phe39, Phe40, Cys41) and D (Ala54, Ile57). This indicated that β -strands C and D constitute a core of Domain V, consistent with the three dimensional structure of Domain V (Hoshino et al. 2000).

To obtain a quantitative measure of stability at each amide site, the relative peak intensities of individual residues were plotted against exchange time. Then each plot was analyzed on the basis of single exponential decay with time to calculate the exchange rate constant (k_{ex}) (Fig. 5). As the exchange rate depends on the amino acid type and neighboring residues, we then expressed k_{ex} as protection factor defined by $PF = k_{int}/k_{ex}$, where k_{int} is the intrinsic exchange rate constant predicted from the amino acid sequence (Bai et al. 1993; Connelly et al. 1993). The protection factor represents the extent of protection against exchange, and is thus a measure of the structural rigidity of each NH site. From the plot of PF against residue number, it was obvious that residues with large PF values ($>10^4$) corresponded well with the distribution of the secondary structure (Fig. 6). In particular, the protection factors confirmed that the core of Domain V consisted of β C and β D strands. In contrast, the absence of protected residues in the region between His70 and Thr78 supported the previous suggestion that this region is flexible.

H^2H exchange in the presence of GroEL

We tried to perform the same H^2H exchange experiment in the presence of GroEL. However, at the same protein concentration used for Domain V (i.e., 1 mM), the protein solutions containing GroEL aggregated significantly. As the aggregation was serious even at low protein concentrations for typical NMR measurements, direct observation of the exchange reaction was difficult. Instead, we performed the H^2H exchange reaction at the same low protein concentration as used for the disulfide bond reduction experiments (i.e., 20 μM). After the exchange reaction, Domain V was separated from GroEL by ion-exchange chromatography, followed by concentration by ultrafiltration. As a control,

Fig. 4. The ^{15}N - ^1H HSQC spectra of Domain V after various exchange periods at p^2H_i 6.0 and 5°C in the absence of GroEL. After dissolving Domain V in $^2\text{H}_2\text{O}$ at p^2H_i 6.0, ^{15}N - ^1H HSQC spectra were recorded over 60 h at 5°C . (A) Reference spectrum in H_2O . Spectrum recorded after (B) 10 min, (C) 15 h, and (D) 60 h of reaction.

we also measured the $\text{H}/^2\text{H}$ exchange reaction of Domain V in the absence of GroEL using the same processes of separation and concentration. Although the salt concentration changed markedly during the process of separation of Domain V from GroEL by ion-exchange chromatography (see Materials and Methods), the apparent exchange rate constants for individual residues were similar to those in the absence of salt (data not shown).

$\text{H}/^2\text{H}$ exchange kinetics of Domain V in the absence (large open symbols with error bars) and presence (large closed symbols) of $10\ \mu\text{M}$ GroEL at $10\ \mu\text{M}$ Domain V were included in Figure 5. Due to the experimental difficulty, only the data at 15 and 18 h were obtained. Most of the data obtained at $10\ \mu\text{M}$ Domain V in the absence of GroEL agreed well with the corresponding data at $1\ \text{mM}$ Domain V (i.e., lines), indicating that the processes of separation and concentration did not perturb the $\text{H}/^2\text{H}$ exchange reaction. The only exception was found for Thr60 (Fig. 5H). Although we do not know the precise reason for this difference, it is possible that the interaction between the negative

charges of CM-sepharose resin and the positive charges neighboring Thr60 affected the results.

For most residues, the extent of exchange after 15 or 18 h incubation in the presence of GroEL was much larger than that in the absence of GroEL. Although we did not measure the time course in the presence of GroEL, this strongly suggested that the $\text{H}/^2\text{H}$ exchange kinetics proceeded much faster in the presence of GroEL than in its absence. For each protected NH, we calculated the ratio of intensities (i.e., intensity in the presence of GroEL divided by intensity in its absence) at the same exchange time of 18 h (Fig. 7A). Many showed values less than 0.5, and intriguingly these residues were distributed throughout the molecule. Ratios higher than 1 were observed for Val15, Val16, Ile23, and Cys86. As the protection factors for these residues were relatively low even in the absence of GroEL (Fig. 6), their errors were larger than the others. Nevertheless, it seems that the effect of GroEL binding varies depending on the residue: While the exchange of many residues was accelerated upon interaction with GroEL, some were not notably affected.

Fig. 5. H^2/H exchange kinetics of representative residues of Domain V at 5°C and p^2H , 6.0. Open small symbols show observed relative proton occupancy at 10 mg mL⁻¹, and the solid lines represent the single exponential fitted to the data. Large open and filled symbols show peak intensity of individual residues obtained with the processes of separation and concentration at 20 μM Domain V in the absence or presence of 10 μM GroEL, respectively.

Discussion

Similarity between GroEL–substrate interaction and membrane–Domain V interaction

Domain V of β2-GPI, a highly basic protein with pI of 10, assumes a unique conformation consisting of a disordered flexible loop of about 10 amino acid residues, K(68)EHSSLAFWKT(78), and a core β-sheet structure

(Figs. 6 and 7). This flexible loop contains several hydrophobic residues (i.e., Leu73, Ala74, Phe75, and Try76) surrounded by many positive charges (i.e., Lys42, Lys44, Lys47, Lys68, His70, and Lys77). This unique conformation is responsible for the binding of β2-GPI to acidic phospholipid membranes. Domain V of β2-GPI interacts with acidic membranes through both electrostatic and hydrophobic interactions. This has been suggested to result in the subsequent association of multiple β2-GPI molecules on the

Fig. 6. Localization of the protected NH residues of Domain V at p^2H_i 6.0 and 5°C. (A) Protection factor as a function of the residue number. The locations of β -strands (filled bars) and helices (open bars) are indicated on the top of the figure. (B) Schematic representation of the secondary structures and the protected amide protons. (C) The protected residues represented in the X-ray crystal structure. In (B) and (C), PF values are indicated by different colors: red: $PF > 10^5$; blue: $10^5 > PF > 10^4$; green: $10^4 > PF > 10^2$; white: $10^2 > PF$ or not assigned in our HSQC spectrum. Secondary structures in (B) are indicated by symbols: β -strand (open squares), helix (open diamonds), or others (open circles). Hydrogen bonds are shown by arrows, with the arrowhead pointing to the carbonyl group. Disulfide bonds are indicated by blue solid lines. X-ray crystal structure of Domain V in (C), and Figure 7B was drawn using Molscript (Kraulis 1991) with PDB file 1C1Z.

Fig. 7. Effects of the interaction with GroEL on the protected NH residues of Domain V at p^2H_i 6.0 and 5°C. (A) Ratio of the peak intensities of 20 μ M Domain V in the absence (I_{free}) and presence (I_{+EL}) of 10 μ M GroEL at same exchange time at 18 h was plotted as a function of residue number. (B) Localization of the residues affected by binding to GroEL represented in the X-ray crystal structure. The effects of GroEL are indicated by different colors: green: $I_{+EL}/I_{free} > 1$; blue: $1 > I_{+EL}/I_{free} > 0.1$; red: $0.1 > I_{+EL}/I_{free}$.

membrane (Glaser et al. 1996; Hinderliter et al. 2001). Alternatively, the interaction of β_2 -GPI with the membrane fragment through Domain V may cause formation of an aggregative complex comprised of membrane and β_2 -GPI molecules (Hong et al. 2001). These complexes are considered to be effective targets of antiphospholipid antibodies present in patients. We considered the above mechanism of interaction between β_2 -GPI and membrane to be analogous to that between GroEL and its substrates, although the aggregation or association of substrates after the initial interaction is not obvious in the case of GroEL. The analogy of the GroEL–substrate interaction with the membrane–protein interaction was previously proposed by us to interpret the interaction between GroEL and apocytochrome *c*, a disordered highly basic protein (Hoshino et al. 1996).

As expected, Domain V was bound to GroEL as demonstrated by the fluorescence of the single tryptophan residue located in the flexible loop. The burial of Trp76 upon in-

teraction with GroEL was similar to the interaction of Domain V with acidic phospholipid membranes, indicating that both hydrophobic and electrostatic interactions contribute to the specific binding of Domain V to the substrate binding site of GroEL.

The affinity of GroEL with various substrate proteins have been suggested to be determined mainly by hydrophobic interactions (Fenton and Horwich 1997; Coyle et al. 1997; Bukau and Horwich 1998; Chen and Sigler 1999; Hartl and Hayer-Hartl 2002). Electrostatic interactions also contribute favorably when the net charge of the substrate is positive (e.g., cytochrome *c*; Hoshino et al. 1996) or adversely when the net charge is negative (e.g., α -lactalbumin, Katsumata et al. 1996; β -lactoglobulin, Yamasaki et al. 1999). In the case of Domain V, hydrophobic residues on the flexible loop are mainly responsible for the interaction, and the presence of several positive charges also contributes to stabilization of the interaction. Indeed, with increasing salt concentration, the apparent affinity of Domain V for GroEL was decreased (data not shown). However, as described in the Results section, stable GroEL-Domain V complex formation was not observed by gel filtration chromatography, probably because the association and dissociation rates are too fast to retain the complex during gel chromatography.

Conformational change induced by GroEL-binding

Although the interaction of Domain V with GroEL is initiated through the flexible loop, it is likely that the conformational stability of the rest of the molecule is also affected. We probed the stability of Domain V by the reactivity of disulfide bonds and H^2H exchange reaction of amide protons. Both can be comprehensively interpreted by assuming scheme 1, a mechanism often used to address the H^2H exchange reaction of amide protons.

where N, X, and D are the native state, state accessible to DTT or D_2O , and the reduced or exchanged state, respectively, K_{NX} ($= [X]/[N]$) and $K_{N'X'}$ ($= [X']/[N']$) are the equilibrium constants of the corresponding processes and k_{int} is the intrinsic reaction rate under the experimental conditions.

Reduction of disulfide bonds.

In the case of the reduction of disulfide bonds, we can assume that the equilibrium between N and X is rapid in comparison with the intrinsic reduction rate, $k_{int}[DTT]$, as the reduction rate increased linearly with increases in the

concentration of DTT (Fig. 2C). Thus, the apparent rate constant of reduction, k_{app} , is represented by $K_{NX} k_{int}[DTT]$. From the comparison in the presence and absence of 8 M urea at 10 mM DTT, K_{NX} was estimated to be 7×10^{-3} . By analogy with H^2H exchange reaction, the protection factor (PF) from the reducing reaction, the inverse of K_{NX} was estimated to be 140. The interaction with GroEL increased the apparent reduction rate by threefold. The acceleration can be most simply interpreted in terms of the shift of $N \rightarrow X$ equilibrium by threefold, or the change in protection factor by a factor of 3. Although the binding may cause the conformational change of N, as represented by N' , the disulfide bond reduction experiments did not specify the possible conformational change.

H^2H exchange of amide protons.

For H^2H exchange reaction of NHs at pH 6, the EX2 mechanism is more likely than the EX1 mechanism because k_{int} is considered to be small in comparison with the refolding rate of conformational change. Thus, as is the case for disulfide bond reduction, the protection factor represents the equilibrium constant between the exchangeable and protected states. The advantage of NMR is that we can discuss conformational fluctuation at each amide site. The high PF values for many residues in β -strands C and D indicated that these regions rarely experience the unfolded conformation accessible to exchange (Fig. 6).

Upon binding to GroEL, the exchange reaction of many protected NHs was notably accelerated. The reduction of the disulfide bonds suggested that the interaction with GroEL shifted the equilibrium to the more disordered conformation by a factor of 3. If the conformational transition follows a strict two-state transition as shown in Scheme 1, this factor of destabilization should be independent of amino acid residues. However, we observed significant variations in the ratio of remaining NHs between the presence and absence of GroEL (Fig. 7). This argued that the effects of GroEL binding are more complicated than the simple shift of equilibrium between N and U. It is likely that, upon interaction with GroEL through the flexible loop, the entire conformation of Domain V was distorted as represented by N' . Alternatively, the shift of the equilibrium constant upon interaction with GroEL may not be uniform. It is possible that the shift of the equilibrium is larger for residues located close to the flexible loop.

Correlation between reduction of disulfide bond and H^2H exchange

As described above, K_{NX} for reduction of the disulfide bond was estimated to be 7×10^{-3} . Therefore, the protection factor of the disulfide bond, PF(SS), was 140. Among the three

disulfide bonds of Domain V (i.e., Cys5–Cys56, Cys41–Cys66, Cys48–Cys86), side chains of Cys48–Cys86 are most exposed to solvent and this disulfide bond may be the one first attacked by DTT (Fig. 6C). The observed PF(SS) value of 140 indicates that the disulfide bond is fairly protected against reduction even if the disulfide bond is exposed to solvent. On the other hand, the PF values of cysteine NH estimated by H^2H exchange varied significantly depending on the residue. The PF values of Cys5, Cys41, Cys48, and Cys86 were estimated to be 3.5×10^4 , 4.3×10^5 , 1.4×10^5 , and 3.8×10^4 , respectively, and those of Cys56 and Cys66 to be less than 10^2 (Fig. 6). While the amide protons of exposed cysteine residues (i.e., Cys48 and Cys86) are protected from exchange, for Cys5–Cys56 and Cys41–Cys66, only one of the two residues forming the disulfide bond was protected from exchange. Most of the protected residues form hydrogen bonds as indicated by X-ray analysis (Fig. 6B). The results indicated that accessibility of the side chain disulfide bond to reducing reagents such as DTT and accessibility of the main chain amide protons to H/D exchange are not correlated. Although not surprising, we considered these results clearly implying the independence of disulfide bond reduction and H/D exchange to be intriguing.

Implication for the unfoldase activity of GroEL

We believe the present results could also be useful to understand the unfoldase activity of GroEL. The role of GroEL as an unfoldase has been proposed in several reports. Zahn et al. (1994) examined the interaction of GroEL with the small protein cyclophilin using NMR, and indicated that the complete secondary structure of cyclophilin was disrupted when bound to GroEL. Zahn et al. (1996) investigated the interaction of barnase with GroEL and SecB by H^2H exchange monitored by NMR, and indicated that both chaperones bound native barnase under physiologic conditions and catalyzed H^2H exchange of deeply buried amide protons. They reported that the presence of GroEL or SecB increased the exchange rate constants of the locally exchanging protons by a factor of 2 or 3 and globally exchanging protons by a factor of 4 to 25. Nieba-Axmann et al. (1997) also studied by NMR the exchange of amide protons of cyclophilin A interacting with GroEL. They reported that a set of highly protected protons in the native state ($PF = 10^5$ – 10^7) were much less protected ($PF = 10^2$ – 10^4) in the complex with GroEL.

In the examples described above, the unfoldase activity can be explained by a passive mass action sense, in which GroEL selectively interacts with the more unfolded form out of an equilibrium mixture of the folded and unfolded conformations. In contrast, Shtilerman et al. (1999) showed by hydrogen exchange that the GroEL–GroES complex can unfold a trapped protein actively using the free energy of

ATP binding and hydrolysis. They suggested that the passive unfolding is less likely for GroEL in active *E. coli*.

Our results were basically consistent with the passive unfolding of substrate proteins by GroEL. However, the results suggest a unique mechanism promoting the interaction of substrates with GroEL. In the Domain V and GroEL interaction, by anchoring the flexible loop, the interaction of the rigid core with GroEL occurs easily because of the increase in effective concentration of the interacting sites. This provides a driving force to destabilize or denature the rigid native conformation of Domain V. In other words, because of the hydrophobic environment of GroEL substrate binding site, once the protein molecules bind through the loop region, the hydrophobic interactions of protein interiors may be weakened effectively, resulting in destabilization of the entire molecule. Thus, the presence of the initial interaction site is important for the passive unfolding by GroEL, and substrate proteins with the flexible and hydrophobic loops might be unfolded or destabilized effectively by GroEL even in active *E. coli*.

Materials and methods

GroEL and GroES

GroEL and GroES were prepared with a GroE-overproducing strain of *E. coli*, DH1/pKY206, a gift from Prof. Kawata (Tottori University). Purification of GroEL/GroES was performed according to the method of Buchner et al. (1991) with some modifications. The entire purification was carried out in 50 mM Tris-HCl buffer (pH 7.8), containing 1 mM EDTA, 1 mM β -mercaptoethanol, and 50 mM NaCl. After lysis of the cells by sonication and removal of the insoluble material by centrifugation for 45 min at $12,000 \times g$, the supernatant was collected. Ammonium sulfate was added to the solution to 55% saturation and stirred for 1 h on ice. The precipitated protein was collected by centrifugation for 30 min at $12,000 \times g$ and dissolved in the buffer. This protein solution was applied to a Sephacryl S-300 gel-filtration column and fractions of GroEL or GroES were obtained. GroEL was further purified by ion-exchange chromatography with a Q-Sepharose column, followed by a reactive red A column (Clark et al. 1998). GroES solution was heat treated for 20 min at 80°C . The supernatant obtained by heat treatment was purified by ion-exchange chromatography with a Q-Sepharose column.

β_2 -GPI Domain V

Human β_2 -GPI Domain V with a signal peptide, Tyr-Val-Glu-Phe-Met-Ile-Glu-Gly-Arg-Thr, added to the amino-terminal Lys2 (i.e., Lys242 of the whole molecule) was expressed in methylotrophic yeast *Pichia pastoris*, and then purified by ion-exchange chromatography, as described previously (Hagihara et al. 1997). For heteronuclear NMR spectroscopy, ^{15}N -labeled β_2 -GPI Domain V was also expressed in *P. pastoris* using ^{15}N -ammonium sulfate.

Fluorescence measurements

Tryptophan fluorescence spectra were measured with a Hitachi fluorescence spectrometer, model F-4500, at 25°C in 20 mM so-

dium phosphate buffer (pH 6.0) with excitation at 295 nm at 2 μ M Domain V in the presence of 0–3 μ M GroEL. As a control acidic protein, we used GroES, and tryptophan fluorescence of Domain V was measured in the same way.

Reduction of disulfide bonds of Domain V

Reduction of disulfide bonds of Domain V was carried out at 20 μ M Domain V in 50 mM Tris-HCl buffer (pH 8.0) at 25°C. The solution of Domain V was incubated in the presence of DTT for various periods and the reaction was quenched by the addition of HCl to decrease the pH to 2. Then, the extent of reduction was monitored by reversed-phase HPLC with absorption at 220 nm.

H^2H exchange measurements

All NMR spectra were recorded on a 500 MHz spectrometer (Bruker DRX500) equipped with a triple-axis gradient and triple-resonance probe.

The exchange of Domain V was performed at 5°C and p^2H_2 6.0 by dissolving the lyophilized protein into 20 mM Na phosphate buffer at a protein concentration 10 mg mL⁻¹. The reaction was monitored by recording a series of ¹⁵N-¹H HSQC spectra over 60 h. Spectra were processed with NMRPipe and analyzed with NMRDraw and PIPP (Delaglio et al. 1995). The exchange kinetics were analyzed assuming single exponential decay with time. Protection factors were calculated as a ratio between intrinsic exchange rate constant predicted from the amino acid sequence and observed rate constant.

The H^2H exchange in the presence of GroEL was carried out at 20 μ M Domain V, 10 μ M GroEL, and 20 mM Na phosphate buffer (pH 6.0) at 5°C. Deuterated GroEL and deuterated Na phosphate buffer were used. After the exchange reaction, CM-Sepharose was added to the protein solution to adsorb Domain V (15 min). CM-Sepharose was washed with 20 mM Na phosphate buffer (30 min) and then Domain V was eluted with the same buffer containing 400 mM KCl (45 min). This Domain V solution was concentrated by ultrafiltration (6 h) for measurements of ¹⁵N-¹H HSQC spectra using NMR.

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