Calorimetric Observation of a GroEL-Protein Binding Reaction with Little Contribution of Hydrophobic Interaction*

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Binding of Escherichia coli chaperonin, GroEL, to substrate proteins with non-native structure, reduced \( \alpha \)-lactalbumin (rLA) and denatured pepsin, were analyzed by isothermal titration calorimetry at various temperatures in the presence of salt (0.2 M KCl). Both proteins bound to GroEL with 1:1 stoichiometry and micromolar affinity at all temperatures tested. However, thermodynamic properties of their binding to GroEL are remarkably different from each other. While heat capacity changes (\( \Delta C_p \)) of rLA-GroEL binding showed large negative values, -4.19 kJ mol\(^{-1} \) K\(^{-1} \), that of denatured pepsin-GroEL binding was only -0.2 kJ mol\(^{-1} \) K\(^{-1} \). These values strongly indicate that the hydrophobic interaction is a major force of rLA-GroEL binding but not so for denatured pepsin-GroEL binding. When salt was omitted from the solution, the affinity and \( \Delta C_p \) of the rLA-GroEL binding reaction were not significantly changed whereas denatured pepsin lost affinity to GroEL. Thus, in the non-native protein-GroEL binding reaction, thermodynamic properties, as well as the effect of salt, differ from protein to protein and hydrophobic interaction may not always be a major driving force.

Chaperonin GroEL from Escherichia coli is an abundant and indispensable protein that assists folding of other proteins in vitro and in vivo (1, 2). As determined by x-ray crystallography, GroEL appears to be a hollow cylinder of 14 identical 57-kDa subunits, which are made of 7-fold rotationally symmetrical rings stacked back-to-back with a dyad symmetry (3). GroEL binds non-native proteins, prevents them from irreversible aggregation, and improves the final yield of productive folding in a co-chaperonin GroES- and ATP-dependent manner (2, 4). A wide range of proteins, unrelated in their primary sequences and native tertiary structures, can be a "substrate" for GroEL (5). Since GroEL cannot interact with proteins in native state, there should exist structural features recognizable by GroEL that are commonly found in many proteins in the non-native, but not in the native, state. However, a search for any specific, definite structural features of substrate proteins responsible for GroEL binding, such as a certain secondary structure and a short peptide sequence motif, has been unsuccessful. Rather, based on the observations that two or more structurally different non-native states of the same protein can bind to GroEL (6–10), the view has been postulated that the conformational state of a protein recognized by GroEL is not necessarily unique. According to this view, driven by hydrophobic interaction, GroEL recognizes and binds to hydrophobic clusters of substrate proteins which are exposed to accessible surface only when they are in non-native state.

To assess the extent of hydrophobic interaction in the GroEL-substrate protein binding by experiment, calorimetric analysis of the binding reaction provides useful information. Especially, temperature dependence of the values of enthalpy change of the binding reaction, designated as heat capacity change (\( \Delta C_p \)), is thought to be a good marker to evaluate the contribution of hydrophobic interaction. Lin et al. (11) reported a large negative \( \Delta C_p \) value, indicative of strong hydrophobic interaction, for the binding of the unfolded mutant of subtilisin to GroEL, and they concluded that the binding of substrate protein to GroEL is determined by hydrophobic interaction. However, their report has been so far the sole report on calorimetric analysis of GroEL-substrate protein binding and more examples have to be analyzed before generalization of their conclusion.

The present study was carried out to obtain the further insight in the thermodynamic aspect of the GroEL-substrate proteins interaction by isothermal calorimetric titration in the presence (0.2 M KCl) or absence of salt. We chose two kinds of non-native proteins, reduced \( \alpha \)-lactalbumin (rLA) and denatured pepsin, as substrate proteins. \( \alpha \)-Lactalbumin (\( \alpha \)-LA) is a small Ca\(^{2+} \)-binding protein (14,200 Da) present in mammalian milk and is one of the best characterized proteins in protein folding study (12). \( \alpha \)-LA without bound Ca\(^{2+} \) (apo-\( \alpha \)-LA) assumes the stable molten globule state at low salt concentration (13) and rLA is known to be more unfolded than the molten globule state although it has some secondary structure as measured by CD spectra (6, 8). Another substrate protein used in this study, pepsin, is a gastric aspartic proteinase (34,550 Da) with pH optimum for catalytic activity at pH < 2. Pepsin contains two conformationally homologous domains and mainly consists of \( \beta \) sheets at acidic pH (14), and when the pH is raised to above pH 7, it loses the activity with its conformation being denatured irreversibly (15). The results of calorimetry show that hydrophobic interaction may not always be the major binding force in GroEL-substrate protein binding. In addition, it is also shown that the salt has a significant effect on the binding.

EXPERIMENTAL PROCEDURES

Materials—GroEL was purified from E. coli cells JM109 bearing the expression plasmid pKY206, which was a kind gift from Drs. Ito and Akiyama (16, 17). The concentration of proteins was determined spec-

The abbreviations used are: rLA, \( \alpha \)-lactalbumin from bovine whose four disulfide bonds are reduced; HPLC, high performance liquid chromatography.
trophotometrally. Extinction coefficients at 280 nm used for GroEL, rLa, and pepsin were 23,800 (18), 25,900 (19), and 38,600 \( \text{m}^{-1} \text{cm}^{-1} \), respectively. The concentration of GroEL is described as tetradecamer concentration throughout this paper. Bovine apo-\( \alpha \)-LA (type III, Ca\(^{2+}\)-depleted) and porcine pepsin were purchased from Sigma. rLa was prepared by dissolving bovine apo-\( \alpha \)-LA in buffer A (25 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA) containing 0.2 mM KCl when indicated. Pepsin was dissolved in buffer A and was used as denatured pepsin. Fluorescently labeled pepsin was prepared as follows. Pepsin (3.5 mg) was dissolved in 180 \( \mu \text{l} \) of 0.1 M sodium carbonate buffer (pH 9), and 20 \( \mu \text{l} \) of 20 mM dansyl chloride was added. The solution was incubated for 18 h at 4 °C, and residual free dansyl chloride was removed by a Sephadex\textsuperscript{TM} G-25 column. Approximately 1 mol of dansyl chloride were coupled per mol of pepsin.

Circular Dichroism—Circular dichroism spectra were recorded at room temperature with a Jasco J720 spectropolarimeter using a 1-mm path length cell for far-UV spectra and a 10-mm path length cell for near-UV spectra. For calculation of the mean residue molar ellipticity \( [\theta] \), the mean residue weights of 115 (rLA) and 106 (pepsin) were used.

HPLC Analysis—Binding of dansyl-labeled pepsin to GroEL was analyzed with size exclusion HPLC analysis. Labeled pepsin (0.5 \( \mu \text{M} \)) was incubated with GroEL (1 \( \mu \text{M} \)) in the elution buffer (50 mM Tris-HCl buffer, pH 7.8, 1 mM dithiothreitol, 0.2 mM KCl) for 20 min at 37 °C. When indicated, Mg-ATP (final concentration, 5 mM ATP and 5 mM MgCl\(_2\)) and GroES (final concentration, 5 \( \mu \text{M} \)) were supplemented to the mixture. The mixtures were applied on a size exclusion HPLC column, Superdex\textsuperscript{TM} 200 HR10/30, that had been equilibrated with the elution buffer with or without 1 mM Mg-ATP. The column was eluted with the same solution used for the equilibration at a flow rate of 0.8 ml/min at room temperature. Elution of labeled pepsin was monitored with an on-line fluorometer (FS-8010, Tosoh; excitation at 340 nm and emission at 540 nm).

Isothermal Titration Calorimetry—Calorimetric measurements were carried out using a MicroCal OMEGA ultramicrocalorimetric titration calorimeter (MicroCal, Inc., Northampton, MA) (20, 21). The reference cell was filled with water, and the instrument was calibrated using standard electrical pulses. The GroEL solution and rLA solution were separately dialyzed in buffer A for more than 12 h at 4 °C. The dialysis medium contained 0.2 mM KCl when indicated. All solutions were thoroughly dialyzed by stirring under vacuum before use. The solutions of GroEL (16–20 \( \mu \text{M} \)) in buffer A were titrated with solutions of rLA (170–200 \( \mu \text{M} \)) or denatured pepsin (157–167 \( \mu \text{M} \)) in the same buffer at various temperatures. The titrations were carried out by injecting 20 identical 15-\( \mu \text{l} \) solutions at 3-min intervals from a syringe into the sample cell (1.31 ml). The injection syringe, on which a stirrer paddle is mounted, stirred the solutions at 400 rpm. Control experiments were performed by making the same injections of the substrate proteins into a cell containing the buffer without GroEL. The peaks of the obtained thermographs were integrated using the ORIGIN software supplied with the instrument by MicroCal, Inc. The resulting isotherms were fitted by nonlinear least-square simulation, as described previously (20), yielding estimates of the dissociation constant \( (K_d) \), the caloricimetric enthalpy change \( (\Delta H) \), and the number of bound substrate protein per GroEL (N).

RESULTS

Substrate Proteins—The structural features of \( \alpha \)-LA have been investigated extensively (6, 8, 12), and CD spectra of our preparation of rLA agreed with the reported ones (6, 8) (data not shown). In contrast, structural features of pepsin denatured at neutral pH have been less characterized. When the pH is raised to above pH 7, pepsin loses the activity due to selective denaturation of N-terminal domain (15, 22). In fact, only a trace amount of casein was digested after a 1-h incubation with pepsin at 37 °C at neutral pH (data not shown). When pepsin was applied on size exclusion HPLC, equilibrated, and eluted at pH 7.8, it was eluted at a retention time corresponding to that of a globular protein with \( M_t \), 65,000, about double of the true \( M_t \) of pepsin (refer to Fig. 2; a 30-kDa globular protein should be eluted at 21 min), indicating an expanded, noncompact molecular shape of pepsin at pH 7.8. CD spectra of pepsin at 200–250 nm at pH 7.8 shows that the trough at 210 nm of native pepsin shifts to 200 nm, but some secondary structure remains (Fig. 1A). It is also shown from CD spectra at 250–300 nm that ordered arrangement of aromatic amino acid residues is largely lost at pH 7.8 (Fig. 1B). The presence of 0.2 mM KCl did not affect the spectra. Therefore, it is safe to conclude that pepsin at pH 7.8 is in the partially unfolded, flexible state, and we termed pepsin in this state as denatured pepsin hereafter.

Binding of denatured pepsin to the polypeptide binding site of GroEL was directly demonstrated using denatured pepsin labeled by a fluorescent dye, dansyl chloride. When the incubated mixture of labeled pepsin and GroEL was applied on size exclusion HPLC, labeled pepsin was coeluted with GroEL (Fig. 2a). The amount of labeled pepsin at the GroEL fraction was slightly decreased when Mg-ATP was present in the mixture (Fig. 2b). Inclusion of GroES in addition to Mg-ATP resulted in a further decrease of the amount of labeled pepsin at the GroEL fraction, and free denatured pepsin increased reciprocally (Fig. 2c). The residual labeled pepsin at the GroEL fraction observed in Fig. 2c was caused from rebinding of released labeled pepsin during chromatography because most GroEL-bound labeled pepsin disappeared when the elution buffer contained Mg-ATP (Fig. 2d) and Mg-ATP + GroES (Fig. 2e). Denatured pepsin, either labeled or non-labeled with dansyl chloride, inhibited the GroEL/ES-mediated folding reaction of guanidine HCl-denatured rhodanese; in the presence of 27 molar excess of denatured pepsin or labeled denatured pepsin over denatured rhodanese, the yield of productive folding of rhodanese was 3.1 or 1.8%, respectively, of the yield in the absence of these denatured pepsins. These results ensure that denatured pepsin binds to the polypeptide binding sites of GroEL as a substrate protein.

Calorimetry of rLA-GroEL Binding—Fig. 3A represents a typical raw trace of calorimetric titration of rLA to the GroEL solution. Titration was carried out at 10.0 °C in the presence of 0.2 mM KCl. Area of each peak corresponds to the amount of heat uptake (upward peak) or release (downward peak) at each injection of rLA. The peak area of the last injection of Fig. 3, upper trace, was very close to that of the simple dilution of rLA into the buffer without GroEL shown in the lower trace, indicating that the binding reaction was almost completed at the last injection. Heat of each injection was integrated, corrected for the dilution heat, and was analyzed by nonlinear least squares fitting of the data (Fig. 3B). The best fit curve shown in Fig. 3B was obtained when the value of each parameter of the binding reaction was given as follows: dissociation constant, \( K_d = 5.88 \times 10^{-6} \text{ M} \); enthalpy change, \( \Delta H = 49.7 \text{ kJ mol}^{-1} \); and saturated molar ratio of bound rLA per GroEL, \( N = 0.91 \text{ mol} \text{mol}^{-1} \). Gibbs energy change (\( \Delta G = -28.4 \text{ kJ mol}^{-1} \))
Calorimetry of GroEL-Protein Binding

entropy change ($\Delta S = 276 \text{ J mol}^{-1} \text{ K}^{-1}$) were calculated from the value of $K_d$ and $\Delta H$ ($\Delta G = RT \ln K_d$, $\Delta G = \Delta H - T\Delta S$). The $K_d$ value obtained here is three orders larger than that obtained from surface plasmon resonance (BIACoreTM) (23). Although the real reason is not known, covalent immobilization of rLA to the measuring tip of BIAcoreTM and/or rebinding of released GroEL to immobilized rLA might be possible reasons.

Similar calorimetric titration and analysis were carried out at 10.5 °C in the absence of KCl (Fig. 3, C and D). $N$, $K_d$, $\Delta H$, $\Delta G$, and $\Delta S$ are calculated to be 1.33 mol/mol, 1.51 × 10⁻⁶ M, 82.2 kJ mol⁻¹, −31.6 kJ mol⁻¹, and 401 J mol⁻¹ K⁻¹, respectively. This result is not consistent with the observation by Okazaki et al. (6) that rLA did not bind to GroEL when KCl was absent. They used size exclusion chromatography for the analysis of the rLA-GroEL binding. It is possible that this method tends to underestimate the binding because, once rLA dissociates from GroEL in the column, it has less chance to rebind to GroEL since both proteins are being physically separated.

Temperature dependence of these parameters was examined by similar titration experiments at various temperatures up to 40.0 °C (Fig. 5, A and B). The saturated molar ratio of bound rLA per GroEL appeared to be independent of temperature, roughly 1 mol/mol (+KCl, 1.12 ± 0.25 mol/mol; −KCl, 1.32 ± 0.28 mol/mol). $K_d$ and, therefore, $\Delta G$ values were also almost constant at all temperatures in the presence (Fig. 5A) or absence of KCl (Fig. 5B). At low temperatures, −22 °C (+KCl) and <35 °C (−KCl), the enthalpy change is positive and is unfavorable to the binding of rLA to GroEL. The binding reaction is, therefore, driven by entropy change at this temperature range. As temperature raised, contribution of entropy change to the binding reaction decreased and that of enthalpy change increased (Fig. 5A and B; $-\Delta T\Delta S$ is plotted instead of $\Delta S$). As a consequence of this reciprocal change of $\Delta H$ and $\Delta S$, at high temperatures, >30 °C (+KCl) and >45 °C (−KCl from extrapolation), enthalpy change becomes the driving force of the binding reaction.

Calorimetry of Denatured Pepsin-GroEL Binding—Fig. 4 represents typical raw traces and analysis of calorimetric titration of denatured pepsin to the GroEL solution. The titrations were carried out in the presence of 0.2 M KCl at 25.1 °C (Fig. 4A) and in the absence of KCl at 25.0 °C (Fig. 4C). In the presence of KCl, values of parameters obtained from analysis of the integrated curve (Fig. 4B), $N$ (1.01 ± 0.16 mol/mol), $\Delta G$ (−32.1 kJ mol⁻¹), and $K_d$ (2.41 × 10⁻⁶ M), are fairly close to those of the rLA-GroEL binding at 10.0 °C. Similar to the case of the rLA-GroEL binding, these values are almost constant at various temperatures (Fig. 5C). The values of $\Delta H$ (47.4 kJ mol⁻¹) and $\Delta S$ (267 J mol⁻¹ K⁻¹) are also close to those observed for rLA at 10.0 °C, but very different from the rLA, these values were not affected by temperature. That is, they were constant, always positive from 11.7 to 30.1 °C (Fig. 5C). This indicates that the binding reaction of denatured pepsin to GroEL is driven by entropy change at all temperatures. Another remarkable difference from rLA is that denatured pepsin cannot bind to GroEL when KCl is absent; no net heat uptake or release was observed at 25 °C (Fig. 4C). This indicates that the binding reaction of denatured pepsin to GroEL is driven by entropy change at all temperatures. Another remarkable difference from rLA is that denatured pepsin cannot bind to GroEL when KCl is absent; no net heat uptake or release was observed at 25 °C (Fig. 4C). The same results were observed at 11 and 35 °C. Therefore, denatured pepsin most likely cannot bind to GroEL in the absence of salt although we cannot eliminate a small possibility that binding occurs without enthalpy change at all temperatures. Pepsin is a highly acidic protein (−38 net charge at pH 7.4), and GroEL is also an acidic protein (−18 net charge per GroEL monomer at pH 7.4). The extraordinary acidic nature of the pepsin may cause strong electrostatic repulsion to GroEL which can be overcome only when electrostatic shielding effect is provided by counter-ion binding to the proteins at high concentration of salt. rLA is also an acidic protein (−7 net charge at pH 7.4), but electrostatic repulsion between them can be overcome or compensated for even in the absence of salt.
DISCUSSION

Forces of GroEL-protein binding are exerted at the GroEL-protein interfaces. In principle, titration calorimetry is reporting energy changes within the entire system rather than reflecting only the local interaction at the GroEL-protein interfaces. The observed energy changes can be considered to be the sum of energy changes derived from the local interaction at the binding sites and those from accompanying conformational changes in GroEL and the substrate protein. Titration calorimetry can provide information on thermodynamic properties of binding reaction itself only if the contribution of the latter fractions is little. Fortunately, by virtue of the recent achievement in structural studies of chaperonin, it seems to be really the case. Buckle et al. (24) determined crystal structure of mini-chaperonin (monomeric polypeptide-binding fragment corresponding to the apical domain of GroEL) with bound polypeptide. It is almost identical to the structure of mini-chaperonin without bound polypeptide that they determined previously (25) and to the structure of the apical domain of the intact GroEL (26). Thus, the conformation within apical domain of GroEL is not changed significantly by the binding of substrate polypeptide. In addition, contrary to the large en bloc movement of apical domain of GroEL induced by GroES binding (26), substrate polypeptide can bind to GroEL without large domain movement (27). In the crystal structure of mini-chaperonin with bound polypeptide, seven substrate residues are bound in a relatively extended conformation (24). This implies that each polypeptide-binding site of GroEL recognizes a short, flexible, extended polypeptide segment, and specific conformational change is not necessary for partially or fully unfolded proteins to bind GroEL. Taken together, it seems reasonable to assume that binding of polypeptide by itself neither induces large conformational change in GroEL nor in substrate protein and that energy changes observed in titration calorimetry are primarily caused from the interaction of GroEL-protein interfaces.

Recently, the heat capacity change ($\Delta C_p = d\Delta H/dT$) of the binding reaction has been taken as a reliable thermodynamic parameter that reflects the contribution of hydrophobic interaction to the reaction; the large negative heat capacity change has been thought to be strong evidence for the role of hydrophobic interactions as the driving force for the binding (28–31). We calculated $\Delta C_p$ from temperature dependence of $\Delta H$ by least-squares fitting of $\Delta H$-temperature plots (Fig. 5D). The calculated $\Delta C_p$ values were $-4.19 \text{ kJ mol}^{-1} \text{ K}^{-1}$ (rLA, +KCl), $-3.12 \text{ kJ mol}^{-1} \text{ K}^{-1}$ (rLA, -KCl), and $-0.20 \text{ kJ mol}^{-1} \text{ K}^{-1}$ (denatured pepsi, +KCl). Therefore, hydrophobic interaction plays a major role in the binding of rLA to GroEL irrespective of the presence of salt, but it is not the case for the binding reaction of denatured pepsi to GroEL. Lin et al. (11) reported calorimetric analysis of the binding of the unfolded mutant of subtilisin BPN to GroEL and obtained the value of $\Delta C_p = -3.6 \text{ kJ mol}^{-1} \text{ K}^{-1}$. Based on this large negative value, they concluded that the binding of substrate protein to GroEL was determined by hydrophobic interaction. According to the results reported here, this conclusion is valid for the rLA-GroEL binding but not for the denatured pepsi-GroEL binding, even though all three model proteins, a mutant BPN (11), rLA, and denatured pepsi have similar micromolar affinities (and hence similar $\Delta G$ values of the binding) to GroEL. Thus, the extent of contribution of hydrophobic interaction in the binding force appears to be different from one protein to another. In the crystal structure of mini-chaperonin with bound heptapeptide (GLVPRGS), binding force appears to be provided from two kinds of interactions: four hydrogen bonds between the main chain of the substrate peptide and the polar side chains of the binding site of GroEL, and hydrophobic interaction between leucine and valine resi-
dues of the substrate peptide and the nonpolar side chains of the binding site (24). Combining the structural information with our results, it seems likely that GroEL-protein binding is stabilized by hydrogen bonds, and an additional, but yet indispensable, binding force is also provided from interactions between side chains of the polypeptide and GroEL. Depending on whether side chains of the bound polypeptide segment are polar or nonpolar, the substrate-binding site of GroEL can present either polar or nonpolar side chains as binding counterparts, and thus extent of hydrophobic interaction in the GroEL-protein binding reaction can be varied depending on substrate proteins.

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