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Effect of Electrostatic Interactions on the Binding of Charged Substrate to GroEL Studied by Highly Sensitive Fluorescence Correlation Spectroscopy

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The binding processes of GroEL with apo cytochrome *c* (apo-cyt *c*) and disulfide-reduced apo α-lactalbumin (rLA) in homogeneous solution at low concentration were analyzed by fluorescence correlation spectroscopy (FCS) with extremely high sensitivity. Although apo-cyt *c*, a positively charged substrate, was tightly bound to GroEL in both the absence and the presence of 200 mM KCl, the strength of the binding was changed with varying salt concentration. Results from experiments when two different salts (KCl or $MgCl_2$) were titrated into a sample solution containing GroEL and apo-cyt *c* clearly showed that the binding strength decreased with increasing salt concentration. On the other hand, the binding affinity of GroEL for rLA, a negatively charged substrate, increased by adding of 200 mM KCl. These results indicate that electrostatic interactions substantially contribute to the binding interactions by manipulating the binding affinity of charged substrates.

Key Words: **fluorescence correlation spectroscopy; GroEL; apo cytochrome *c*; electrostatic interaction; homogeneous analysis.**

Chaperonin GroEL from *Escherichia coli* is an indispensable protein that binds non-native proteins to prevent their irreversible aggregation. It has also been shown to promote their efficient folding *in vivo* and *in vitro* (1–4). Although there has been considerable progress in unveiling the mechanism of GroEL-mediated refolding, the details of its recognition mechanism and function at the molecular level are still unclear (3, 4). For example, how chaperonin is able to bind to a variety of substrates of very different sequence, structure and function is an interesting question. Hydrophobic interactions have been shown to be important in the binding of substrates to GroEL (5–7). On the other hand, it has recently been reported that the GroEL binding to substrates is also affected by electrostatic interactions (8–15) and that the electrostatic interactions are involved in the initial binding process (10). However, experiments involving some substrates, such as apo cytochrome *c* (apo-cyt *c*) and disulfide-reduced apo α-lactalbumin (rLA), have barely shown the effect of ionic interaction (13) or have produced inconsistent results depending on the methods of measurement (11, 14). In fact, detailed consideration of GroEL–substrate interactions in view of hydrophobic and electrostatic aspects is important for understanding the recognition mechanism of GroEL for substrate.

The present study was carried out to obtain new insight into the electrostatic aspect of the GroEL–substrate interactions using fluorescence correlation spectroscopy (FCS). FCS is an extremely sensitive method based on fluctuation analysis of fluorescence intensity in order to detect and characterize fluorophores at the single molecular level in homogeneous solution without any physical separation step required in other biochemical methods (16–20). The method provides two important physical parameters for biochemistry: the average number of fluorescent molecules in the detection volume and the translational diffusion time of the molecules through the small volume of detection. Since the diffusion time of a small fluorescent molecule increases upon its binding to much lager molecules, measuring the change of its diffusion time by FCS allows analysis of binding prop-

Abbreviations used: FCS, fluorescence correlation spectroscopy; FAF, fluorescence autocorrelation function; Tris, 2-amino-2-hydroxymethyl-1, 3-propanediol; EDTA, ethylenediaminetetraacetic acid; apo-cyt *c*, apo cytochrome *c* from horse heart; rLA, disulfide-reduced apo α-lactalbumin from bovine milk.

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erties of biomolecules in solution. More detailed description of the theory and other possible applications can be found elsewhere (20–23).

MATERIALS AND METHODS

Preparation of proteins. Cytochrome *c* from horse heart containing a heme group were purchased from Wako Pure Chemical Industries (Osaka, Japan). Apo-cyt *c* was prepared by removing the heme group linked to the two cystine residues by reaction with silver sulfate according to a published procedure (24). Fluorescently labeled apo-cyt *c* was prepared as follows; The prepared solution of apo-cyt *c* in 0.05 M ammonium acetate buffer (pH 5.0) was equilibrated with 0.2 M sodium carbonate buffer (pH 9.0) and then concentrated to a volume of 1.5 ml (concentration of the protein was about 5.5×10^{-5} M) by a centrifugal filter device (Ultrafree-15, Millipore, Bedford, MA). Tetramethylrhodamine-5-isothiocyanate (Molecular Probes, Eugene, OR) solution (75 μl, 0.9 mg/ml in dimethylformamide) was added to 1.5 ml apo-cyt *c* solution in sodium carbonate buffer. The reaction mixture was kept at 4°C for 24 h, and subsequently free tetramethylrhodamine was removed by a Sephadex G-25 column (12 mm × 560 mm) equilibrated with a binding buffer (25 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA). The eluted fraction of labeled apo-cyt *c* was immediately used for titration experiments. Bovine α-lactalbumin (type III, Ca^{2+}-depleted) was purchased from Sigma (St. Louis, MO). GroEL and labeled rLA was prepared as described previously (14, 15), and was used for titration experiments in the binding buffer. The dye-to-protein ratio was estimated to be about 2 for each molecule of rLA and apo-cyt *c*.

FCS measurements and data analysis. FCS measurements were carried out using a ConfoCor spectrometer (Carl Zeiss, Jena GmbH, Jena, Germany). The details of the measurement system was described previously (15). The sample was excited with a sharply focused laser beam of 50 μW power. The pinhole diameter for confocal detection was adjusted to 30 μm. All measurements were performed on Lab-Tek chambered coverglass with eight wells (Nalge Nunc International, Naperville, IL). To prevent nonspecific adsorption of proteins, the sample chamber was treated with a protein blocker prior to the FCS measurement (Block Ace; 25 ~ 50%, Snow Brand, Tokyo, Japan) (15). The measured fluorescence autocorrelation functions (FAF), $G(\tau)$, were fitted with FCS Access Fit software (EVOTEC BioSystems GmbH, Hamburg, Germany) by one or two-component (molecular species) model as follows:

$$G(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2} = 1 + \frac{1}{N}\left[\frac{1-y}{\left(1+\frac{\tau}{\tau_{\text{free}}}\right)\left(1+s^2\frac{\tau}{\tau_{\text{free}}}\right)^{1/2}} + \frac{y}{\left(1+\frac{\tau}{\tau_{\text{bound}}}\right)\left(1+s^2\frac{\tau}{\tau_{\text{bound}}}\right)^{1/2}}\right] \quad [1]$$

where y denotes the bound ratio of labeled molecule to GroEL, N is the number of molecules in the detection volume element defined by radius w_0 and length $2z_0$, s is the structure parameter (18, 19), and τ_{free} and τ_{bound} are the translational diffusion times of the free and the bound molecules, respectively. The radius was experimentally estimated by the relation of $D = w_0^2/4\tau_{\text{rho}}$ using the measured diffusion time, τ_{rho}, and the diffusion coefficient of rhodamine 6G ($D = 2.8 \times 10^{-10}$ m^2 s^{-1}) as a reference. The structure parameter was obtained by fitting the measured FAF of rhodamine 6G to Eq. [1], and then the size of the detection volume was determined. The structure parameter, s, and the size of the detection volume were estimated to be 0.1–0.2 and 0.4–0.6 fl, respectively. Since fluorescence intensity per one molecule of labeled substrate was constant, we insist that the fluorescence quantum yield did not change upon binding to GroEL.

In titration experiments of GroEL or salt solution, the duration of the measurement was 60 or 120 s, and each point on the titration curve is represented by the average of three measurements in the same sample solution.

We typically titrated a sample solution (20 μl) of labeled apo-cyt *c* or rLA with appropriate volumes (microliters) of diluted GroEL solutions of 134 or 78 μM. In the case of salt titration, each salt solution (1 M KCl, 1 M $MgCl_2$) was added to a sample (20 μl) containing GroEL (0.64 μM) and apo-cyt *c* (2.4 nM), respectively. For the analysis of the binding of substrate from the titration experiments of GroEL, we used a bimolecular reaction model in equilibrium as (9, 13, 14, 25)

$$[\text{GroEL}] + [\text{S*}] \rightleftarrows [\text{S*GroEL}] \quad [2]$$

with the relation

$$[\text{S*GroEL}] = [\text{GroEL}][\text{S*}]/K_d, \quad [3]$$

where [GroEL], [S*], and [S*GroEL] are the concentration of free GroEL, free labeled substrate, and the bound complex, respectively, and K_d is the equilibrium dissociation constant. The bound ratio, y, in Eq. [1] is given as a function of [GroEL] by:

$$y = [\text{S*GroEL}]/([\text{S*}] + [\text{S*GroEL}]) \quad [4]$$

Using Eq. [3], the following form is obtained after rearrangement:

$$y = ([\text{GroEL}]/K_d)/(1 + [\text{GroEL}]/K_d). \quad [5]$$

The titration curves obtained in this study were fitted to Eq. [5], and subsequently K_d was estimated. The estimation of K_d values in salt titration was carried out by the procedure as described in a previous report (15).

RESULTS

A labeled substrate was used as a fluorescent reporter for titration experiments using FCS, because the diffusion time of the labeled small protein changes by forming a complex with a much lager protein, GroEL (800 kDa). There was no detectable aggregation of the labeled substrate in the sample solution. The molecular weights of the labeled proteins were calculated from the relation: $MW_f \approx (\tau_f/\tau_{\text{rho}})^3 \times MW_{\text{rho}}$, assuming a spherical shape. MW_f, τ_f are the molecular weight and diffusion time of the fluorescent molecule, respectively, and MW_{rho} is the molecular weight of rhodamine 6G (0.479 kDa). Apo-cyt *c* (12 kDa) showed a diffusion time of about 0.12 ms in the FCS experiment that corresponds to a calculated molecular weight of about 13 kDa. This calculated value is in good agreement with the reported molecular weight of the protein (13). The diffusion times of GroEL and rLA were estimated to be about 0.53 and 0.12 ms, respectively. These values of diffusion time also reflect well the molecular weights of the proteins (14, 15). The number, N, and bound ratio, y, were changed as free parameters, and the measured diffusion times of labeled substrates and GroEL were set as a fixed parameter in the

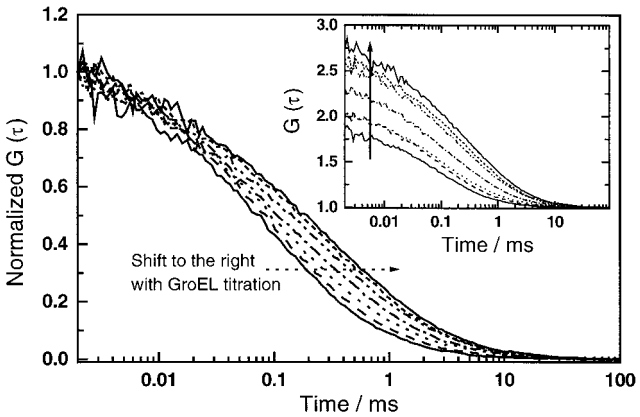

FIG. 1. Typical fluorescence autocorrelation functions (FAFs) in titration of diluted GroEL solutions (microliters) of 134 μM to sample solution (20 μl, 4 nM) of labeled apo-cyt *c*. To compare the FAFs of different titrations, the ordinate scale is normalized to unity at the origin, G(0). The FAF shifts to the right due to the increase of the fraction of bound complex with GroEL (dotted arrow). (Inset) Raw data of FAFs of the titration experiment are shown. The amplitude of autocorrelation function increases upon the dilution of the sample when titrated by homogeneous solution of GroEL (solid arrow).

fitting procedure of titration experiments. In fact, FAFs measured in titration experiments were well fitted with these fixed parameters.

Figure 1 shows typical FAFs obtained from the titration of GroEL to labeled apo-cyt *c*. The correlation curve shifted to the right during the titration, indicating an increase of the fraction of bound complex with GroEL. The bound ratios of the substrates were obtained from the two-component fit of Eq. [1], and then titration curves were obtained. The titration curves of apo-cyt *c* and rLA in the absence and in the presence of 200 mM KCl are plotted in Fig. 2. The bound ratio of apo-cyt *c* in the absence of KCl increased promptly with the titration of GroEL, and then saturated at almost the maximum value of 1.0 (solid circles in Fig. 2a). In the presence of 200 mM KCl, however, the bound ratio of apo-cyt *c* increased with GroEL concentration, but reached a plateaue at about 0.8 (open circles in Fig. 2b). Another titration experiment with apo-cyt *c* showed that the bound ratio in the presence of 200 mM KCl also reached the maximum value at higher concentration (18 μM) of GroEL (data not shown). The average value of K_d obtained from the entire titration curve of apo-cyt *c* increased from 0.14 to 0.42 μM when the KCl concentration was changed from 0 to 200 mM (Fig. 2a). This result implies that apo-cyt *c* binding to GroEL has a preference for low salt concentration. In contrast, the K_d values of rLA decreased from 2.85 to 0.75 μM when the KCl concentration was changed (Fig. 2b). These values of K_d are slightly lower in the case of rLA, and slightly higher in the case of apo-cyt *c* than the values obtained in our previous study (15). It seems likely that increasing the number of labeled fluorophor as described above can contribute to the binding affinity by reducing the net charge on the protein since the NH_2 group is blocked by the label. Moreover, unremoved free dyes (about 20%) might affect the evaluation of K_d in fitting procedure in our previous study (15). Nevertheless, this result also shows that rLA binding to GroEL is clearly dependent on the salt concentration.

To clarify the effect of electrostatic interaction on the binding of apo-cyt *c* with GroEL, two kinds of salt solutions (KCl and $MgCl_2$) with different concentrations (0–400 mM) were chosen for the salt titration experiment. Figure 3 shows the dependence of K_d of apo-cyt *c* on salts and their concentration. In all cases, the value of K_d in the absence of salt was lower than that in the presence of salt, and increased with salt concentration. At the higher salt concentrations (100 mM of $MgCl_2$, and 150 mM of KCl), K_d saturated and

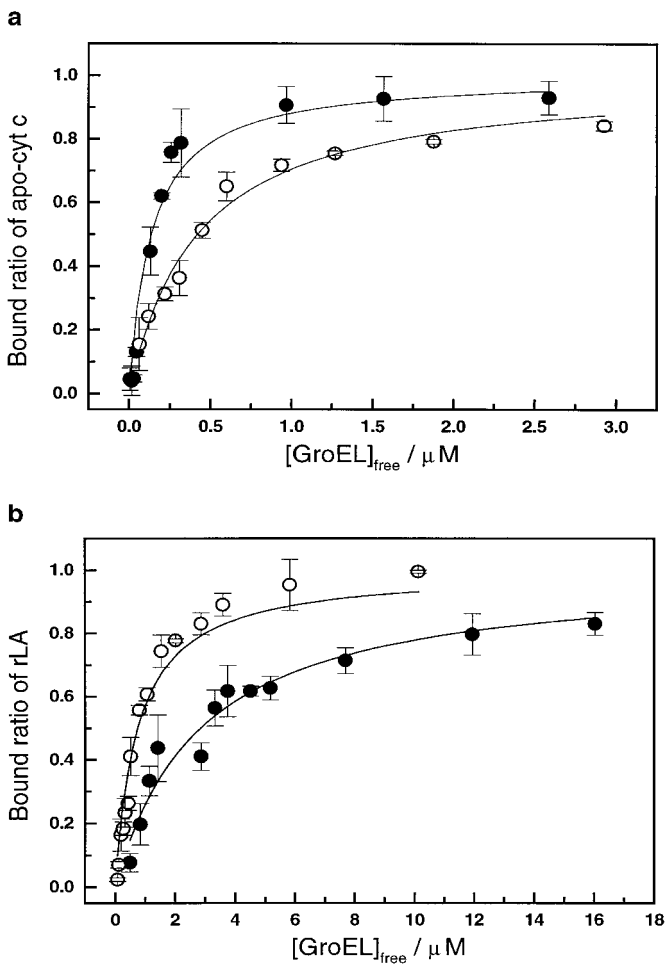

FIG. 2. (a) Bound ratios of apo cytochrome *c* and (b) disulfide-reduced apo α-lactalbumin to GroEL as a function of GroEL concentration in the absence (solid circles) and in the presence of 200 mM KCl (open circles). Titration curves were measured by adding appropriate volumes (microliters) of diluted GroEL solutions of 134 or 78 μM (0 or 200 mM KCl) to each sample (20 μl) of apo-cyt *c* (4 nM) and rLA (0.2 μM) to increase the GroEL concentration.

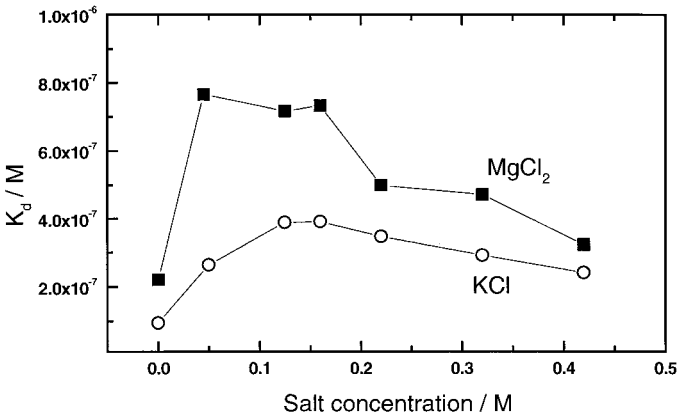

FIG. 3. Dependence of the dissociation constant of apo-cyt *c* on the concentration of two different salts (KCl and $MgCl_2$). Data points were obtained from adding appropriate volumes (microliters) of each salt solution (1 M) to samples (20 μl) containing GroEL and labeled apo-cyt *c* (0.64 μM:2.4 nM), respectively.

then slowly decreased with increasing salt concentration. Although K_d decreased at the higher salt concentration, the value was still higher than that in the absence of salt. This result shows clearly that the binding strength of apo-cyt *c* with GroEL is dependent on the salt concentration. Moreover, the value of K_d was more affected by a divalent ion, $MgCl_2$ (solid squares in Fig. 3) rather than by a monovalent ion, KCl (open circles in Fig. 3) at salt concentrations below 50 mM.

DISCUSSION

The binding processes of apo-cyt *c* with GroEL in a very dilute solution have been quantitatively measured by FCS. It has been shown that GroEL (p*I* 4.7) binding with apo-cyt *c* (p*I* 10.1), one of the positively charged substrates, depends on the salt concentration, even though GroEL binds tightly to the substrate both in the absence of salt and in the presence of 200 mM KCl. Moreover, the binding affinity of apo-cyt *c* substantially decreased with concentration of $MgCl_2$ as well as KCl. On the contrary, the binding of negatively charged protein, rLA (p*I* 4.7), to GroEL was clearly strengthened by increased salt concentration. In addition, the result presented in this study suggests that positively charged protein strongly binds to GroEL rather than negatively charged protein.

In a previous report, it was shown using size-exclusion chromatography that apo-cyt *c* and three kinds of fragment from cytochrome *c* bind to GroEL with a strength which is in proportion to the length of the amino acid sequence of the fragments. The binding strength of the three fragments to GroEL substantially decrease with increasing KCl concentration in the range of 50 to 200 mM (13). However, this dependence was not clearly observed in the case of apo-cyt *c* with full length of sequence using the size-exclusion chromatography in the same range of salt concentration. This seems to be in contrast to our result obtained with apo-cyt *c* at KCl concentrations in the range of 0 to 400 mM. The result of the previous report is probably explained by the fact that the effect of salt on apo-cyt *c* binding to GroEL is not elucidated over the concentration of 50 mM salt as shown in present study (Fig. 3). Recently, it was reported using CD method that the K_d value between barnase (13 nM), which is another positively charged substrate with p*I* 8.8, and GroEL in low salt concentration (5 mM 2-(*N*-morpholino)-ethanesulfonic acid) was increased by two orders of magnitude in high salt concentration (50 mM 2-(*N*-morpholino)ethanesulfonic acid and 200 mM NaCl) (10). Consequently, it is suggested that the binding affinity of a positively charged substrate with GroEL is substantially affected by salt concentration, even though the extent of the salt effect can be varied depending on the substrate proteins.

It was reported that the binding strength of GroEL to several negatively charged substrates such as denatured pepsin (p$I < 1$) (14, 15), apo α-lactalbumin (9, 12, 25), and rLA (11, 15) increases with increasing salt concentration. It was also suggested that ionic interactions affect the binding of GroEL by shielding the negative charge on GroEL and its substrates. Interestingly, rLA did not show the dependence on salt concentration at all as measured by isothermal titration calorimetry (14). However, another report using size-exclusion chromatography found that rLA bound to GroEL only when KCl was present (11). These observations with rLA are not consistent with our results showing that rLA binds to GroEL whether the salt is present or not, and that the binding affinity of rLA with GroEL increases with increasing salt concentration. In the calorimetric method, measuring samples with very high concentration might be a reason for diminishing the salt effect. On the other hand, the strong dependence on salt concentration observed by size-exclusion chromatography seems to be due to the physical separation of GroEL and the substrate, which reduce the chance of rebinding by the proteins.

Recent studies with polylysine and mitochondrial malate dehydrogenase (mMDH) as substrates showed that the polypeptide strongly binds to GroEL (K_d = 50 nM) and inhibits the GroEL-assisted refolding of mMDH (26). In addition, the results from fluorescence titration experiment showed that the binding affinity of the polypeptide and GroEL is not affected by salt at all but affected by the size of the lysine polymer. From these results, it was suggested that hydrophobic interactions may not always be the major binding force in the GroEL–substrate binding and the polar side chains of the binding site of GroEL contribute to the stabilization of GroEL–substrate complexes. This result indicates that, regardless of salt in solution, the positive

charges on a substrate protein could be an important factor for strengthening the binding of GroEL to the substrate. In addition, it has been reported that positively charged hydrophobic structures, such as signal sequences of β-lactamase (6) and N-terminal sequence of rhodanese (27, 28) are preferentially bound to GroEL. This result, combined with the studies on the binding of polylysine with GroEL could explain our present and previous results (15) indicating that the positively charged protein, apo-cyt *c* has a much stronger affinity to GroEL than the negatively charged protein, rLA and denatured pepsin have. It is supposed that the positive charges on substrate might be an important for the recognition of GroEL for charged substrates.

On the other hand, several studies using hydrophobic fluorescent probes suggested that the ionic interaction of salt can expose restricted hydrophobic surfaces on the binding site of GroEL and strengthen the binding interaction (29, 30). Combining these reports with our results in this paper, it seems that salt can influence the binding affinity of substrate with GroEL by both shielding the charges on the proteins and by exposing the hydrophobic surfaces in the binding site of GroEL.

In summary, based on the results presented herein, it is suggested that the binding interactions of GroEL to charged substrate proteins in a state of equilibrium is susceptibly manipulated by electrostatic interactions derived from both the positive charges on substrate and cations in solution. Moreover, it is noted that FCS is a very promising tool for analyzing the protein-protein binding interactions systematically in homogeneous solution with very high sensitivity and simplicity that may not be accessible by other methods as shown in this study.

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