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Thermodynamics of nucleotide binding to the chaperonin GroEL studied by isothermal titration calorimetry: evidence for noncooperative nucleotide binding

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Abstract

We characterized the thermodynamics of binding reactions of nucleotides ADP and ATPγS (a nonhydrolyzable analog of ATP) to GroEL in a temperature range of 5°C to 35°C by isothermal titration calorimetry. Analysis with a noncooperative binding model has shown that the bindings of nucleotides are driven enthalpically with binding constants of 7×10^3 M^{-1} and 4×10^4 M^{-1} for ADP and ATPγS, respectively, at 26°C and that the heat capacity change ΔC_p is about 100 cal/mol•K for both the nucleotides. The stoichiometries of binding were about 8 and 9 molecules for ADP and ATPγS, respectively, per GroEL tetradecamer at 5°C, and both increased with temperature to reach about 14 (ADP) and 12 (ATPγS) for both nucleotides at 35°C. The absence of initial increase of binding heat as well as Hill coefficient less than 1.2, which were obtained from the fitting to the model curve by assuming positive cooperativity, showed that there was virtually no positive cooperativity in the nucleotide bindings. Incorporating a difference in affinity for the nucleotide (ADP and ATPγS) between the two rings of GroEL into the noncooperative binding model improved the goodness of fitting and the difference in the affinity increased with decreasing temperature.

Keywords: Chaperonin; Protein folding; Isothermal titration calorimetry; Nucleotide binding; Cooperativity; Allosteric effect

1. Introduction

Molecular chaperones are a class of ubiquitous proteins which prevent unproductive aggregation of a target protein and facilitate its folding and assembly both in vitro and in vivo. The best studied among them is the chaperonin GroEL from *Escherichia coli*, which is a tetradecameric protein of 14 identical 57-kDa subunits arranged in two heptamer rings stacked back-to-back with a central cavity [1–5]. The atomic structure determined by X-ray crystallography [6] has shown that each subunit of GroEL consists of three domains: an equatorial domain that forms a central core of the ring structure, an apical domain that contains a binding site for a target protein, and an intermediate domain that connects the equatorial and apical domains. Target proteins in the nonnative state are bound to GroEL mainly by hydrophobic interactions [7–9], but electrostatic interactions are also shown to be important [10–15]. GroEL has a weak ATPase activity [16,17], and the 14 nucleotide-binding sites are located in the

Abbreviations: AMP-PNP, 5′-adenylylimidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ITC, isothermal titration calorimetry

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equatorial domain of each subunit [18]. It has been suggested that multiple rounds of binding of a target protein to and release from GroEL, which are controlled by both the ATP hydrolysis and the co-chaperonin GroES binding, are occurring during the GroEL-mediated folding [19,20]. However, numerous questions remain to be addressed for a molecular description of this complex process.

The binding reactions of ATP and other adenine nucleotides (ADP and ATP analogs) to GroEL have been studied by measuring the ATPase activity of GroEL [16,17,21–24] and the nucleotide-induced fluorescence changes of pyrene-labeled GroEL [25–27]. These studies have shown that the nucleotide binding to GroEL exhibits positive cooperativity within a GroEL ring [16,17,21–27] and negative cooperativity between the two rings [21–24]. The results have been interpreted in terms of an allosteric model, in which each ring of GroEL is in equilibrium between two conformational states, a T (tense) state that has low affinity for the nucleotide but high affinity for a target protein and an R (relaxed) state that has high affinity for the nucleotide but low affinity for the target protein [16,21–24]. The affinities for target proteins of GroEL are known to be decreased not only by ATP but also by its nonhydrolyzable analogue AMP-PNP and ADP [28–31], and the allosteric model has been thought to be important in understanding the molecular mechanisms of GroEL function. In support of this model, a cryo-electron microscopic study has revealed the nucleotide-induced conformational transitions in GroEL [32]. However, the X-ray crystallographic structure of the GroEL complexed with ATPγS, an ATP analog, has also shown that there are no such conformational changes when the structure is compared with the structure of GroEL without the nucleotide [33], and the reasons for this discrepancy in the GroEL structure remain unclear. Therefore, the effect of the nucleotide binding on the GroEL structure and its role in the GroEL function have not yet been fully understood.

In the present study, we have characterized the binding reactions of two nucleotides, ADP and ATPγS, to GroEL quantitatively by isothermal titration calorimetry (ITC). The ITC measurements have been performed at five different temperatures (between 5°C and 35°C), from which we estimate a heat capacity change ΔC_p, which is a measure of a change in the hydrophobic accessible surface area of GroEL caused by nucleotide binding. The ITC is thus a powerful technique that allows us to determine simultaneously the binding constant, stoichiometry and changes in the thermodynamic parameters of the protein–ligand interaction [34–36], and would be particularly useful in characterizing the allosteric nucleotide binding by GroEL. In contrast to this expectation, however, no trace of the positive cooperativity has been observed in the ITC data of the present study, and the difference in affinity for the nucleotides between the two rings must be small, if any, at room temperature. The ΔC_p value associated with nucleotide binding is also small, suggesting that GroEL may not undergo a substantial conformational change. Comparison of the present study with the previous ones that have shown the cooperative nucleotide binding [16,21–25] indicates that the only remarkable difference in the experimental condition is a difference in GroEL concentration; the concentrations used in those previous studies were about 170 to 360 times lower than the concentration used in this study (9 μM of GroEL tetradecamer). It is concluded that the allosteric effect of the nucleotide binding is absent at a high GroEL concentration because the allostery is linked with dissociation of the GroEL two rings.

2. Materials and methods

2.1. Materials

GroEL was purified from *E. coli* cells TG1 bearing an expression plasmid pKY206, which was a generous gift from Dr. K. Ito [37], and purified according to the method of Buchner et al. [38] with slight modifications. Cells were grown overnight at 37°C in LB media containing 12.5 μg/ml tetracycline. After collecting the cells by centrifugation at 20 000 × *g* for 60 min at 4°C, they were resuspended in buffer A (50 mM Tris–HCl (pH 7.5) and 2 mM EDTA), and lysed by sonication at room temperature. The cell debris was removed by centrifugation at 20 000 × *g* for 1 h at 4°C, then 0.1 mg/ml DNase and 2 mM $MgCl_2$ were added to the clear lysate and the solution was stirred for 30 min at room temperature. The lysate

was fractionated by ammonium sulfate precipitation. The fraction soluble at 30% saturation but precipitated at 60% saturation was collected and resuspended in buffer A and heated for 30 min at 50°C. The insoluble portion was discarded by centrifugation at 20 000 × *g* for 1 h at 4°C. The supernatant was filtered and then applied on a Sephacryl S-300 gel filtration column equilibrated with buffer A. The elution position of GroEL was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the protein was further purified by Q-Sepharose Fast Flow anion-exchange column chromatography in buffer A with a linear gradient from 0.3 to 0.5 M NaCl. The fractions of purified GroEL were pooled. The other fractions that contained GroEL were incubated with 1 mM ATP, 10 mM $MgCl_2$, and 10 mM KCl for 30 min at room temperature to remove peptides bound to GroEL. The latter sample was further purified by repetition of the same anion-exchange chromatography step. The purified GroEL was stored at −20°C in buffer A containing 15% (v/v) glycerol. The concentrations of GroEL was determined spectrophotometrically at 280 nm with an extinction coefficient of $E^{1cm}_{0.1\%} = 0.210$. GroEL concentrations are given in tetradecamer throughout this paper.

The nucleotides ADP and ATPγS were purchased from Sigma (catalog nos. A2754 and A1388) and purified by anion-exchange chromatography. Because commercially available nucleotides often contain contaminants [39,40], we purified the nucleotides and used the purified nucleotides throughout the present experiments. For the purification, each nucleotide in 100 mM NH_4HCO_3 was applied on a Q-Sepharose anion-exchange column at 4°C and eluted with a linear gradient from 100 mM to 600 mM NH_4HCO_3. The fractions containing the nucleotide were collected and lyophilized. The concentrations of both the nucleotides were determined using a same extinction coefficient of 0.0154 $\mu M^{-1}\,cm^{-1}$ at 259 nm.

2.2. Isothermal titration calorimetry (ITC)

Calorimetric measurements were carried out with a MCS calorimeter for ITC (MicroCal, Northhampton, MA, USA) interfaced with a personal computer. Prior to titration, GroEL solution was dialyzed against buffer B (50 mM sodium cacodylate, 10 mM KCl, and 10 mM $MgCl_2$, pH 7.0). The cacodylate buffer was chosen because of its little temperature dependence of pH as well as little heat effect of the proton exchange between GroEL and the buffer. The outer solution of the dialysis was an experimental buffer, which was used to dissolve the nucleotides and also for a control titration (see below). All solutions were filtered using membrane filters (pore size 0.45 μM) and thoroughly degassed under vacuum for 5 min with gentle stirring immediately before use. The ambient temperature of the system was kept about 10°C below the experimental temperature by circulating thermostatted water to improve baseline stability.

The nucleotide solution (typically 2 mM for ADP and 0.8 mM for ATPγS) in a 250-μl injection syringe was titrated into the GroEL solution (typically 9 μM) placed in the sample cell of 1.35 ml volume. The reference cell, which acts only as a thermal reference to the sample cell, was filled with water. Each titration consisted of a preliminary 2-μl injection followed by 24 subsequent 10-μl injections of 25 s durations with a 6-min interval between injections. Data for the preliminary injection, which were affected by diffusion of the solution from and into the injection syringe during the initial equilibration period, were discarded.

The raw data correspond to the power per unit time required to maintain a constant temperature of the sample cell after each injection. The calorimeter was calibrated beforehand with electrically generated heat pulses of a known amount to yield heat exchange per unit time in units of μcal/s. Manual peak-by-peak integration was found to yield better representations of the data than did the automatic baseline determination provided by MicroCal Origin for ITC analysis software package which was provided with the instrument. The integrated peak area produced by each injection generally decreases as the titration progresses because of the saturation of ligand binding sites. The heat effects which are not directly related to the binding reaction, such as dilution of the injectant and viscous mixing, were measured by a control titration of the injectant into the experimental buffer and subtracted from the heat of whole reaction for each injection. Binding isotherms are generated by plotting heats of reaction normal-

ized by the moles of injectant versus the ratio of total injectant to total protein per injection. Binding isotherms thus obtained were fitted to the models indicated in Section 3 by Marquardt nonlinear least-squares analysis [41,42]. The heat effects associated with the volume displaced by each injection were also taken into account (see below).

3. Results

3.1. Calorimetric titration curves

We carried out ITC experiments on the interactions of GroEL and two nucleotides ADP or ATPγS in buffer B at five different temperatures, 5°C, 10°C, 15°C, 26°C, and 35°C. Fig. 1A,B shows representative profiles at 5°C and 26°C for the titrations of ADP into a GroEL solution (lower trace) and into a buffer without GroEL (upper trace). Downward peaks indicate exothermic reactions accompanied by heat releases, whereas upward peaks indicate endothermic reactions accompanied by heat uptakes. By subtracting the control dilution data from the binding data, we obtained the binding isotherms of the nucleotides to GroEL, which are shown in Fig. 1C,D and will be fitted to several different binding models (see below).

Fig. 1A,B also shows that there is a slight baseline drift in some of the titrations. This drift occurs only at a stoichiometry around 10 when ADP was titrated, and at a stoichiometry beyond 10 when ATPγS was titrated (data not shown). The drift became more significant as the temperature was increased, and was found to be reproducible, so that it was not such an artifact as due to an environmental temperature change. The baseline drift might be caused by some very slow process which might have occurred after the binding of the nucleotides to GroEL, but no convincing explanation for such a slow process has been found so far. We merely assumed linear baseline for each peak manually.

3.2. Analysis based on a noncooperative binding model

The observed binding isotherms were first fitted to a simplest model, in which GroEL has n identical binding sites for the nucleotide, which are independent of each other and have a uniform binding constant, K_b, and an enthalpy change ΔH. The fractional saturation, Θ, of the binding sites relates to K_b and the free nucleotide concentration [X] as

$$\Theta = \frac{K_b[X]}{1 + K_b[X]} \quad (1)$$

Because the [X] was not known beforehand, the following relation was used to calculate Θ:

$$[X] = X_t - n\Theta M_t \quad (2)$$

where X_t and M_t are the total concentrations of the nucleotide and GroEL (tetradecamer), respectively. Once the Θ has been obtained, the total heat, Q, released by the binding reaction in the cell with a volume, V_0, is given by

$$Q = n\Theta M_t \, \Delta H V_0 \quad (3)$$

Table 1
Best-fit parameters obtained by noncooperative binding model

Nucleotide	Temperature (°C)	n	K_b (10^3 M^{-1})	ΔH (kcal/mol)	ΔG (kcal/mol)	$-T\Delta S$ (kcal/mol)
ADP	5.1	7.64 ± 0.36	21.9 ± 1.5	−13.0 ± 0.8	−5.52 ± 0.04	+7.51 ± 0.76
	10.3	7.86 ± 0.50	15.5 ± 1.1	−13.0 ± 1.0	−5.43 ± 0.04	+7.57 ± 0.99
	15.3	9.85 ± 0.36	13.3 ± 0.7	−10.1 ± 0.5	−5.44 ± 0.03	+4.65 ± 0.46
	25.6	13.7 ± 0.4	7.15 ± 0.29	−8.62 ± 0.36	−5.27 ± 0.02	+3.35 ± 0.36
	35.5	15.9 ± 3.5	3.04 ± 0.54	−10.3 ± 2.9	−4.92 ± 0.11	+5.36 ± 2.92
ATPγS	5.1	8.62 ± 0.11	49.3 ± 2.7	−8.84 ± 0.18	−5.97 ± 0.03	+2.87 ± 0.18
	10.3	9.26 ± 0.07	45.6 ± 1.7	−7.53 ± 0.10	−6.04 ± 0.02	+1.49 ± 0.10
	15.2	10.1 ± 0.1	43.8 ± 1.7	−6.10 ± 0.08	−6.12 ± 0.02	−0.02 ± 0.08
	25.8	11.0 ± 0.2	37.5 ± 2.7	−4.70 ± 0.11	−6.25 ± 0.04	−1.55 ± 0.12
	35.3	12.3 ± 0.3	13.7 ± 1.3	−4.98 ± 0.26	−5.83 ± 0.06	−0.85 ± 0.27

Errors indicate standard deviations of estimated parameter values in the nonlinear least-squares fitting.

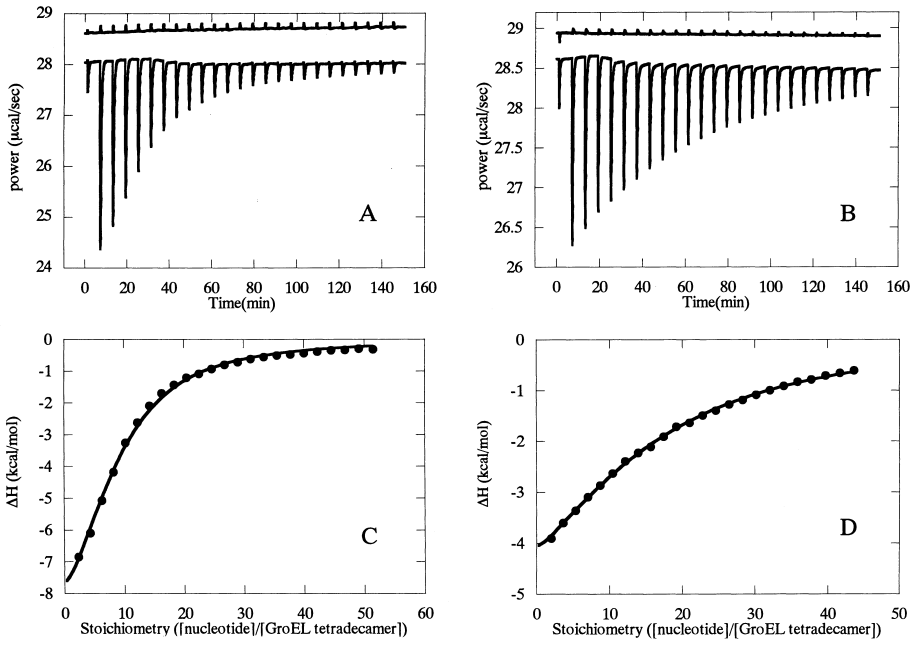

Fig. 1. Calorimetric titration of GroEL with ADP. (A,B) Representative profiles of the titration of GroEL with ADP at 5°C (A) and 26°C (B). Ten-μl aliquots of 2 mM ADP solution were injected at time intervals of 6 min to a 9-μM solution of GroEL (lower trace) or buffer B without GroEL (upper trace). (C,D) Integrated data of net heat exchange of panel A (C) and panel B (D) after subtraction of control data for the titration into the buffer without GroEL. Thick solid lines represent theoretical curves generated by fitting the experimental data to the noncooperative binding model by Marquardt nonlinear least-squares analysis [41,42].

where ΔH is the molar enthalpy change of the nucleotide binding to GroEL. The experimentally observed heat release by the ith injection, $\Delta Q_{\mathrm{exp}}(i)$, is related to the calculated Q values after the $(i-1)$th and ith injections, $Q(i-1)$ and $Q(i)$, respectively, by the equation

$$\Delta Q_{\mathrm{exp}}(i) = Q(i) - Q(i-1) + \frac{dV_i}{V_0}\,\frac{Q(i)+Q(i-1)}{2}. \quad (4)$$

Here, the last term represents the correction for a heat released by a small volume ($\mathrm{d}V_i$) of the solution that is displaced out of the cell by the ith injection. We carried out nonlinear least-squares fitting of the experimental data to the above equations [41,42]. Sufficient convergence of the three variables (n, K_b, ΔH) was obtained in all titrations, and the resultant parameter values are listed in Table 1. The results show that the stoichiometry n increases from 8 to 14 (ADP) or from 9 to 12 (ATPγS) gradually as the temperature is increased and that ATPγS is bound to GroEL more tightly (with a binding constant in the order of $1–5\times10^4$ M^{-1}) than ADP ($0.3–2\times10^4$ M^{-1}) at all temperatures investigated.

The results also give us a measure of the appropriateness of the experimental condition. A dimensionless parameter

$$c = nK_bM_t \quad (5)$$

determines the experimental sensitivity to the variable parameters [34,36] and the c values of the present experiments were 1 and 4 for the ADP and ATPγS bindings, respectively. Although the c value between 10 and 100 is recommended for an ideal experiment, the GroEL concentration could not be increased more than 9 μM because of a possible aggregation, so that the above c values are the best realized in the present systems.

Thick solid lines in Fig. 1C,D are the theoretical curves drawn using the parameter values of Table 1. The experimental data of ADP binding at 26°C scatter uniformly along the theoretical curve, whereas the data at 5°C show small but significant deviations from the theoretical curve. They tend to be higher than the theoretical curve at an n between 10 and 25 and lower than that at an n larger than 25. This behavior, though less pronounced, was also observed

in ADP binding data at 10°C, but not in ATPγS binding. It will be shown that the behavior is due to negative cooperativity of the nucleotide binding between the two heptamer rings of GroEL (see below).

The Gibbs free energy change, ΔG, and the entropy change, ΔS, of binding were calculated from K_b and ΔH using the equations,

$$\Delta G = -RT \ln K_b = \Delta H - T\Delta S \quad (6)$$

and the resultant thermodynamic parameters are also listed in Table 1. The binding reactions of both the nucleotides are driven enthalpically. The ΔS is unfavorable for the ADP binding at all temperatures examined. The ΔS is also unfavorable for the ATPγS binding at 5°C, whereas it becomes favorable at a higher temperatures above 15°C. The ADP binding to GroEL is accompanied by a more favorable ΔH but a more unfavorable ΔS than the ATPγS binding. Interestingly, the ΔH and ΔS contributions compensate each other to yield similar ΔG values for the two nucleotide bindings.

The heat capacity change of the nucleotide binding, ΔC_p, can be estimated from the ΔH values at different temperatures. The ΔH showed a small increase with an increase of temperature for both the nucleotide bindings. Assuming that the ΔC_p is independent of temperature in the temperature range examined, the ΔC_p values were estimated at 120 cal/mol•K and 130 cal/mol•K for ADP and ATPγS bindings, respectively. These values are surprisingly small compared with reported values of ΔC_p in other protein–ligand interactions (see Section 4).

3.3. Analysis based on a cooperative binding model (Hill analysis)

The binding isotherms were fitted to the cooperative binding model, in which the Θ is given by:

$$\Theta = \frac{K_{app}[X]^{n_H}}{1 + K_{app}[X]^{n_H}} \quad (7)$$

where K_{app} is the apparent binding constant in the cooperative binding model, and n_H is the Hill constant that is an index of the cooperativity [43]. As in Eqs. 2 and 3, [X] and Q are given by

$$[X] = X_t - n_H \Theta M_t \quad (8)$$

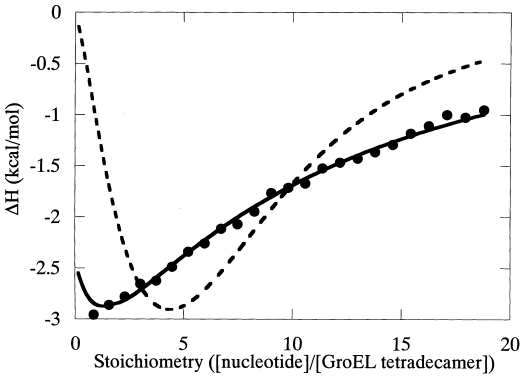

Fig. 2. Analysis based on the positively cooperative binding model (Hill analysis). Experimental data points are those for the binding of ATPγS to GroEL at 35°C, which gives an example of the relatively small value of resultant n_H. A thick line is the best-fit theoretical curve of Hill's saturation function [43] with $n_H = 1.08$. Although an increased binding at the beginning of the titration is expected if positive cooperativity exists, no such increase has been observed in any of the nucleotide-binding experiments at all temperatures. The best-fit curve with a fixed Hill constant $n_H = 2$ is also drawn, which indicates that the Hill constant of 2 does not give satisfactory agreement with the experimental data.

$$Q = n_H \Theta M_t \Delta H V_0 \quad (9)$$

The equation for $\Delta Q_{exp}(i)$ is identical to Eq. 4, and the three variable parameters (n_H, K_{app}, and ΔH) were evaluated by the nonlinear least-squares calculations. Since each data point represents a differential heat release accompanied with a single injection of a small volume of the nucleotide solution, the titration curve in Fig. 2 corresponds to a differential saturation curve, so that there must be a differential increase in the binding, which should be represented by a minimum in Fig. 2, at a beginning of the titration if the positive cooperativity is present. A solid line in Fig. 2 is the best-fit theoretical curve with an n_H of 1.08, which indicates that this minimum should be seen when n_H is as small as 1.08. However, we observed no such phenomenon in any of the titrations we performed, and the best-fit n_H values obtained were not more than 1.2 for the binding reactions of both the nucleotides at all the temperatures examined (data not shown). Thus we conclude that there is virtually no positive cooperativity in the nucleotide binding to GroEL under the present experimental condition.

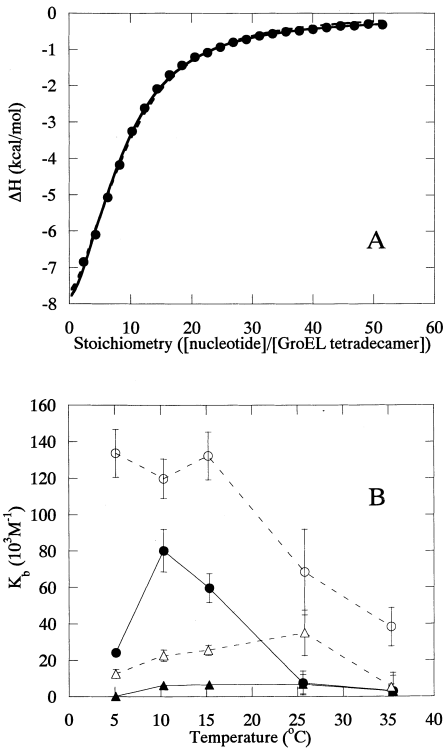

Fig. 3. Analysis incorporating negative cooperativity between the two rings of GroEL. (A) Data points for the ADP binding to GroEL at 5°C are shown. The theoretical curves of this model (solid line) fit better to the experimental data points than the noncooperative binding curve that is the same as in Fig. 1C (dashed line). (B) Temperature dependence of the binding constants of the two rings for nucleotide binding to GroEL. The two binding constants for the nucleotide binding in the two rings of GroEL obtained by the model incorporating negative cooperativity are plotted against temperature. Filled circles and triangles are K_1 and K_2 for ADP binding and open circles and triangles are those for ATPgS, respectively. The difference becomes smaller with increasing temperature.

3.4. *Analysis incorporating negative cooperativity between two rings of GroEL*

GroEL tetradecamer consists of two heptamer rings both of which have seven nucleotide-binding sites [6], and besides, we have observed the stoichiometry of nucleotide binding to GroEL varies from 8 to 14 (ADP) or from 9 to 12 (ATPγS) as the temperature is increased. Considering these, we assume that there are seven identical binding sites in each of the two rings with the binding constants, K_{b1} and K_{b2}, and the enthalpy changes of binding, ΔH_1, and ΔH_2, for the first and the second rings, respectively. Using the same definition of symbols as above, the fractional saturations, Θ_1 and Θ_2, for the first and the second rings were calculated by the following equations

$$\Theta_1 = \frac{K_{b1}[X]}{1 + K_{b1}[X]},\ \Theta_2 = \frac{K_{b2}[X]}{1 + K_{b2}[X]} \tag{10}$$

$$[X] = X_t - 7(\Theta_1 + \Theta_2)M_t \tag{11}$$

and the total heat release is calculated by

$$Q = 7M_t V_0(\Theta_1 \Delta H_1 + \Theta_2 \Delta H_2) \tag{12}$$

We fitted the experimental data to this model by the nonlinear least-squares. The best-fit parameter values are summarized in Table 2.

The theoretical curve by this model is illustrated in Fig. 3A, which indicates that the model gives a better agreement with the experimental data at low temperatures than the noncooperative binding model shown in Fig. 1C. The data at higher temperatures which were well fitted to the noncooperative binding model were also decomposed into the two binding curves with different affinities, although there was no significant improvement in the fitting. The binding constants of the two rings are plotted against temperature in Fig. 3B. The difference in binding constants of the two rings became larger as temperature was decreased. This behavior is interpreted in terms of an

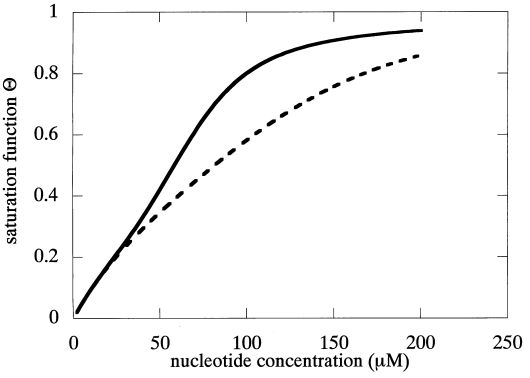

Fig. 4. Saturation curve simulated based on the ring-dissociation model calculated at two GroEL tetradecamer concentrations 1 mM (dashed line) and 10 nM (solid line). Positive cooperativity appears by lowering GroEL concentration when other parameters are fixed constant as follows: dissociation constant for two heptamer rings $K_d = 1.0 \times 10^{-8}$, binding constants in the T and R states $K_T = 1.0 \times 10^4$ and $K_R = 1.0 \times 10^5$, and allosteric constant $L = 3.0 \times 10^{-5}$.

Table 2
Best-fit parameters obtained by negatively cooperative model

Nucleotide	Temperature (°C)	K_1 and K_2 (10^3 M^{-1})	ΔH (kcal/mol)	ΔG (kcal/mol)	$-T\Delta S$ (kcal/mol)
ADP	5.1	24.2 ± 1.6	−13.2 ± 0.4	−5.58 ± 0.04	+7.61 ± 0.39
		0.10 ± 0.01	−28.3 ± 11.4	−2.56 ± 0.08	+25.7 ± 11.4
	10.3	80.2 ± 11.7	−7.42 ± 0.27	−6.36 ± 0.08	+1.06 ± 0.28
		6.13 ± 0.35	−8.04 ± 0.32	−4.91 ± 0.03	+3.13 ± 0.32
	15.3	59.6 ± 7.9	−6.31 ± 0.22	−6.30 ± 0.08	+0.09 ± 0.23
		6.48 ± 0.25	−8.49 ± 0.29	−5.03 ± 0.02	+3.46 ± 0.29
	25.6	7.42 ± 6.59	−11.3 ± 59.1	−5.29 ± 0.53	+6.0 ± 59.1
		6.73 ± 5.36	−5.6 ± 59.2	−5.23 ± 0.47	+0.3 ± 59.2
	35.5	2.93 ± 10.4	−8 ± 339	−4.90 ± 2.17	+3 ± 339
		2.77 ± 8.70	−16 ± 338	−4.86 ± 1.93	+11 ± 338
ATPγS	5.1	134 ± 13	−7.99 ± 0.22	−6.52 ± 0.05	+1.47 ± 0.22
		12.6 ± 2.5	−3.55 ± 0.58	−5.22 ± 0.11	−1.67 ± 0.59
	10.3	120 ± 11	−7.38 ± 0.30	−6.58 ± 0.05	+0.80 ± 0.30
		22.7 ± 3.1	−2.33 ± 0.45	−5.65 ± 0.08	−3.31 ± 0.45
	15.2	132 ± 13	−5.89 ± 0.21	−6.75 ± 0.06	−0.86 ± 0.22
		25.6 ± 2.8	−2.71 ± 0.31	−5.81 ± 0.06	−3.10 ± 0.31
	25.8	68.4 ± 23.6	−6.15 ± 2.95	−6.61 ± 0.20	−0.46 ± 2.96
		35.0 ± 12.4	−0.84 ± 3.09	−6.21 ± 0.21	−5.38 ± 3.10
	35.3	38.3 ± 10.6	−3.75 ± 0.29	−6.47 ± 0.17	−2.72 ± 0.34
		5.27 ± 1.51	−6.39 ± 1.28	−5.25 ± 0.18	+1.14 ± 1.29

increased negative cooperativity with decreasing temperature.

4. Discussion

We have characterized the thermodynamics of binding reactions of the two adenine nucleotides, ADP and ATPγS, to GroEL using ITC. This technique has a potential advantage in that we are measuring directly the heat effect of binding, which allows us to obtain the binding isotherm and the ΔH of binding simultaneously [34–36]. It was expected that the technique would be useful for investigating the allosteric nucleotide binding of GroEL, which had been observed by many other researchers using techniques such as GroEL ATPase activity measurements [16,17,21–24] or fluorescence spectroscopy of pyrenyl GroEL [25–27]. On the contrary, however, there was no positive cooperativity in the nucleotide-binding reactions observed by the present technique.

Here, we first describe the thermodynamic parameters of the nucleotide binding by GroEL, then discuss the apparent discrepancy in the binding behavior, i.e., the noncooperative versus cooperative binding reactions, observed in the present and the previous studies. We show that a ring-dissociation model assuming the dissociation of the two rings of GroEL at its very low concentration (<10 nM of the tetradecamer) reasonably interpret both the noncooperative and cooperative behavior of the GroEL nucleotide binding.

4.1. Thermodynamic parameters

The binding reactions of ADP and ATPγS to GroEL were both well represented by the noncooperative binding model, and the Hill coefficient of the nucleotide binding must be smaller than 1.2 under any conditions used in this study. Thus, the binding parameters for the two nucleotides were estimated by this model (Table 1). The binding constants are 7.2×10^3 and 3.8×10^4 M^{-1} for ADP and ATPγS, respectively, at 26°C, and the stoichiometry n is about 12 for both nucleotides. The previous ATPase activity studies of GroEL followed the allosteric model of the ATP binding and reported the binding constant for ATP in the R state of GroEL ranging

from 2.1×10^4 to 1.0×10^5 M^{-1} [16,21,22]. These values are, however, not directly compared with the binding constants in this study because the present data do not follow the allosteric model. Table 1 also shows that the binding reactions of both ADP and ATPγS are driven mainly by the enthalpy changes (ΔH), which are ascribable to hydrogen bonding, van der Waals interactions and electrostatic interactions, within and between GroEL, nucleotide and solvent molecules.

From the temperature dependence of the ΔH, we have evaluated the ΔC_p values for the binding reactions. Both the ADP and ATPγS bindings to GroEL are accompanied by a small ΔC_p of about 100 cal/mol•K. The ΔC_p is known to be approximately proportional to a change in nonpolar accessible surface area accompanied by a protein conformational change [44–46], and the ΔC_p values for protein–ligand interactions are usually negative in sign and typically in a range of −100 to −550 cal/mol•K [47] because the ligand binding stabilizes the protein and reduces the overall extent of the hydrophobic-surface exposure. The small and positive ΔC_p values for the nucleotide bindings to GroEL thus make a contrast to the ΔC_p values for other protein–ligand interactions and suggest that the nucleotide binding induces slight exposure of hydrophobic surface. This is probably correlated with an unusual character of GroEL, i.e., it has flexible exposed hydrophobic residues in the apical domains in the native state [6]. The positive ΔC_p is consistent with the fact that the presence of ADP or other adenine nucleotides destabilizes GroEL in urea-induced dissociation and thermal denaturation and makes the hydrophobic surfaces more exposed to solvent as probed by proteolytic digestion [48–50].

4.2. Absence of positive cooperativity

The absence of the positive cooperativity in the nucleotide binding by GroEL (Fig. 2) is apparently in conflict with the current view of the allosteric nucleotide binding by GroEL, in which the binding occurs with positive cooperativity within the GroEL heptameric ring that switches the conformations of the seven molecules between the T and R states concertedly [51]. The presence of a second level of allostery, i.e., negative cooperativity between two rings of GroEL that undergoes a sequential transition from the TT state via TR to the RR state has also been put forward in the allosteric model [52]. Such a view has been deduced from the experimentally measured dependence on nucleotide concentration of the GroEL ATP-hydrolysis rate [16,17,21–24] and of the fluorescence intensity of pyrene-labeled GroEL [25]. The positive cooperativity in the nucleotide binding to GroEL has been regarded as an important factor for the protein folding activity, providing concerted conformational changes in the apical domains to regulate affinities for target proteins [53]. Therefore, a question arises as to why the cooperative nucleotide binding has not been observed by the ITC that must be sensitive to such cooperative effect if present.

Comparison in the experimental conditions between the present study and the previous studies that have reported the cooperative nucleotide binding of GroEL [16,17,21–25] has indicated that the only remarkable difference is a difference in GroEL concentration; all of the experiments in those previous studies were performed at GroEL tetradecamer concentrations in a range of 25 to 54 nM, 170 to 360 times lower than the concentration used in this study. Therefore, it is very likely that this difference in the GroEL concentration has led to the difference in the nucleotide-binding behavior of GroEL. In support of this view, Burston et al. [26] also observed noncooperative bindings of ATP and ADP to GroEL, when they repeated the measurement of the nucleotide binding at a GroEL tetradecamer concentration of 2 μM. The binding constant for ATPγS (4×10^4 M^{-1}) in Table 1 agrees with the value for ATP reported by Burston et al. (1×10^5 M^{-1}), considering the difference between ATPγS and ATP.

4.3. Possible model for describing the cooperative and noncooperative nucleotide bindings

As the difference in GroEL concentration is the only difference which brings about the transition between the cooperative and noncooperative nucleotide-binding reactions, it is quite natural to ascribe this transition to association or dissociation of GroEL tetradecamer, because only such an association or dissociation reaction must depend on GroEL concentration. Considering the destabilization of

GroEL by the presence of nucleotides, which has been reported as mentioned above [48–50] and confirmed by the positive ΔC_p obtained in the present study, we propose that the nucleotide binding at a low GroEL concentration, which shows the positive cooperativity, is linked with the GroEL dissociation. In support of this view, Goloubinoff et al. reported the dissociation of the GroEL tetradecamer to a species of lower relative molecular mass observed in their native gel electrophoresis in the presence of ATP, although the number of the GroEL subunits in this lower molecular mass component was not identified [54]. Although such dissociation of GroEL might ultimately give rise to a monomeric species, the dissociation into the monomer is not consistent with the cooperative nucleotide binding because the monomeric species does not show the cooperativity as far as it has only a single nucleotide-binding site. Therefore, the dissociation of GroEL tetradecamer into a smaller oligomeric species is most likely to be connected with the cooperative nucleotide binding at a low concentration of GroEL. We assume the heptameric ring of GroEL as such smaller oligomeric species, which is consistent with the reported ATP-dependent heptamer exchange between GroEL and the chaperonin from *Thermus thermophilus* [55].

Thus, a ring-dissociation model is considered, in which the dissociation of the GroEL two rings is promoted by the nucleotide binding and leads to a conformational state (the R state) of the heptameric ring that has higher affinity for the nucleotide. Therefore there are two equilibria, the dissociation and the conformational transition of GroEL, taking place in addition to the nucleotide binding as

$$\mathrm{TT} \rightleftarrows 2\mathrm{T},\ \mathrm{T} \rightleftarrows \mathrm{R} \tag{13}$$

where TT represents a GroEL tetradecamer that shows always low affinity for the nucleotide, and T and R represent heptameric rings with low (T) and high (R) affinities, respectively, for the nucleotide. The total molar concentration (M_t) of GroEL with respect to tetradecamer is thus given by

$$M_t = \sum_i [\mathrm{TT}]_i + (1/2)\left(\sum_j [\mathrm{T}]_j + \sum_k [\mathrm{R}]_k\right) \tag{14}$$

where $[\mathrm{TT}]_i$, $[\mathrm{T}]_j$ and $[\mathrm{R}]_k$ are the molar concentration of TT, T and R that bind i, j and k nucleotide molecules. Eq. 14 is expressed by the use of the dissociation constant, K_d, for TT, the equilibrium constant between T and R, L ($=[\mathrm{R}]_0/[\mathrm{T}]_0$), and the binding constants, K_T and K_R, in the T and R states, respectively, as

$$M_t = \frac{1}{2}[\mathrm{T}]_0\left[\left(\frac{2[\mathrm{T}]_0}{K_d}\mathrm{X}(1+K_T[\mathrm{X}])^7 + 1\right)\right.$$
$$\left.\times(1+K_T[\mathrm{T}])^7 + L(1+K_R[\mathrm{X}])^7\right] \tag{15}$$

The sum of the terms within the bracket of the right-hand side is taken as a partition function, Z, and expressed as a sum of the statistical weights, Z_T and Z_R, for the T state (TT and T) and the R state, respectively, as

$$Z = Z_T + Z_R \tag{16}$$

$$Z_T = \left(\frac{2[\mathrm{T}]_0}{K_D}(1+K_T[\mathrm{X}])^7 + 1\right)(1+K_T[\mathrm{X}])^7$$

$$Z_R = L(1+K_R[\mathrm{X}])^7 \tag{17}$$

Because the fractional saturations for the T and R states, Θ_T and Θ_R, are given by $\Theta_T = K_T[\mathrm{X}]/(1+K_T[\mathrm{X}])$ and $\Theta_R = K_R[\mathrm{X}]/(1+K_R[\mathrm{X}])$, the total fractional saturation Θ is:

$$\Theta = \frac{1}{Z}\left(\frac{Z_T K_T[\mathrm{X}]}{1+K_T[\mathrm{X}]} + \frac{Z_R K_R[\mathrm{X}]}{1+K_R[\mathrm{X}]}\right) \tag{18}$$

Fig. 4 shows simulation curves based on the ring-dissociation model which describes appearance of sigmoidal positively cooperative curve when GroEL concentration is lowered from 1 mM to 10 nM. This model also reasonably interprets why the X-ray structure of GroEL tetradecamer complexed with ATPγS has not reveal the expected conformational differences for the T→R transition, relative to the structure of the uncomplexed GroEL [33]. The tetradecameric state of the protein in the crystal may force the ring structure into the T state because the ring dissociation is required for the conformational transition.

4.4. Negative cooperativity between two rings of GroEL

We have meant a difference between the two rings of GroEL in the affinity for the nucleotide by the

term negative cooperativity, and the incorporation of the negative cooperativity into the nucleotide-binding model improved the agreement with the experimental data (Fig. 3). Because the two rings of GroEL in the absence of the nucleotide must be identical, the difference in the affinity may be best interpreted in terms of the negative cooperativity in the nucleotide binding between the rings. Our data suggest that the negative cooperativity is stronger at a low temperature with a more than 10-fold difference in the binding constant at 5°C (Table 2) but this difference in the binding constant diminishes with increasing temperature. At 26°C, about 14 (ADP) or 11 (ATPγS) nucleotide molecules are bound to GroEL on the basis of the noncooperative binding model, and the difference in the binding constant between the two rings seems only marginal (Table 2). This result thus questions the validity of the simplification that nucleotide binding occurs exclusively to the ring in R state [16,21–24]. However, it may not be inconsistent with the asymmetric ATP hydrolysis in GroEL with a stoichiometry of the seven ATP molecules per one tetradecamer at a high GroEL oligomer concentration (7 μM) [26]. The recently reported noncompetitive inhibition of ATP hydrolysis by ADP [27] also suggests the binding of more than seven nucleotides at a time even if it may be transient and asymmetric.

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