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Analysis of Peptides and Proteins in Their Binding to GroEL

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Abstract

The GroEL-GroES is an essential molecular chaperon system that assists protein folding in cell. Binding of various substrate proteins to GroEL is one of the key aspects in GroEL-assisted protein folding. Small peptides may mimic segments of the substrate proteins in contact with GroEL, and allow detailed structural analysis of the interactions. A model peptide SBP has been shown to bind to a region in GroEL that is important for binding of substrate proteins. Here, we investigated whether the observed GroEL-SBP interaction represented those of GroEL-substrate proteins, and whether SBP was able to mimic various aspects of substrate proteins in GroE-assisted protein folding cycle. We found that SBP competed with substrate proteins, including α-lactalbumin, rhodanese, and malate dehydrogenase, in binding to GroEL. SBP stimulated GroEL ATP hydrolysis rate in a manner similar to that of α-lactalbumin. SBP did not prevent GroES from binding to GroEL, and GroES association reduced the ATPase rates of GroEL/SBP and GroEL/α-lactalbumin to a comparable extent. Binding of both SBP and α-lactalbumin to apo GroEL was dominated by hydrophobic interaction. Interestingly, association of α-lactalbumin to GroEL/GroES was thermodynamically distinct from that to GroEL with reduced affinity and decreased contribution from hydrophobic interaction. However, SBP did not display such differential binding behaviors to apo GroEL and GroEL/GroES, likely due to the lack of a contiguous polypeptide chain that links all of the bound peptide fragments. Nevertheless, studies using peptides provide valuable information on the nature of GroEL-substrate protein interaction, which is central to understand the mechanism of GroEL-assisted protein folding.

Introduction

The paradigm molecular chaperone GroEL, along with co-chaperone GroES, assists protein folding in cell in an ATP-dependent manner [1–7]. GroEL is a homo-tetradecamer whose fourteen subunits are arranged into two homo-heptameric rings that stack back-to-back [8, 9]. The cylindrical structure contains two separate central cavities. Each subunit consists of three domains. The apical domains are situated at both ends of the cylinder, forming the opening of the central cavities, and contain the binding sites for substrate proteins [10–13] and GroES [14]. The equatorial domains are located in the middle of the cylindrical assembly, providing all the interring contacts and most of intra-ring interactions. The equatorial domains are also the location of the chaperone's nucleotide binding sites [15]. The apical and the equatorial domains are linked by the intermediate domains. GroES binds to Helix H and I of the GroEL apical domains *via* seven symmetry-related loops (termed the GroES mobile loops), and large structural changes are observed in GroEL upon its association with GroES [14]. The intermediate domains swing about 25° downward to the equatorial domains, closing

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the nucleotide binding sites. The apical domains rotate about 90° along their domain axis and about 60o upward away from the central cavity. As a result of such domain movements, inter-subunit interface formed by the apical and intermediate domains is disrupted, and the surface lining the GroEL central cavity changes from hydrophobic in unliganded GroEL (termed apo GroEL in this study) to hydrophilic in GroEL-GroES. The volume within the enclosed GroEL-GroES central chamber is twice as that in apo GroEL. These domain movements are initiated and promoted by binding of nucleotides (e.g., ATP or ADP) [16, 17], and are obligatory for binding of GroES as the presence of nucleotides is required for GroES to associate with GroEL.

One of the most intriguing aspects of GroEL in assisting protein folding is its substrate promiscuity. GroEL interacts with a large number of substrate proteins of widely ranging sizes. Many of these proteins play important roles in cellular activities including transcription and translation, and in biosynthetic pathways [18–20]. Since GroEL interacts with the nonnative states of the substrate proteins [18], sequence-independent hydrophobic interactions are generally believed to be the main feature of GroEL-substrate interactions. Most residues that are important for substrate binding are hydrophobic [10], and these residues, located in Helix H and I, are on the rim of the central cavity facing into the cylinder. Electron microscopic and small angle neutron scattering studies on GroEL-substrate complexes have found extra density, presumably from the substrate protein, at the opening of one of the central cavities where Helix H and I are situated [21–25]. However, further structural characterizations at the atomic level are hindered by the intrinsic disordered conformation of the bound substrate.

This inherent yet important issue has been tackled using small peptides in place of substrate proteins, and this reduction approach has allowed precise structural analysis on GroEL-substrate interaction to be performed. Since substrate proteins bind to GroEL in a multi-valent attachment manner [26], the GroEL-substrate protein interaction may be simplified as a collection of interactions between GroEL subunits and individual segments of the substrate proteins, which may be represented by a spectrum of GroEL-peptide interactions. So far, structural studies on GroEL-peptide interactions have revealed that Helix H and I and the groove between them in GroEL is the peptide-binding site, that the peptides adopt various conformations when bound to GroEL, and that the GroEL-peptide interactions are largely hydrophobic [11–13, 27]. In our earlier studies, we identified a strong binding peptide (SWMTTPWGFHLP, termed SBP) using a phage display method, and found that the GroEL-bound SBP adopts a β-hairpin structure [12, 13]. The observations that both SBP and the GroES mobile loop bind to the same region on GroEL and their GroEL-bound conformations are similar have generated concerns that peptide SBP may not have been selected to represent substrate proteins in their interactions with GroEL but rather to mimic GroES in its association with GroEL [28, 29]. In this report, we further examined the efficacy of peptide SBP in emulating GroEL substrate proteins. We compared various aspects of substrate proteins and SBP in the functional cycle of GroE-assisted protein folding, including the binding site on GroEL, their effect on GroEL ATP hydrolysis rate, their response to GroES binding, and the thermodynamic nature of their interactions with GroEL.

Materials and Methods

Proteins

GroEL and GroES were purified as described previously [14], except for the additional steps to further purify GroEL by removing the bound residual substrate proteins as follows. Following the gel filtration purification, GroEL (at 1mg/ml) was dialyzed against 50 mM TrisCl pH 7.5, 1 mM EDTA and 30% methanol, loaded onto a FastQ column (GE Healthcare), and eluted with 0–1 M NaCl gradient. The GroEL-containing fractions were

combined, dialyzed (against 50 mM TrisCl pH 7.5, 150 mM NaCl and 1 mM EDTA), and further purified via a Superdex 200 column. GroEL thus purified was confirmed to have low Trp fluorescence.

Purification of rhodanese was similar to a published procedure [30]. Bovine apo α-lactalbumin and malate dehydrogenase (MDH) were purchased from Sigma. Reduced apo α-lactalbumin was prepared by incubating the purified apo α-lactalbumin (in 25 mM TrisCl pH 7.8, 200 mM KCl and 1 mM EDTA) with 5 mM dithiothreitol (DTT) for 30 minutes at 4 °C. To prepare the GroEL/α-lactalbumin complex, purified GroEL was incubated with freshly prepared reduced apo α-lactalbumin in 50 mM TrisCl pH 7.5, 200 mM KCl, 5 mM DTT and 1 mM EDTA to a final molar ratio of GroEL tetradecamer: α-lactalbumin = 1:250. The solution was incubated at room temperature for 1 hour and purified by a Superdex 200 column (GE Healthcare). The GroEL/GroES complex was prepared and isolated as previously [14] using the ultra-pure GroEL. SDS-PAGE was used to confirm the components of the complexes.

MDH refolding

MDH was unfolded in 3 M GdmHCl, 50 mM triethanolamine (TEA), pH 7.4, 50 mM KCl, and 20 mM $MgCl_2$. To initiate MDH refolding, 2.5 μL unfolded MDH was diluted at 1:100 (vol:vol) to a final volume of 250 μL refolding solution (at 30 °C) containing 50 mM TEA, pH 7.4, 50 mM KCl, 1 mM ATP, and 1 μM tetradecameric GroEL. GroES was added to the refolding solution to a final concentration of 4 μM prior to the addition of the unfolded MDH. To perform GroEL/SBP complex, SBP was added into the refolding solution five minutes before additions of GroES and unfolded MDH. An experiment in which SBP, GroES and unfolded MDH were added into the refolding solution at the same time was also performed. At desired time intervals, 20 μL of reaction solution was removed, mixed with 1 ml NADH assay solution (50 mM TrisCl, pH7.4, 10 mM DTT, 0.2 mM NADH, 1 mM Ketomalonate), and absorption at 340 nm was taken to monitor the decrease of NADH. Refolded and active MDH converts NADH to NAD^+.

Rhodanese aggregation assay

Rhodanese was denatured in buffer (7 M GdmHCl, 30 mM TrisCl, pH 7.4, 50 mM KCl, and 5 mM DTT) for 30 minutes and diluted 100-fold into a buffer containing 30 mM TrisCl, pH7.4, and 50 mM KCl in absence or presence of GroEL. The final concentration of rhodanese was 0.43 μM and GroEL concentration varied from 0.5, 1 and 1.5 folds of 0.43 μM. Aggregation was monitored at 320 nm using a Cary 100 Bio UV-Vis spectrophotometer for 30 minutes at room temperature. When SBP was included in the experiment, the final concentration of SBP was 60.2 μM. SBP was either incubated with GroEL for 30 minutes prior to mixing with rhodanese or directly into GroEL-rhodanese solution. When α-lactalbumin was included in the experiment, the final concentration of the preformed GroEL/α-lactalbumin complex was 0.43 μM.

Steady state ATPase assay

Steady state ATP hydrolysis rate was measured using the malachite green assay [31]. GroEL was added to a buffer containing 50 mM TEA, pH 7.5, 50 mM KCl, and 20 mM $MgCl_2$ to a final concentration of 0.125 μM. Where desired, the final concentrations of GroES, SBP, and freshly reduced α-lactalbumin were 0.3 μM, 17.5 μM, and 5 μM, respectively. 5 mM DTT was included when using α-lactalbumin. The solution was incubated at 25 °C for 10 minutes. The hydrolysis was initiated by addition of 100 mM ATP (pH 7.0) to a final concentration of 10 mM, and followed every 2 minutes for 12 minutes using the malachite green assay.

Peptide synthesis and purification

Peptide SBP (SWMTTPWGFHLP) was synthesized by solid-phase synthesis using an ABI 433A peptide synthesizer (Applied Biosystems), and purified by C18 reversed-phase HPLC (Vydac) with an acetonitrile gradient of 0–80% in 0.1 TFA, and confirmed by mass spectrometry. The purified peptide was lyophilized and stored at −20 °C.

Isothermal titration calorimetry (ITC)

ITC experiments were carried out using a VP-ITC instrument (MicroCal). The experimental conditions are described in the appropriate figure legends. Complexes of GroEL/α-lactalbumin and GroEL/GroES were formed and purified as described earlier. The ternary complex of GroEL/GroES/α-lactalbumin was prepared by recovering ITC samples of temperature-dependent studies of GroEL/GroES with α-lactalbumin (final molar ratio GroEL/GroES:α-lactalbumin = 1:4), followed by concentration and purification via a Superdex200 column. The composition of the ternary complex was verified by SDS-PAGE, and the concentration was quantified using a Bradford method (Bio-Rad). Time intervals between each injection varied from 240 s to 600 s in different experiments. Thermodynamic parameters of the binding process were derived using ORIGIN ITC software (Origin Lab) by fitting the corrected binding isotherm to different binding models. The single-site binding model appeared to give the best fitting results, which are presented here.

Results and Discussion

SBP competes with substrate proteins in binding to GroEL

The peptide SBP was identified from the bio-panning of a phage display peptide library against the apical domain of GroEL [12]. It binds to the groove formed by Helix H and I in the apical domain of GroEL, which is the main binding site for substrate proteins, *via* both hydrophobic and hydrogen bonding interactions [12, 13]. To validate the relevance of SBP with the GroEL substrate proteins, we set out to examine if both SBP and substrate proteins (α-lactalbumin, rhodanese, and malate dehydrogenase (MDH)) bound to the same region of GroEL.

We first compared the binding sites of SBP and α-lactalbumin on GroEL. α-lactalbumin is a commonly used GroEL substrate protein [32–35]. It contains eight Cys residues that form four disulfide bonds, and in the absence of Ca^{2+} (apo form) and under reducing conditions, α-lactalbumin adopts a molten globular conformation that is sufficiently stable to allow the protein to be isolated [36]. Only the reduced form of apo α-lactalbumin has been shown to interact with GroEL [34], and was used in our studies here. (Unless otherwise stated, the reduced apo α-lactalbumin is referred to as α-lactalbumin in this report.) α-lactalbumin bound tightly to only one of two apo GroEL rings by isothermal titration calorimetry (ITC) with dissociation constant (K_d) of 0.027 μM at 20 °C (Table 1, and [37, 38]). When GroEL/α-lactalbumin was titrated into SBP solution, seven SBP molecules were found to bind to one GroEL/α-lactalbumin complex Fig. 1A, Table 2), suggesting that SBP bound only to the unoccupied open GroEL ring and did not interact with the α-lactalbumin-bound GroEL ring. When SBP was titrated with GroEL/GroES/α-lactalbumin where both GroEL rings were occupied with either GroES or α-lactalbumin, little heat exchange was observed (Fig. 1B), consistent with that SBP did not interact with the GroEL ring once the ring was occupied with α-lactalbumin. The observation that the bound α-lactalbumin prevents SBP from binding to GroEL suggests that the binding sites of α-lactalbumin and SBP on GroEL at least overlap.

Next, we studied the effect of SBP on the interactions of GroEL with rhodanese, another commonly used GroEL substrate protein. When unfolded rhodanese was introduced into

folding buffer, the protein precipitated readily as revealed by the rapid increase in scattering intensity at 320 nm (Fig. 2A). In the presence of GroEL, rhodanese precipitation was suppressed (Fig. 2A) and in a stoichiometric manner (data not shown), suggesting that rhodanese was stabilized by forming a specific complex with GroEL. When SBP was added to this stable GroEL/rhodanese complex, scattering intensity at 320 nm was instantaneously observed, which, given that addition of SBP to GroEL did not increase scattering intensity (data not shown), suggested that rhodanese was displaced from GroEL and aggregated nonspecifically. Increase in scattering was also observed when reduced apo α-lactalbumin was added to GroE/rhodanese (data not shown). When unfolded rhodanese was brought into a folding buffer containing the GroEL/SBP complex, scattering intensity at 320 nm was immediately and drastically increased (Fig. 2A), suggesting that SBP blocked the binding site for rhodanese. In summary, these experiments indicate that SBP and rhodanese bind to the same region on GroEL.

Finally, we investigated whether SBP influenced the GroE-assisted refolding of malate dehydrogenase. As shown in Fig. 2B, preincubation of SBP with GroEL slowed the MDH refolding kinetics, and prolonged the time it took to achieve the maximal MDH recovery from less than 20 minutes to ~50 minutes. Noticeably, the MDH folding yield decreased significantly with the presence of SBP (from ~80% to less than 50%). We also found reductions in both MDH refolding kinetics and yield when SBP was introduced to the folding solution at the same time as MDH (Fig. 2B). These observations are consistent with the hypothesis that SBP competes with MDH in binding to GroEL.

SBP stimulates GroEL ATPase activity

Since binding of substrate proteins stimulates ATP hydrolysis rate of GroEL [39–43], we examined if SBP affected GroEL ATPase activity. As shown in Table 3, like reduced apo α-lactalbumin, SBP enhanced ATPase rate of GroEL and in a similar magnitude. More remarkably, the inhibitory effect by GroES on the SBP- and α-lactalbumin-stimulated GroEL ATPase rates were comparable (Table 3). The significance of GroES's ability to suppress the ATP hydrolysis rate of GroEL/SBP will be discussed next.

Binding of SBP does not prevent GroES from binding to GroEL

In GroE-assisted protein folding, GroES binds to the substrate-bound GroEL to form the GroEL/GroES complex, displacing the GroEL-bound substrate protein from GroEL into the enclosed central chamber and initiating protein folding. To examine whether SBP interfered with GroES binding to GroEL, GroEL was incubated with excess amount of SBP (GroEL subunit:SBP = 1:10, a condition favoring formation of symmetric GroEL-SBP_{14}) for 30 min, GroES was then added at a molar ratio of GroEL subunit:GroES subunit=1:1 and the solution was incubated at 37 °C for 10 min. The mixture was directly separated using a size exclusion chromatographic column, and the content of elution peaks was analyzed by SDS-PAGE. GroES was detected in the GroEL-containing fractions, and the ratio of Coomassie-stained band intensities of GroEL to GroES was comparable to that seen for an isolated GroEL/GroES complex (data not shown), suggesting that GroES was able to interact with SBP-bound GroEL to form the GroEL/GroES complex. A bullet-shape structure (data not shown) revealed by negative stained electron microscopy further confirmed the canonical 1:1 asymmetric GroEL/GroES complex.

Association of GroES with GroEL suppresses the GroEL ATP hydrolysis rates (both the intrinsic and the substrate-enhanced rates) by 35–50% [38, 40, 44, 45]. Here we observed that GroES reduced ATPase rate of GroEL by ~60%, and that of GroEL in the presence of α-lactalbumin by ~40% (Table 3). Notably, the presence of GroES resulted in ~28% reduction in the ATP hydrolysis rate of GroEL/SBP (Table 3), suggesting that the inhibitory function

of GroES on GroEL ATPase rate was not obstructed by the bound SBP. Since GroES regulates GroEL's ATP hydrolysis rate by forming a direct complex with GroEL, the observations that GroES was capable to suppress the ATP hydrolysis rate of GroEL/SBP suggest that GroES can associate with GroEL in the presence of SBP. Furthermore, the comparable reduced magnitudes by GroES on samples of GroEL/α-lactalbumin and GroEL/SBP argue that GroES binds to GroEL in the presence of SBP in the same manner as it does in the presence of α-lactalbumin. Taken together, results shown here argue that SBP does not prevent GroES from binding to GroEL, and that GroES associates with GroEL in the presence of SBP and α-lactalbumin in a similar manner.

Previously, the structural resemblance between SBP and the GroES mobile loop has prompted concerns that SBP may not have been selected to represent GroEL substrate proteins in general [28]. In their experiments, Ashcroft et al covalently linked the C-terminus of SBP to a GroEL mutant (GroELN229C) *via* a Lys-maleoyl moiety, termed GroELN229C-SBP, and based on the observations that GroELN229C-SBP could not associate with GroES using surface plasmon resonance (SPR), they concluded that SBP competed with GroES for binding to GroEL [29]. However, the inability of GroELN229C-SBP to associate with GroES can also be explained by the restraints imposed by the linker (the Lys-Mal moiety) on the obligatory domain movements in GroEL as observed in the GroEL/GroES structure [14, 46]. N229 is buried within the inter-subunit interface in apo GroEL and forms direct contact with I270 of the neighboring GroEL subunit, and this interface becomes completely disrupted upon GroES binding. Any modifications at this position may interfere with the inherent structural flexibility of the apical domain and the functional consequence of domain movement required for GroES binding. In this study, we demonstrated that SBP did not prevent GroES from binding to GroEL because GroES associated with GroEL in the presence of excess amount of SBP present, and that SBP did not interfere with GroES function because GroES suppressed ATPase rate of GroEL/SBP in the similar manner as that of GroEL/α-lactalbumin.

Thermodynamic studies of SBP and α-lactalbumin binding to GroEL

A polypeptide substrate has been shown to interact with GroEL *via* multiple attachments to various binding sites, in addition to the groove formed by Helix H and I where SBP binds [24]. To investigate the significance of these additional binding sites to the overall GroEL-substrate protein interaction and the importance of the cooperativity among the different substrate binding sites due to a contiguous polypeptide chain, we used ITC to compare thermodynamic aspects of α-lactalbumin and SBP in their binding to GroEL.

The ITC derived dissociation constant (K_d) for SBP/apical domain interaction is 1.4 μM, consistent with the result (K_d of 2 μM) from our previous fluorescence polarization study [12]. As shown in Table 2, SBP had the similar affinity for apo GroEL (K_d of 1.2 μM, Fig. 3A), the GroEL/GroES complex (1.1 μM), and GroEL/α-lactalbumin (1.0 μM) as that for the isolated apical domain, indicating that binding of SBP to each of the substrate binding sites within tetradecameric apo GroEL (14 sites), the GroEL/GroES complex (7), and GroEL/α-lactalbumin (7) was independent and non-cooperative. Binding of SBP to GroEL proteins, including GroEL and GroEL/GroES, was mainly enthalpy driven, releasing large amount of heat (Table 2 and Fig. 3A&B).

Binding of α-lactalbumin to GroEL was endothermal (Fig. 3C) as reported before [37]; the unfavorable enthalpic requirement was overcome by a large favorable entropic change (Table 1). α-lactalbumin bound tightly with GroEL with K_d of 20–60 nM in the temperature tested (Table 1), comparable with the reported K_d of 50 nM also by ITC [38]. Only one α-lactalbumin binds to GroEL as indicated by the near unity binding stoichiometry, leaving one GroEL ring unoccupied with the substrate protein. The inability of α-lactalbumin to

occupy both GroEL rings is consistent with the ineffectiveness of GroEL/α-lactalbumin complex to suppress rhodanese aggregation or to assist MDH refolding (data not shown). The negative cooperativity in binding substrate proteins suggests that effect of the bound substrate protein is transmitted across the ring and alters the substrate binding function of the open *trans* ring. In contrast, the negative effect on substrate binding to the second ring in GroEL is not observed in SBP: SBP can bind to both GroEL rings (ITC of this study and crystal structure studies [12, 13]), and to the preformed GroEL/α-lactalbumin complex. Very likely, a contiguous polypeptide chain that tethers all the interacting sites in a substrate-bound GroEL ring, including those besides the SBP-binding site, confers the additional influence on the structure and function of the opposite open GroEL ring. Due to their size, small peptides, like SBP, do not mimic this important allosteric function.

Thermodynamic parameters of SBP-GroEL interaction at different temperatures are listed in Table 2, and the temperature dependence of enthalpic change is shown in the insert of Fig. 3A. The derived heat capacity change (ΔC_p), was large negative, $\Delta C_p = -251.9$ cal mol^{-1} K^{-1}. Given that a large negative ΔC_p is correlated with the dominant contribution of hydrophobic interaction in a macromolecular interaction reaction [47–49], the ITC studies indicated that SBP-GroEL interaction was mainly hydrophobic, which is in agreement with the largely hydrophobic SBP-GroEL interface in the GroEL/SBP complex revealed by our crystallographic studies [12, 13]. Table 2 shows that thermodynamically SBP interacted with GroEL/GroES in a similar fashion as with GroEL, for example, with the comparable affinities. Notably, ΔC_p for SBP interaction with GroEL/GroES was negative ($\Delta C_p = -200.5$ cal mol^{-1} K^{-1}) and comparable to that with GroEL, suggesting that, like in SBP binding to GroEL, hydrophobic interaction was dominant in SBP binding to GroEL/GroES.

Table 1 lists thermodynamic parameters of α-lactalbumin and GroEL interaction. Fig. 3C insert shows that the required enthalpic intake decreased drastically with increasing temperature, and the large negative heat capacity change (this study and Aoki et al [37]), $\Delta C_p = -766$ cal mol^{-1} K^{-1}, indicate that the interaction between α-lactalbumin and GroEL in the GroEL/α-lactalbumin complex was mainly hydrophobic. Large negative heat capacity change was reported for the association of either subtilisin or α-Casein with GroEL [50].

Interestingly, binding of α-lactalbumin to the GroEL/GroES complex was strikingly different than that to apo GroEL. The association of α-lactalbumin to GroEL/GroES was exothermal (Fig. 3D and Table 1), and the favorable large enthalpic release was responsible for the formation of GroEL/GroES/α-lactalbumin ternary complex. Furthermore, the temperature dependence of enthalpic change was positive (Fig. 3D insert) with $\Delta C_p = 44.2$ cal mol^{-1} K^{-1}: the absence of negative ΔC_p for α-lactalbumin binding to the GroEL/GroES complex suggests that hydrophobic effect might not play the dominant role in association of α-lactalbumin with the open GroEL ring *trans* to the bound GroES in the GroEL/GroES complex. Finally, α-lactalbumin had a ten-fold reduced affinity to the *trans* GroEL ring in GroEL/GroES when compared to apo GroEL (Table 1). Taken together, these marked differences in thermodynamic properties indicate that α-lactalbumin interacts with the *trans* GroEL ring of GroEL/GroES in a very different way than with the apo GroEL. The distinct binding behaviors observed in our thermodynamic studies here are in line with previous studies. In their work, Rye and coworkers reported that the conformation of Rubisco is more compact when bound to the *trans* GroEL ring of GroEL/GroES than that to apo GroEL based on results of fluorescence (FRET), proteolytic digestion, and Cys chemical reactivity studies [51].

Conclusions

In elucidating GroEL-substrate protein interaction, small peptides are valuable in providing information, which is otherwise unfeasible using the substrate proteins, on the substrate binding site, the GroEL-bound substrate conformation, and the nature of GroEL-substrate protein interactions. Complementing our previous structural work, our current biochemical and biophysical studies show that peptide SBP was effective in competing with substrate proteins in binding to GroEL and stimulating GroEL ATP hydrolysis, and did not interfere with GroES function. While our results validate SBP as an effective mimic of GroEL substrate proteins, peptide mimics also have their limitations. First, SBP binding to GroEL did not display negative cooperativity of substrate proteins in binding to the two GroEL central cavities. The inherent uncoupling feature among the individual GroEL-bound SBP molecules due to the lack of a contiguous polypeptide chain may forestall this important allosteric attribute in GroEL. Second, binding of substrate proteins to the *trans* ring of GroEL/GroES was markedly different from that to apo GroEL, and small peptide SBP did not reveal such distinctive modes of association. Substrate proteins have been shown to bind to regions other than the hydrophobic SBP binding interface [10, 24], however, binding of SBP does not reflect the integration of the additional sites with the main hydrophobic site. Nevertheless, despite the limitations, peptides as mimics for substrate proteins play an important role in elucidating the interactions of GroEL with the substrate proteins, which is central to GroEL-assisted protein folding.

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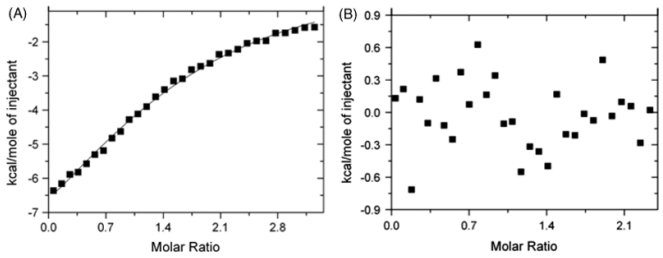

Fig. 1.

ITC data of: **A)** SBP and GroEL/α-lactalbumin interaction; **B)** SBP and GroEL/GroES/α-lactalbumin interaction. Shown are the integrations of heat exchange (after background correction) for each injection, and the line represents the fit to a single-site binding model. Concentration of GroEL/α-lactalbumin is normalized to the substrate-unliganded monomeric GroEL subunit, assuming that α-lactalbumin binds to one GroEL ring. For comparison, concentration of GroEL/GroES/α-lactalbumin is expressed in the same manner as that in **A)**. For GroEL/α-lactalbumin/SBP studies, 10.8 μM isolated GroEL/α-lactalbumin (Methods) was titrated into 5.4 μM SBP in 50 mM HEPES, pH 7.2, 200 mM KCl, 5 mM DTT, and 1 mM EDTA. For GroEL/GroES/α-lactalbumin/SBP studies, 7.2 μM isolated GroEL/GroES/α-lactalbumin (Methods) was titrated into 5 μM SBP in 50 mM TrisCl, pH 7.5, 10 mM $MgCl_2$, 200 mM KCl, 1 mM EDTA, 0.05% NaN_3, 5 mM DTT, and 50 μM ADP.

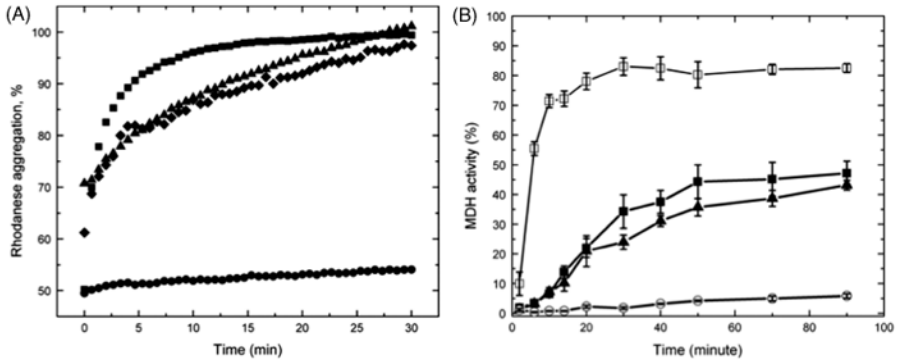

Fig. 2.

Effect of SBP on GroEL-substrate protein interactions. **A)** Suppression of rhodanese aggregation by GroEL. The final rhodanese concentration was 0.43 μM. Squares, unfolded rhodanese was added to a solution without GroEL; circles, unfolded rhodanese was added to a GroEL solution with a molar ratio of GroEL:rhodanese = 1:1; diamonds, unfolded rhodanese was added into a GroEL-SBP (GroEL subunit:SBP=1:10) solution at a molar ratio of rohdanese:GroEL/SBP_{14}=1:1; triangles, SBP was added into a preformed GroEL/rhodanese (GroEL:rhodanese=1:1) solution at a molar ratio of SBP:GroEL subunit = 10:1. Turbidity, due to protein aggregation, was monitored at 320 nm. **B)** Refolding of MDH assisted by GroEL. The final concentrations of MDH, tetradecameric GroEL, heptameric GroES, and SBP were 0.18, 1, 4, and 70.7 μM, respectively (with the final molar ratio of GroEL subunit:GroES subunit:SBP=1:2:5). Open circles, unfolded MDH was added to a solution without GroEL and GroES; open squares, unfolded MDH and GroES were added to a GroEL solution; filled squares, unfolded MDH, GroES and SBP were added to a GroEL solution; filled triangles, unfolded MDH and GroES were added to a preformed GroEL/SBP solution. The errors represent standard deviations of three experiments.

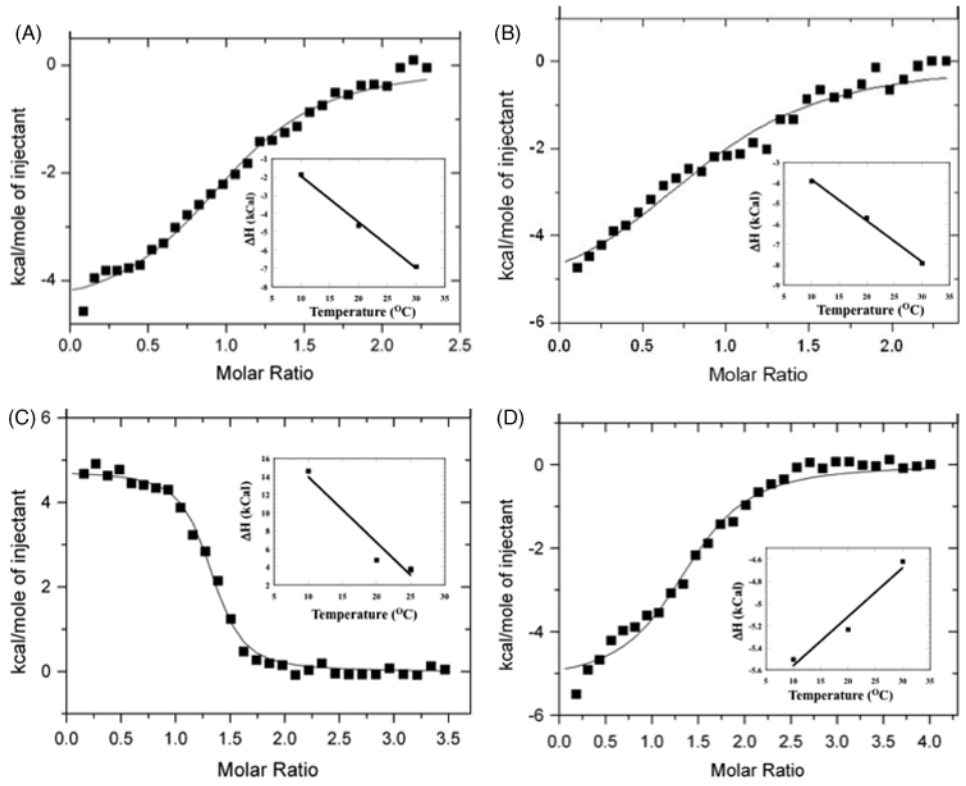

Fig. 3.

ITC data at 20 °C and the temperature dependence of enthalpic change (insert) of: **A)** SBP association with GroEL; **B)** SBP association with GroEL/GroES; **C)**α-lactalbumin association with GroEL; **D)**α-lactalbumin association with GroEL/GroES. Shown are the integrations of heat exchange (after background correction) for each injection during titration, and the line represents the fit to a single-site binding model. A), 21.4 μM GroEL was titrated into 30 μM SBP in 50 mM TrisCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA; B), 21.4 μM GroEL/GroES was titrated into 30 μM SBP in 50 mM TrisCl, pH 7.5, 10 mM $MgCl_2$, 200 mM KCl, 1 mM EDTA 0.05% NaN_3, 5 mM DTT, and 50 μM ADP; C), 50 μM α-lactalbumin was titrated into 21.4 μM GroEL in 50 mM Hepes, pH 7.2, 200 mM KCl, 5 mM DTT and 1 mM EDTA; D), 50 μM α-lactalbumin was titrated into 21.4 μM GroEL/GroES in 50 mM TrisCl, pH 7.5, 10 mM $MgCl_2$, 200 mM KCl, 1 mM EDTA 0.05% NaN_3, 5 mM DTT, and 50 μM ADP.

Table 1

Thermodynamic parameters of α-lactalbumin binding to GroEL and GroEL/GroES under different temperatures

Temp (°C)	K_d (μM)	ΔH (cal/mol)	ΔS (cal/mol)	Stoichiometry (N)
GroEL				
10	0.022	1.5×10^4	86.6	1.16
20	0.027	4762	50.8	1.26
25[a]	0.057	3799	45.9	0.76
GroEL/GroES				
10	0.203	−5504	11.2	1.38
20	0.28	−5235	12.1	1.39
30	0.63	−4620	13.1	1.19

[a] the heat exchange of α-lactalbumin binding to GroEL at 30 °C was small and not reliable, so the experiment was not carried out at temperatures higher than 25 °C.

Table 2

Thermodynamic parameters of SBP binding to various GroEL proteins. Results of GroEL, GroEL/GroES, and GroEL/α-lactalbumin are normalized to the monomeric concentration of the unliganded open GroEL subunit

Samples	K_d (μM)	ΔH (cal/mol)	ΔS (cal/mol)	Stoichiometry (N)
Apical domain	1.4	−5295	8.7	0.91
GroEL (20°C)	1.2	−4643	11.3	1.04
GroEL-GroES (20°C)	1.1	−5707	7.8	0.94
GroEL/α-lactalbumin (20°C)	1.0	−6429	−57.0	1.04
GroEL (10°C)	0.55	−1858	22.1	1.18
GroEL (30°C)	2.0	−6896	3.32	0.77
GroEL/GroES (10°C)	1.24	−3906	13.2	0.98
GroEL/GroES (30°C)	0.31	−7916	3.67	0.85

Table 3

ATP hydrolysis rates of GroEL under various conditions. The rates are expressed as ATP min^{-1} per GroEL tetradecamer

Sample	ATP hydrolysis rate (min^{-1})
GroEL	34.72 ± 7.84
GroEL + GroES	13.6 ± 6.16
GroEL + α-lactalbumin	57.84 ± 5.52
GroEL + α-lactalbumin + GroES	34.88 ± 6.08
GroEL + SBP	57.60 ± 6.40
GroEL + SBP + GroES	41.52 ± 5.68