J. Biochem. **140**, 591–598 (2006)
doi:10.1093/jb/mvj188

Probing Dynamics and Conformational Change of the GroEL-GroES Complex by ^{13}C NMR Spectroscopy

Noritaka Nishida[1,2], Fumihiro Motojima[3], Mayu Idota[2], Hiroshi Fujikawa[1], Masasuke Yoshida[3], Ichio Shimada[1] and Koichi Kato[2,*]

[1]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033; [2]Graduate School of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467-8603; and [3]The Chemical Research Laboratory, Tokyo Institute of Technology, Midori-ku, Yokohama 226-8503

Received July 5, 2006; accepted September 6, 2006

Bacterial chaperonin GroEL with a molecular mass of 800 kDa was studied by ^{13}C NMR spectroscopy. Carbonyl carbons of GroEL were labeled with ^{13}C in an amino acid specific manner in order to reduce the number of signals to be observed in the spectrum. Combination of selective labeling and site-directed mutagenesis enabled us to establish the sequence specific assignment of the ^{13}C resonances from GroEL. ADP-binding induced a chemical shift change of Tyr478 in the equatorial domain and His401 in the intermediate domain, but little of Tyr203 in the apical domain. Upon complex formation with co-chaperonin GroES in the presence of ADP, Tyr478 exhibits two peaks that would originate from the *cis* and *trans* rings of the asymmetric GroEL-GroES complex. Comparison between the line width of the GroEL resonances and those from GroES in complex with GroEL revealed broadening disproportionate to the size of GroEL, implying the existence of conformational fluctuations which may be pertinent to the chaperone activity. Based on these results, we concluded that ^{13}C NMR observation in combination with selective labeling and site-directed mutagenesis can be utilized for probing the conformational change and dynamics of the extremely large molecules that are inaccessible with current NMR methods.

Key words: amino acid selective labeling, ^{13}C NMR, conformational fluctuation, GroEL, GroES.

Abbreviations: ATPγS, Adenosine 5′-*O*-(3-thio)triphosphate; CRIPT, cross-correlated relaxation-induced polarization transfer; TROSY, transverse relaxation optimized spectroscopy.

Protein folding both *in vivo* and *in vitro* is facilitated by a variety of molecular chaperones. GroEL, the best-characterized molecular chaperone from *Escherichia coli*, transiently captures nascent polypeptides or denatured proteins and protects them from undesirable interactions that result in misfolding and aggregation. The chaperone activity of GroEL is further enhanced in the presence of the co-chaperonin GroES which facilitates the folding cycle involving ATP binding and hydrolysis (reviews in Refs. *1* and *2*). GroEL is composed of 14 identical subunits, forming a barrel-like structure in which two seven-membered rings are stacked in a back-to-back manner. Several crystal structures of GroEL and its mutants representing distinctive steps of folding cycle, including the ligand-free state (*3–5*), nucleotide-bound state (*6, 7*), and GroES-bound state (*8*), have been reported. These atomic structures revealed a dramatic conformational rearrangement of GroEL induced upon complex formation with GroES, producing a sequestered chamber inside of the GroEL where substrate peptides fold into the native state. In addition to those crystal studies, structural characterization under the physiological condition should be required since local structural perturbations and suppression of dynamics may arise as a consequence of crystal contacts. Solution state NMR spectroscopy has been be utilized to study protein structure and dynamics under physiologically relevant conditions. However, the molecular size of GroEL was generally considered to exceed the size limitation of conventional solution NMR.

During the past decade, novel pulse schemes such as TROSY (transverse relaxation optimized spectroscopy) have been invented in order to extend the utility of solution NMR to larger proteins (*9, 10*). In addition, the development of the ultra-high magnetic field spectrometers and advances in isotope labeling techniques has enabled the investigations of high-molecular weight proteins and protein complexes by NMR. A milestone NMR study on GroEL-GroES complex performed by Fiaux *et al.* (*11*) demonstrated that the combination of the CRIPT (cross-correlated relaxation-induced polarization transfer) (*12*) and TROSY pulse schemes could be used to observe resonances in a 900 kDa protein complex. In their study, ^{1}H-^{15}N CRIPT-TROSY spectra were observed for the GroES heptamer with a molecular mass of 72 kDa composed of the seven identical 97-residue subunits in the complex with the GroEL tetradecamer with a molecular mass of 800 kDa or its single ring variant SR1 heptamer. As a result, they successfully mapped the GroES-residues responsible for GroEL binding on the basis of the chemical shift comparison between free and GroEL-bound states. On the other hand, only 20% of the potential crosspeaks from [^{2}H,^{15}N]-labeled GroEL were detected on the

*To whom correspondence should be addressed. Tel: +81-52-836-3447, Fax: +81-52-836-3447, E-mail: kkato@phar.nagoya-cu.ac.jp

CRIPT-TROSY spectrum. Insufficient peaks on the spectrum might be ascribed to the reasons including incomplete N-D/N-H exchange, overlapping resonances, and/or global conformational changes, and thus indicate that direct observation of GroEL is inaccessible by the CRIPT-TROSY technique. Furthermore, additional advances in NMR technology are required to obtain resonance assignments of the 900-kDa protein given the inapplicability of current triple resonance protocols to study such massive proteins.

An alternative NMR approach for large proteins is the amino acid selective labeling method which avoids the complications of grossly overlapped spectra. For example, using amino acid selective labeling of the ^{13}C carbonyl, significant structural information was obtained of the interaction of immunoglobulin G (150 kDa) and Fc fragment (50 kDa) with Fcγ receptor (*13*) or *Staphylococcus* protein A (*14*).

In this study, we applied the amino acid type–specific labeling technique for the GroEL-GroES complex for observation of ^{13}C NMR signals. Sequence specific assignments of GroEL could be achieved by the site-directed mutagenesis method combined with selective ^{13}C labeling. On the basis of the spectral assignments, functionally relevant dynamics and conformational rearrangement of GroEL were observed.

MATERIALS AND METHODS

Materials—ADP was purchased from Sigma. [1-^{13}C]Tyr and [1-^{13}C]His were purchased from ICON. Other isotopically labeled and unlabeled amino acids were from Masstrace and Wako, respectively. Other chemicals were purchased from Nacalai Tesque.

Protein Preparation—GroEL and GroES expression plasmid pET-EL and pET-ES were transformed into BL21(DE3). Cells culture was carried out with 1 liter of M9 minimum medium which supplemented with following amino acids (Ala 400 mg, Arg 400 mg, Asp 200 mg, Cys 50 mg, Glu 400 mg, Gly 400 mg, His 100 mg, Ile 100 mg, Leu 100 mg, Lys 150 mg, Met 50 mg, Phe 50 mg, Pro 150 mg, Ser 1,000 mg, Thr 100 mg, Trp 50 mg, Tyr 100 mg and Val 400 mg), vitamins (biotin 1 mg, choline chloride 1 mg, niacinamide 1 mg, pyridoxal 1 mg, riboflavin 0.1 mg, thiamine 5 mg), minerals (1 μM of $FeCl_3$, 50 μM $ZnSO_4$, 100 μM $CaCl_2$, 2 mM $MgSO_4$) and 4 g of D-glucose (partially modified the composition described in Ref. *15*). For stable isotope labeling, ^{13}C enriched amino acids were added to the medium instead of the unlabeled corresponding amino acids. Antibiotic ampicilin was added to the medium at 50 mg/liter. Cells were grown to an OD_{600} of 0.6 at 37°C, induced with 0.4 mM IPTG, and incubated for additional 3 h. Protein purification was performed as described previously (*16*). GroEL mutants were produced by Kunkel methods.

NMR Experiments—For NMR measurements, purified proteins were concentrated to a final volume of 2 ml in 10 mM sodium phosphate, pH 7.3, containing 100 mM KCl and 3 mM NaN_3 in 90% H_2O/10% D_2O, and transferred to the 10-mm NMR sample tube (Shigemi). The final concentration of GroEL was approximately 0.07–0.1 mM (56–80 mg/ml calculated as a tetradecamer with a molecular mass of 800 kDa). Protein concentration was determined by Bradford assay (*17*).

All ^{13}C NMR spectra were recorded at 303 K using Bruker AMX400 spectrometer equipped with the probehead specialized for ^{13}C observation with 10-mm bore size. ^{13}C NMR spectra were recorded at 100 MHz with proton decoupling by WALTZ. A thirty degrees pulse was used for excitation of the ^{13}C Z-magnetization. The free induction decay (FID) was recorded with 32 K data points and spectral width of 24,000 Hz. Scans were repeated 30,000–100,000 times accordingly depending on the protein concentration with a recycle delay of 1.0 s. For sensitivity enhancement, the accumulated FID was manipulated with the exponential window function (20 Hz) before Fourier transformation. Chemical shift values are given in parts per million (ppm) calibrated with internal reference 1,4-dioxane at 67.8 ppm. Data processing, including spectral subtraction, was performed by XWIN-NMR (Bruker). In order to subtract the signal from natural isotope abundance properly, the spectrum of the unlabeled GroEL was subtracted from the labeled one so that the signal from the methyl and methylene carbons that appears at higher magnetic filed region (0 ppm to 70 ppm) would almost be completely cancelled out.

Resonance Assignment of GroES—Carbonyl resonances of His7 and Tyr71 were assigned by observing the ^{13}C NMR spectra of [1-^{13}C-His]- and [1-^{13}C-Tyr]-labeled GroES, respectively, since only one His and Tyr residues are contained in the GroES amino acid sequence. Resonance assignment of carbonyl signals of Thr residues (Thr19, Thr28, and Thr36) was established by double labeling method (*18, 19*). Briefly, [1-^{13}C-Thr, ^{15}N-Lys]-labeled GroES was prepared to assign Thr19 signal. Only the signal of Thr19 was observed as a doublet due to the scalar coupling between carbonyl ^{13}C of Thr19 and amide ^{15}N of Lys20. Likewise, the signal of Thr28 was assigned by acquiring the spectrum of [1-^{13}C-Thr, ^{15}N-Gly]-labeled GroES. Remaining signal on the spectrum of [1-^{13}C-Thr]-labeled was assumed as the signal of Thr36.

RESULTS

Observing the Signals of GroEL Using Amino Acid Type–Specific Labeling—Each subunit of the GroEL contains three domains; the apical, the intermediate, and the equatorial domains (*3*). The apical domain comprising the inlet rim of the cylindrical GroEL, provides a binding site for both nascent polypeptides and co-chaperonin GroES (*20*). The equatorial domain has a binding pocket for adenosine nucleotides (ATP or ADP). ATP-binding and hydrolysis are known to regulate the binding and release of GroES during the reaction cycle of GroEL (*21, 22*). The intermediate domain is thought to transmit the conformational rearrangement between the apical and equatorial domains.

His401, which is located in the intermediate domain, is the only one histidine out of 546 residues comprising the GroEL subunit (Fig. 1). To observe the resonance from His401, we prepared the GroEL in which the carbonyl carbon of histidine was selectively labeled with ^{13}C, and recorded its ^{13}C NMR spectrum (Fig. 2a). In order to cancel out the signals derived from the 1 % natural isotope abundance of the ^{13}C, the spectrum of the unlabeled GroEL (Fig. 2b) was subtracted from that of the [1-^{13}C-His]GroEL (see "MATERIALS AND METHODS" section for detail). As shown

Fig. 1. **Distribution of the residues used as a probe throughout the GroEL structure.** Single subunit structure of the unliganded GroEL (PDB code 1OEL) is displayed as Cα-trace. Carbonyl atoms of Tyr and His residues are indicated by green and cyan spheres, respectively.

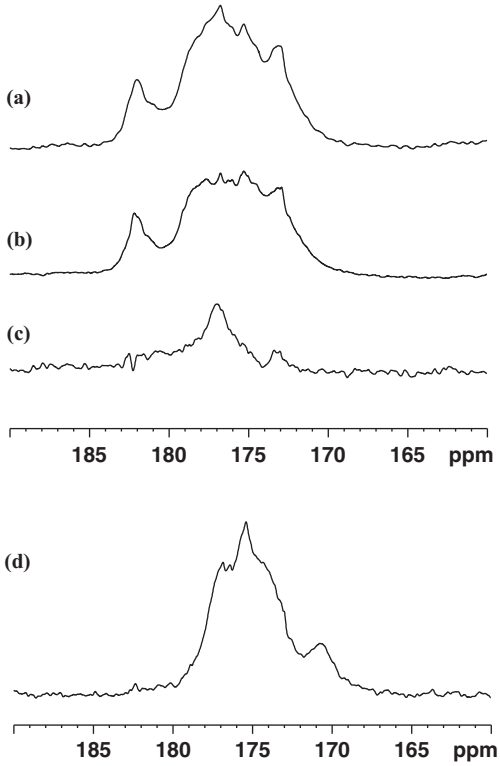

Fig. 2. **^{13}C NMR signal observation of 800 kDa GroEL with amino acid selective labeling.** ^{13}C NMR spectra of the GroEL homotetradecamer (a) labeled with [1-^{13}C]His and (b) without isotope labeling. Only the region corresponding to the carbonyl carbon is displayed. (c) Difference spectrum between (a) and (b). (d) ^{13}C NMR spectra of the [1-^{13}C-Tyr]-labeled wild-type GroEL. The spectrum of the unlabeled GroEL was already subtracted.

in Fig. 2c, a single peak arising at 177.0 ppm could be assigned unambiguously as the resonance from His401.

GroEL subunit contains 7 tyrosine resides in the apical (Tyr199, Tyr203, and Tyr360) and equatorial (Tyr476, Tyr478, Tyr485, and Tyr506) domains (Fig. 1). The ^{13}C NMR spectra measurements of the [1-^{13}C-Tyr]-labeled GroEL and subtraction of the unlabeled GroEL allowed us to observe the resonance originating from 7 tyrosine residues of GroEL (Fig. 2d). In this spectrum, one extremely broad signal can be separately recognized at 170.6 ppm with approximate half line width of 150 Hz. However, other signals are extensively overlapped in the region between 172 ppm and 178 ppm, indicating that amino acid selective labeling alone is insufficient to resolve the individual signals in the ^{13}C NMR spectrum of the 800 kDa GroEL.

Resonance Assignment Using Site-Directed Mutagenesis—In the previous ^{13}C NMR studies on IgG antibodies, amino acid selective labeling of carbonyl ^{13}C and amide ^{15}N was performed in order to make the resonance assignment by observing the 15-Hz splitting of the signal caused by scalar coupling between neighboring carbonyl ^{13}C and amide ^{15}N (*13, 14, 19*). However, this method seems to be unsuitable for the GroEL because the half line width of the GroEL signals exceed at least 150 Hz and extensive signal overlap is observed in the spectrum (Fig. 2d). Therefore, in this study, we utilized an alternative approach by making use of site-directed mutagenesis in order to establish residue-specific resonance assignments of GroEL. The point mutants of the GroEL were produced for each seven tyrosine residues (Y199E, Y203E, Y360F, Y476F, Y478F, Y485F, and Y506E). Structural integrity of some of these mutants was well addressed in the previous functional analysis (*20*). All mutants showed a comparable expression level with wild-type (80 mg/liter), and ^{13}C NMR spectrum was measured for each mutant labeled with [1-^{13}C]Tyr. For example, the GroEL mutant Y478F labeled with [1-^{13}C]Tyr contains the identical ^{13}C labeling as wild-type except for the carbonyl carbon corresponding to Tyr478, which is replaced by phenylalanine in the Y478F mutant. Subtraction of the spectrum of the [1-^{13}C-Tyr]-labeled Y478F (Fig. 3b) from that of GroEL (Fig. 3a) provides only the resonance derived from Tyr478 on the difference spectrum (Fig. 3c). Therefore the peak that appears at chemical shift value of 170.6 ppm can be assigned as the carbonyl ^{13}C resonance of Tyr478. Likewise, the signals originating from all other tyrosine residues were observed. As summarized in Fig. 4a, the assignments of Tyr203, Tyr360, Tyr485, and Tyr506 were clearly established since they yielded a virtually single peak in the corresponding difference spectra. However, assignments of Tyr199 and Tyr476 could not be obtained because multiple peaks appeared on the difference spectra of the Y199E and Y476F mutants (Fig. 4b).

Chemical Shift Perturbation Induced by ADP and GroES Binding—Five tyrosine residues and one histidine residue, whose chemical shifts has been unambiguously assigned as described above, are widely distributed throughout the GroEL structure; two residues in the apical domain (Tyr203, and Tyr360), one in the intermediate

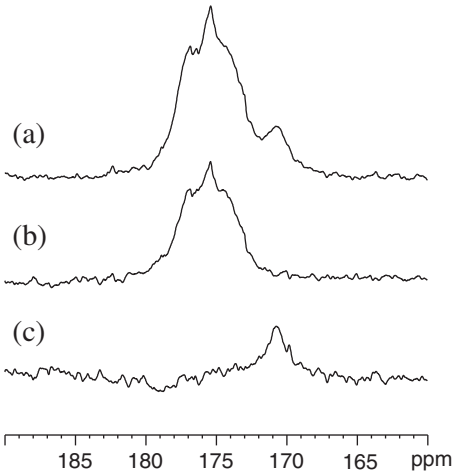

Fig. 3. **The NMR spectral assignment of GroEL by using site-directed mutagenesis.** ^{13}C NMR spectrum of (a) wild-type GroEL and (b) the GroEL mutant Y478F labeled with [1-^{13}C]Tyr. (c) Difference spectrum between (a) and (b).

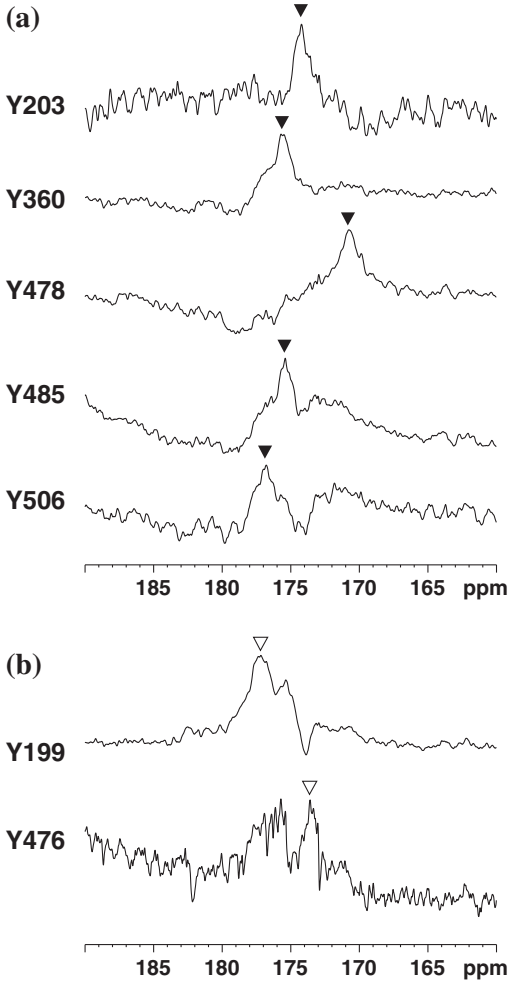

Fig. 4. **Summary of the resonance assignments of the seven tyrosine residues of GroEL.** (a) Difference spectra between [1-^{13}C-Tyr]-labeled wild-type GroEL and [1-^{13}C-Tyr]-labeled GroEL mutant containing the single residue substitution from Tyr to Phe or to Glu. ^{13}C NMR signal of Tyr203, Tyr360, Tyr478, Tyr485, and Tyr506 was unambiguously observed. Filled triangles indicate the peak top of the assigned signal. (b) Difference spectra of [1-^{13}C-Tyr]-labeled wild-type and [1-^{13}C-Tyr]-labeled GroEL mutants Y199E and Y476F. Open triangles indicate tentatively assigned signals.

residue (His401), and three in the equatorial domain (Tyr478, Tyr485, and Tyr506). Using these residues as probes, we investigated conformational changes induced upon nucleotide- or GroES-binding, representing the distinctive step of the reaction cycle of the GroEL.

The difference spectrum between [1-^{13}C-Tyr]-labeled wild-type and Y478F GroEL in the presence of excess ADP shows a single peak at the chemical shift of 171.8 ppm (Fig. 5b). Comparing with the spectrum obtained in the absence of ADP (Fig. 5a), more than 1 ppm difference was detected. According to the crystal structure of GroEL in complex with ATPγS, Tyr-478 is located in the vicinity of the aromatic moiety of the ATPγS (*6*). Thus, the chemical shift perturbation induced by ADP can be explained by the direct effect of the ring current due to the close proximity of the ADP molecule. It has been known that adenosine nucleotide binding and hydrolysis of GroEL is governed by allosteric regulations so-called positive intra-ring cooperativity and negative inter-ring cooperativity (*23*). In the presence of ADP, only one side of the ring is assumed to be occupied with ADP due to intra-ring cooperativity and the other side of the ring is empty because of the inter-ring cooperativity. The fact that only a single peak but not multiple peaks appear on the difference spectrum (Fig. 5b) suggests that association and dissociation of ADP to the GroEL is rapid in the NMR time scale such that the exchange peak was observed at the average of the unliganded and the ADP-bound states. We also recorded the ^{13}C NMR spectrum of the [1-^{13}C-His]-labeled GroEL in the presence of ADP, and found that chemical shift value of the His401 was slightly but significantly perturbed (Fig. 5d). By contrast, little chemical shift change was observed for the difference spectrum of [1-^{13}C-Tyr]Y203E (Fig. 5f).

Heptameric co-chaperonin GroES can form a stable 1:1 stoichiometric complex with GroEL in the presence of either ADP or ATP, forming the asymmetric "bullet" shaped 900 kDa complex involving drastic conformational rearrangements (*8*). We observed the difference spectrum of the [1-^{13}C-Tyr]-labeled wild-type and Y478F GroEL in the presence of 1 mM ADP and excess GroES relative to GroEL (Fig. 6b). Two peaks appeared in this spectrum are expected to correspond to the signals from the Tyr478 residues of the "*cis*" ring to which the GroES binds and from the Tyr478 residues of the "*trans*" ring of GroEL. Considering the fact that ADP-binding would cause the significant chemical shift perturbation for Tyr478 by its ring current effect, we assumed that the peak at 176.3 ppm could be assigned as the resonance of Tyr478 of the *cis* ring. Importantly, the other peak at 172.0 ppm presumably considered as the resonance from Tyr478 of the *trans* ring, also showed a significant chemical shift change.

NMR Signal from GroES in Complex with GroEL—The NMR signals of GroES complexed with GroEL or single-ring GroEL have been previously investigated (*11*). We also observed the ^{13}C NMR signals of GroES

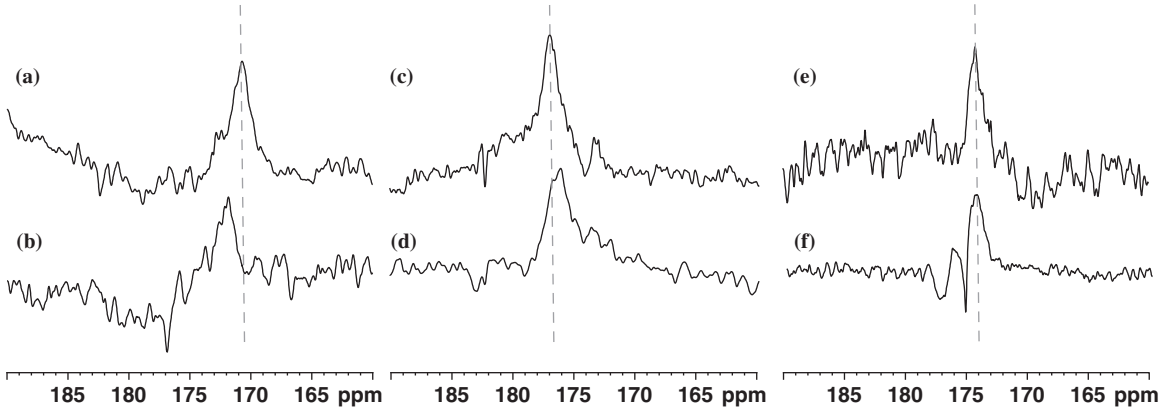

Fig. 5. **Chemical shift perturbation induced upon ADP binding.** Difference spectra between [1-^{13}C-Tyr]-labeled wild-type GroEL and Y478F in the (a) absence and (b) presence of 1 mM ADP. ^{13}C NMR spectra of the GroEL labeled with [1-^{13}C]His in the (c) absence and (d) presence of 1 mM ADP. The spectrum of unlabeled GroEL recoded in the same conditions was already subtracted. Difference spectra between [1-^{13}C-Tyr]-labeled wild-type GroEL and Y203E in the (e) absence and (f) presence of 1 mM ADP.

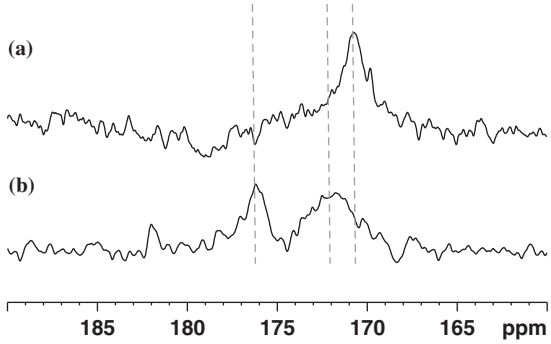

Fig. 6. **Chemical shift perturbation induced upon the GroEL-GroES complex formation.** Difference spectra between [1-^{13}C-Tyr]-labeled wild-type GroEL and Y478F in the (a) absence and (b) presence of 1 mM ADP and 1.2 equimolar GroES.

both in the free and GroEL-bound states in order to discuss the results obtained from the different NMR method. We first observed carbonyl ^{13}C signals of free-GroES, and established the assignment of those resonances as described in the "MATERIALS AND METHODS" section. The GroES segment (residues 16–32), responsible for the GroEL interaction, is called "mobile loop" since the electron density of this region could not be observed in the crystal structure of free GroES (*24*). As expected, the signals from the mobile-loop comprising residues Thr19 and Thr28 (Fig. 7a) were extremely sharp in the [1-^{13}C-Thr]-labeled GroES spectrum (Fig. 7b). By contrast, the line width of the signal from Thr36 that is positioned at out side of the mobile loop is much broader than those of Thr19 and Thr28 (Fig. 7b). We also observed the ^{13}C signals from His7 and Tyr71 (Fig. 7d). Although the His7 signal had a moderate line width, Tyr71 unexpectedly yielded a peak with narrow line width. These results indicate that GroES contains divergent local mobility throughout its structure.

Finally ^{13}C NMR spectra of GroES in the complex with GroEL were recorded and background signals were subtracted. Although the NMR signals from His7 and Tyr71 exhibited a line shape broadening due to complex formation with GroEL, no chemical shift difference was observed (Fig. 7e). On the other hand, Thr19 and Thr28 that form the mobile loop and Thr36 proximal to the mobile loop showed a large chemical shift change and the line shape broadening (Fig. 7c). These results are in accordance with the result of chemical shift mapping done by CRIPT-TROSY experiment for the GroES-SR1 complex (*25*), and is consistent with the fact that only mobile loop interacts with the GroEL apical domain in the crystal structure of the GroEL-ES-ADP$_7$ (*8*). It should be noted that the GroES residues away from the mobile loop have much smaller line width (70 Hz for His7 or 50 Hz for Tyr71) than unliganded GroEL (150 Hz for Tyr478 or 140 Hz for Tyr203). A similar phenomenon was also observed in the previous GroEL NMR study in which 89 out 94 signals of GroES are detectable in complex with GroEL whereas the only 20% signals were detectable from the [^{2}H,^{15}N]-labeled GroEL (*11*). This implies that the aberrant signal broadening of GroEL is not only because of its molecular weight but also because of the conformational exchange in the aqueous solution.

DISCUSSION

In this study, we performed ^{13}C NMR observation of the molecular chaperone GroEL. It was demonstrated that amino acid specific labeling was an effective technique for the 800 kDa GroEL, avoiding the spectral complexity caused by overlapping of multiple resonances. The use of the carbonyl ^{13}C is more advantageous than other nuclei for several reasons. First, the line width of the carbonyl carbon is much narrower than that of the α carbon due to the absence of a relaxation pathway through a directly attached hydrogen atom. Second, ^{13}C isotope of the carbonyl carbon position is considered to be less likely to be metabolically scrambled during protein biosynthesis. For the amide ^{15}N labeling, the incorporation of ^{15}N to the undesirable site would be more likely since the transfer of the amide group is the primary step of the amino acid metabolism *in vivo*. However, the susceptibility of cross-labeling strongly depends on the type of the amino acid. In fact, a small amount of the ^{13}C cross-labeling from threonine to glycine was observed in GroES labeled with [1-^{13}C]Thr (asterisks in Fig. 7b), but not for [1-^{13}C-Tyr]- or [1-^{13}C-His]-labeling of GroEL and GroES.

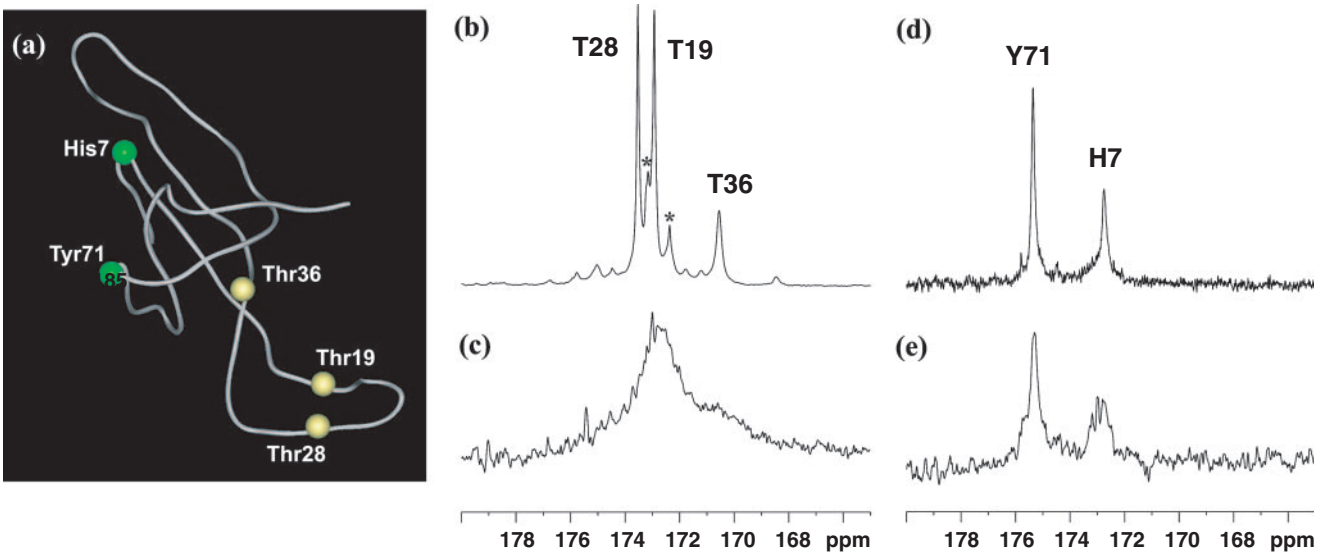

Fig. 7. **^{13}C NMR signal observation of the chaperonin GroES.** (a) Single subunit structure of the GroES taken from the GroEL-ES-ADP_7 complex coordinates (PDB code 1AON). The carbonyl atoms utilized as probes in this study are shown as spheres. ^{13}C NMR spectra of [1-^{13}C-Thr]-labeled GroES in the (b) absence and (c) presence of GroEL. ^{13}C NMR spectra of [1-^{13}C-Tyr, His]-labeled GroES in the (d) absence and (e) presence of GroEL. Two molar excess of GroEL and 1 mM ADP were added for complex formation. In (b), asterisk indicates the signal from Gly residues of GroES due to isotope scrambling.

In this study, the site-directed mutagenesis played a key role in the resonance assignments. NMR signal assignment of the specific residues of GroEL was successfully achieved by comparing spectra between wild-type and the point mutant for which the assignment was intended. To our knowledge, the 800 kDa GroEL is the largest protein that the sequence specific resonance assignment has been established by solution NMR spectroscopy. In principle, any residue can be assigned using the point mutant as described in this study. However, it is obligatory for this approach that the substitution of the side-chain does not affect the microscopic environment of the adjacent residues. In fact, we could not achieve unambiguous resonance assignments for Tyr199 and Tyr476 since multiple peaks were observed in the difference spectrum (Fig. 4b). In the difference spectrum of Y199E mutant, the chemical shift of a negative peak observed at 184ppm is almost identical with that of Tyr203, suggesting that the amino acid substitution at Tyr199 is likely to induce a change in the environment around Tyr203. Multiple peaks in the difference spectrum of Y476F also may suggest that Y476F mutation have caused significant perturbations for neighboring Tyr residues (Tyr478 and Tyr485).

The chemical shift perturbation experiment was performed using the difference spectrum between wild-type and the point mutant of GroEL in the presence and absence of ADP or ADP/GroES. It would be prerequisite that the function of the GroEL mutants is maintained as intact for residue specific observation of the ADP and GroES binding. We confirmed that the Tyr478 mutant has equal ADP binding activity ($K_{1/2}$ 10 μM for wild-type, 20 μM for Y478F) by fluorescent measurements using pyrenylated GroEL (data not shown). Y203E was reported to maintain the same ATP binding activity (*20*). In the presence of excess ADP, chemical shift perturbation was observed for Tyr478 in the equatorial domain and His401 in the intermediate domain, but not for Tyr203 in the apical domain. The chemical shift change of Tyr478 should be due to the direct effect of ADP-binding since Tyr478 is located in the vicinity of the adenine ring of ATPγS according to the crystal structure (*6*). In the previous study, the effect of nucleotide-binding was detected using Trp mutant of the Tyr485, which is located around the ATP binding pocket (*26*). We also found that the chemical shift of His401 was perturbed by addition of ADP despite of the fact that His401 resides far away from the ATP binding site. A cryoEM study of ATP-hydrolysis deficient GroEL (D398A) showed that ATP binding causes a 20° downward movement of the intermediate domain involving the disruption of the interaction with the apical domain of the neighboring subunit (*27*). The chemical shift perturbation of His401 observed in this study may reflect conformational rearrangement induced by nucleotide binding in the equatorial domain. Our NMR study of GroEL/ES complex in the presence of ADP observed the two distinct peaks of Tyr478, which would be derived from the *cis* and *trans* rings of GroEL, and those peaks exhibited significant chemical shift changes from the unliganded state. Recent cryoEM structures of GroEL(D398A)/ES complex in the presence of either ATP or ADP revealed that ATP hydrolysis causes significant conformational rearrangements of *trans* ring domains, which are transmitted through an inter-ring contact (*28*). Thus, chemical shift perturbation of Try478 *trans* ring may reflect those allosteric domain rearrangements described in the cryoEM structure. Our NMR study showed that the line width of the peak from Tyr476 of *trans* ring is broader than that from Tyr476 of *cis* ring or from Tyr476 in unliganded state. This may reflect an exchange broadening due to a local conformational flexibility around the Tyr476 residue in *trans* ring.

The observation of the resonance from GroES in complex with GroEL revealed that the mobile loop forming residues

are responsible for GroEL binding consistent with previous NMR study (*11*). By comparing the resonance line widths between the GroEL residues and GroES residues in complex with GroEL, it was revealed that the resonance from GroEL is much broader than that from GroEL-bound GroES despite of the similar molecular weight. The line width of ^{13}C signal from seven Tyr residues of GroEL all exceeded at least 140 Hz, while GroES resonances out side of the mobile loop exhibited narrower line width (70 Hz for His7) even when it forms a complex with GroEL. Thus it was suggested that the resonances of GroEL are broadened than expected from its molecular weight. This result is consistent with the NMR study of [^{2}H, ^{15}N]-labeled GroEL in which only 20% of the expected peaks were observed on the CRIPT-TROSY spectrum (*11*). A recent NMR study of the [^{2}H,^{15}N-Leu]-labeled GroEL cultured in the H_2O medium showed only 15 out of 41 potential crosspeaks, indicating that insufficient peaks on the GroEL spectrum were not caused by incomplete N-H/N-D exchange or spectral overlapping, and suggesting that the signals might be broadened by conformational averaging (*25*). Our study further demonstrated that the line width of six assigned GroEL resonances is broader than the GroES signals away from the mobile loop, and suggested that the existence of the intrinsic conformational fluctuation within the GroEL molecule. The recently reported crystal structure of wild-type GroEL indicates a significant degree of freedom in internal motion of each subunit (*5*). In addition, the higher B factors in the refined crystal structure of the unliganded GroEL mutant and the incomplete seven-axis symmetry of the ring structure (*3*), also imply the internal movements within the seven-membered ring of GroEL in the crystal lattice. Thus, the unusual broadening of GroEL resonances could be explained by conformational fluctuations of the GroEL molecule. This nature might be implicated not only in regulating cooperative nucleotide-binding to GroEL, but also in chaperone activities of GroEL because such fluctuations would be beneficial for effectively capturing the unfolded polypeptides and preventing the substrates proteins from aggregation.

In conclusion, this study showed that the ^{13}C NMR observation of the amino acid specific labeled protein in combination with site-directed mutagenesis enables us to establish the residue-specific assignments of the GroEL chaperonin with the size of 800 kDa. In addition, by using the NMR signals thus assigned, it was possible to detect the local conformational change upon the binding to ligands on the basis of the chemical shift perturbation data. This study demonstrated that the ^{13}C NMR method will continue to be utilized as an important tool for studying the biological macromolecules in parallel with the development of the cutting-edge NMR methodologies.

We thank Dr. Nathan Astrof for critical reading of this manuscript. This work was supported by Grant-in-Aid for Scientific Research on Priority Area (15032249).

REFERENCES

1. Fenton, W.A. and Horwich, A.L. (2003) Chaperonin-mediated protein folding: fate of substrate polypeptide. *Q. Rev. Biophys.* **36**, 229–256
2. Bukau, B. and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* **92**, 351–366
3. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D.C., Joachimiak, A., Horwich, A.L., and Sigler, P.B. (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* **371**, 578–586
4. Braig, K., Adams, P.D., and Brunger, A.T. (1995) Conformational variability in the refined structure of the chaperonin GroEL at 2.8 Å resolution. *Nat. Struct. Biol.* **2**, 1083–1094
5. Bartolucci, C., Lamba, D., Grazulis, S., Manakova, E., and Heumann, H. (2005) Crystal structure of wild-type chaperonin GroEL. *J. Mol. Biol.* **354**, 940–951
6. Boisvert, D.C., Wang, J., Otwinowski, Z., Horwich, A.L., and Sigler, P.B. (1996) The 2.4 Å crystal structure of the bacterial chaperonin GroEL complexed with ATP γ S. *Nat. Struct. Biol.* **3**, 170–177
7. Wang, J. and Boisvert, D.C. (2003) Structural basis for GroEL-assisted protein folding from the crystal structure of $(GroEL\text{-}KMgATP)_{14}$ at 2.0 Å resolution. *J. Mol. Biol.* **327**, 843–855
8. Xu, Z., Horwich, A.L., and Sigler, P.B. (1997) The crystal structure of the asymmetric GroEL-GroES-$(ADP)_7$ chaperonin complex. *Nature* **388**, 741–750
9. Pervushin, K., Riek, R., Wider, G., and Wüthrich, K. (1997) Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc. Natl. Acad. Sci. USA* **94**, 12366–12371
10. Riek, R., Pervushin, K., and Wüthrich, K. (2000) TROSY and CRINEPT: NMR with large molecular and supramolecular structures in solution. *Trends Biochem. Sci.* **25**, 462–468
11. Fiaux, J., Bertelsen, E.B., Horwich, A.L., and Wüthrich, K. (2002) NMR analysis of a 900K GroEL GroES complex. *Nature* **418**, 207–211
12. Riek, R., Wider, G., Pervushin, K., and Wüthrich, K. (1999) Polarization transfer by cross-correlated relaxation in solution NMR with very large molecules. *Proc. Natl. Acad. Sci. USA* **96**, 4918–4923
13. Kato, K., Sautès-Fridman, C., Yamada, W., Kobayashi, K., Uchiyama, S., Kim, H., Enokizono, J., Galinha, A., Kobayashi, Y., Fridman, W.H., Arata, Y., and Shimada, I. (2000) Structural basis of the interaction between IgG and Fcγ receptors. *J. Mol. Biol.* **295**, 213–224
14. Kato, K., Gouda, H., Takaha, W., Yoshino, A., Matsunaga, C., and Arata, Y. (1993) ^{13}C NMR study of the mode of interaction in solution of the B fragment of staphylococcal protein A and the Fc fragments of mouse immunoglobulin G. *FEBS Lett.* **328**, 49–54
15. Hochuli, M., Szyperski, T., and Wüthrich, K. (2000) Deuterium isotope effects on the central carbon metabolism of *Escherichia coli* cells grown on a D_2O-containing minimal medium. *J. Biomol. NMR* **17**, 33–42
16. Motojima, F., Makio, T., Aoki, K., Makino, Y., Kuwajima, K., and Yoshida, M. (2000) Hydrophilic residues at the apical domain of GroEL contribute to GroES binding but attenuate polypeptide binding. *Biochem. Biophys. Res. Commun.* **267**, 842–849
17. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
18. Kainosho, M. and Tsuji, T. (1982) Assignment of the three methionyl carbonyl carbon resonances in *Streptomyces* subtilisin inhibitor by a carbon-13 and nitrogen-15 double-labeling technique. A new strategy for structural studies of proteins in solution. *Biochemistry* **21**, 6273–6279
19. Kato, K., Matsunaga, C., Igarashi, T., Kim, H., Odaka, A., Shimada, I., and Arata, Y. (1991) Complete assignment of the methionyl carbonyl carbon resonances in switch variant

anti-dansyl antibodies labeled with [1-^{13}C]methionine. *Biochemistry* **30**, 270–278
20. Fenton, W.A., Kashi, Y., Furtak, K., and Horwich, A.L. (1994) Residues in chaperonin GroEL required for polypeptide binding and release. *Nature* **371**, 614–619
21. Rye, H.S., Burston, S.G., Fenton, W.A., Beechem, J.M., Xu, Z., Sigler, P.B., and Horwich, A.L. (1997) Distinct actions of cis and trans ATP within the double ring of the chaperonin GroEL. *Nature* **388**, 792–798
22. Rye, H.S., Roseman, A.M., Chen, S., Furtak, K., Fenton, W.A., Saibil, H.R., and Horwich, A.L. (1999) GroEL-GroES cycling: ATP and nonnative polypeptide direct alternation of folding-active rings. *Cell* **97**, 325–338
23. Gray, T.E. and Fersht, A.R. (1991) Cooperativity in ATP hydrolysis by GroEL is increased by GroES. *FEBS Lett.* **292**, 254–258
24. Hunt, J.F., Weaver, A.J., Landry, S.J., Gierasch, L., and Deisenhofer, J. (1996) The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature* **379**, 37–45
25. Fiaux, J., Bertelsen, E.B., Horwich, A.L., and Wüthrich, K. (2004) Uniform and residue-specific ^{15}N-labeling of proteins on a highly deuterated background. *J. Biomol. NMR* **29**, 289–297
26. Cliff, M.J., Kad, N.M., Hay, N., Lund, P.A., Webb, M.R., Burston, S.G., and Clarke, A.R. (1999) A kinetic analysis of the nucleotide-induced allosteric transitions of GroEL. *J. Mol. Biol.* **293**, 667–684
27. Ranson, N.A., Farr, G.W., Roseman, A.M., Gowen, B., Fenton, W.A., Horwich, A.L., and Saibil, H.R. (2001) ATP-bound states of GroEL captured by cryo-electron microscopy. *Cell* **107**, 869–879
28. Ranson, N.A., Clare, D.K., Farr, G.W., Houldershaw, D., Horwich, A.L., and Saibil, H.R. (2006) Allosteric signaling of ATP hydrolysis in GroEL-GroES complexes. *Nat. Struct. Mol. Biol.* **13**, 147–152