The Roles of Conserved Amino Acids on Substrate Binding and Conformational Integrity of ClpB N-Terminal Domain

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ABSTRACT: *Escherichia coli* heat shock protein ClpB disaggregates denatured protein in cooperation with the DnaK chaperone system. Several studies showed that the N-terminal domain is essential for the chaperone activity, but its role is still largely unknown. The N-terminal domain contains two structurally similar subdomains, and conserved amino acids Thr7 and Ser84 share the same position in two apparent sequence repeats. T7A and S84A substitutions affected chaperone activity of ClpB without significantly changing the native conformation [Liu, Z. et al. (2002) *J. Mol. Biol. 321*, 111−120]. In this study, we aimed to better understand the roles of several conserved amino acid residues, including Thr7 and Ser84, in the N-terminal domain. We investigated the effects of mutagenesis on substrate binding and conformational states of ClpB N-terminal domain fragment (ClpBN). Fluorescence polarization analysis showed that the T7A and S84A substitutions enhanced the interaction between ClpBN and protein aggregates. Interestingly, further analyses suggested that the mechanisms by which they do so are quite different. For T7A substitution, the increased substrate affinity could be due to a conformational change in the hydrophobic core as revealed by NMR spectroscopy. In contrast, for S84A, increased substrate binding would be explained by a unique conformational state of this mutant as revealed by pressure perturbation analysis. The thermal transition temperature of the S84A mutant, monitored by DSC, was 6.1 °C lower than that of wild-type. Our results revealed that conserved amino acids Thr7 and Ser84 both participated in maintaining the conformational integrity of the ClpB N-terminal domain.

Heat shock protein ClpB is a member of the HSP100 family of *Escherichia coli* heat shock proteins and works in cooperation with the DnaK molecular chaperone system to reactivate protein aggregates (*1*−*3*). The detailed structure of *E. coli* ClpB has been determined, and a hexameric (*4*) or heptameric (*5*) ring-shape structure has been proposed for the ClpB assembly. The ClpB sequence includes five domains; it has two ATP-binding domains between the N-terminal and the C-terminal domains. The two ATP-binding domains are separated by a linker domain that is important for chaperone activity (*6*, *7*). It has been suggested that the N-terminal domain (*8*, *9*), the C-terminal domain (*10*, *11*), and the linker domain (*6*, *7*) may be involved in substrate binding. The crystal structure of *Thermus thermophilus* ClpB showed that the linker domain is an 85 Å long, leucine-rich, coiled-coil peptide resembling a two-bladed propeller (*6*). It has been suggested that large protein aggregates bind between the linker domains of adjacent ClpB molecules and the ATP-induced conformational change of ClpB causes dissociation of the aggregates into smaller-size aggregates. Another study proposed that the mechanism of protein disaggregation mediated by ClpB involves ATP-dependent threading of substrate through the central channel in the oligomeric ring (*12*).

The *E. coli* ClpB N-terminal domain contains about 150 amino acid residues, and includes two repeated structural domains each of about 70 amino acid residues with a pseudo-two-fold symmetry (*13*) (Figure 1). Several studies showed that the N-terminal domain is essential for the chaperone activity and may be involved in substrate binding, but its role is still largely unknown and is a subject of intense discussion (*8*, *9*, *13*−*19*). Several amino acids that are important in this domain have been identified by site-directed mutagenesis. For example, the L93Q, L97Q, and L110Q substitutions, located in the hydrophobic groove of helices A5, A6, and A8, resulted in severe defects in its chaperone activity for luciferase (*13*). The same substitutions made on a fragment of the ClpB, the N-terminal domain (ClpBN),[1] resulted in a loss of affinity to denatured malate dehydrogenase (MDH), indicating that these residues are involved in substrate binding (*14*). The substitutions of conserved amino acids T7A, S84A, D103A, and E109A (Figure 1) in the N-terminal domain of ClpB affected chaperone activity for luciferase without changing its secondary structure, self-association, or ATPase activity (*15*). A study using the substrate trap variant of ClpB showed that the D103A/E109A substitutions reduced interaction to denatured glucose-6-phosphate dehydrogenase (G6PDH) (*16*).

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[1] Abbreviations: ClpBN, N-terminal domain of ClpB; G6PDH, glucose-6-phosphate dehydrogenase; MDH, malate dehydrogenase; NTCB, 2-nitro-5-thiocyanobenzoic acid; TNB, 2-nitro-5-thiobenzoic acid.

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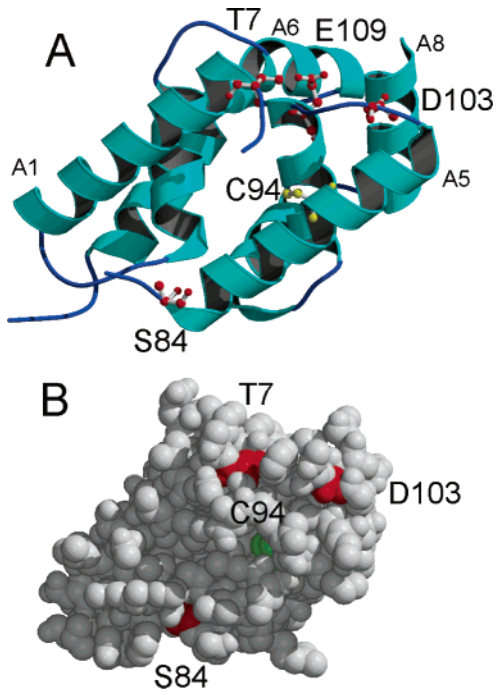

FIGURE 1: Schematic illustration of 3D structure of ClpBN. (PDB code 1KHY (*13*)) drawn with MOLSCRIPT (*33*) and Raster 3D (*34*). Thr7, Ser84, Asp103, and Glu109 are colored in red, and Cys94 is colored in yellow. (A) Ribbon model, the side chains of the amino acids are shown by the ball-and-stick model; (B) CPK model.

In two apparent sequence repeats within the N-terminal domain (Figure 1), the position of Thr7 in the first repeat (helices A1−A4) corresponds to the position of Ser84 in the second repeat (helices A5−A8). It is anticipated that these residues would have important roles in ClpB, though their exact roles are still unclear. In the present study, we aimed to better our understanding of the roles played by four conserved amino acid residues (T7, S84, D103, and E109). In previous studies, we have investigated the substrate recognition of *E. coli* molecular chaperones GroEL, DnaK, and ClpB using isolated fragments of their substrate binding domains. The fluorescence polarization measurement of these fragments enabled estimation of the binding constant between these domains and their substrate polypeptides (*14*, *20*, *21*). Physicochemical analysis for the conformational states of the DnaK fragment revealed detailed mechanism for substrate recognition (*22*). Applying these methods to ClpBN mutants, we investigated the role of four conserved amino acids in the ClpB N-terimnal domain. In addition, we also used a pressure perturbation approach (*23*−*30*) to study the effect of these substitutions on the conformational state of ClpBN. The reactivity of the unpaired Cys94 buried inside the protein (Figure 1B) is suppressed due to steric hindrance. When the applied pressure increases the level of conformational modulation, it will allow the reagent to react with the cysteine. We used this process to monitor the conformational state of ClpBN under high pressure.

MATERIALS AND METHODS

Proteins. Expression vectors for ClpBN were kindly provided by Prof. Zolkiewski (Kansas State University). A Quickchange site-directed mutagenesis kit (Strategene) was used to generate the desired substitutions in target genes (using a pair of complementary primers) and to amplify the full-length plasmid. ClpBN and its mutants were expressed in *E. coli* BL21(DE3)plys, purified by affinity chromatography on a Ni−NTA−agarose column, and the histidine tail was cleaved by thrombin digestion, as described previously (*9*). The cleaved protein was further purified by size-exclusion HPLC (Superdex 200, Pharmacia) in 50 mM Tris, pH 7.5, and 200 mM KCl. Four alanine mutants T7A, S84A, D103A, and E109A were constructed. We confirmed that these substitutions did not affect the native secondary structure of ClpBN by far CD spectroscopy. Firefly (*Photinus pyralis*) luciferase was purchased from Promega. Thermally denatured luciferase was obtained by incubating at 45 °C for 12 min in PBS buffer as described previously (*15*). BSA was omitted in our experiment, since enzymatic activity was not measured. G6PDH from *Lueconostoc mesenteroides* was purchased from Sigma. The preparation of denatured G6PDH was performed as previously described (*16*). A total of 300 μM G6PDH in denaturation buffer (5 M urea, 8% glycerol, and 20 mM DTT) was incubated at 47 °C for 5 min and diluted 10 times in the refolding buffer (50 mM triethanolamine/Cl, pH7.5, 20 mM $Mg(Ac)_2$, 30 mM KCl, 1 mM β-mercaptoethanol, and 1 mM EDTA). The sample was analyzed at 5 °C after a 1 min incubation at 47 °C and 2 min on ice.

Spectroscopy. The CD spectra were measured using a Jasco J-720 (Tokyo, Japan). Fluorescence anisotropy measurements were measured with an excitation wavelength of 490 nm and an emission wavelength of 520 nm using a Shimadzu RF-5000 (Kyoto, Japan). The ClpB N-terminal amino group was labeled with fluorescein isothiocyanate (FITC) for 4 h at pH 8.0, and excess FITC was removed using a PD10 column (Amersham Pharmacia). The final protein concentration was determined with BCA protein assay, and the concentration of FITC was determined by absorbance at 490 nm (68 000 M^{-1} cm^{-1}). The labeling ratio for fluorescent derivatives of ClpBN was confirmed to be 1.0. The fluorescence measurement in the presence of thermally denatured luciferase was performed at 25 °C, and that in the presence of denatured G6PDH was at 5 °C. Proton NMR spectra were measured using an ARX500 spectrometer at 47 °C. One-dimensional NMR spectra were recorded with a sweep width of 8064.5 Hz with 16 K data points, with 256 scans and an 11.1 μs acquisition pulse. NOESY spectra were acquired in the phase-sensitive mode using a mixing time of 150 ms, and 512 t_1 increments of 2 K data points were collected.

DSC. Calorimetric measurements were performed by Nano-DSC II model 6100 (Calorimetry Science Co., UT). All experiments were done at a scan rate of 2.0 °C/min and protein concentrations of 0.7−1.5 mg/mL. Data analyses including baseline subtraction, concentration normalization, and deconvolution were performed with software from Calorimetry Science.

High Pressure Apparatus. Light absorbances at elevated pressures were obtained using a high-pressure cell with two optical windows (Teramecs Co, Kyoto, Japan), together with a Shimadzu Multispec-1500 spectrometer (Kyoto, Japan). The reaction of 2-nitro-5-thiocyanobenzoic acid (NTCB) with Cys94 was monitored by the absorbance of 2-nitro-5-thiobenzoic acid (TNB). Solutions of 60 μM ClpBN with excess NTCB were incubated at a specified pressure at 37 °C

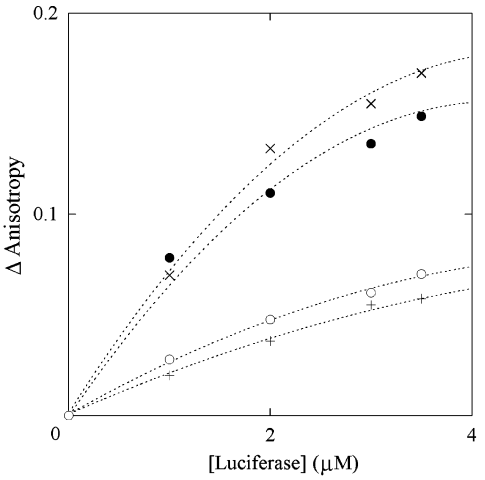

FIGURE 2: Effect of thermally denatured luciferase on fluorescence anisotropy of FITC−ClpBN. Different concentrations of thermally denatured luciferase were diluted 100-fold in refolding buffer. Wild-type ClpBN (○), the T7A mutant (●), the S84A mutant (×), and the D103A mutant (+). The measured increase from the initial anisotropy value (Δ Anisotoropy) was plotted as a function of the final luciferase concentration.

Table 1: Effect of the Single Substitutions on Substrate Binding of ClpBN Measured Using Fluorescence Anisotropy[a]

	Changes in substrate binding as compared to wild-type			
substrate	T7A	S84A	D103A	E109A
luciferase	++	++	−	+/−
G6PDH	+	+	+/−	−
MDH[b]	+/−	+/−	+/−	+/−

[a] +, substrate binding was increased; ++, substrate binding was significantly increased; −, substrate binding was decreased; +/−, change in substrate binding was within limit of the experimental error. [b] Results from ref *14*.

in the presence of 2 mM EDTA, and the time course of the reaction at elevated pressure was obtained by in situ measurements of the absorbance of TNB at 412 nm (ϵ_{412} = 13 600 M^{-1} cm^{-1}). Limited proteolysis under high pressure was performed as described previously (*25*).

RESULTS

The substrate binding of ClpBN and its mutants (T7A, S84A, D103A, and E109A) was studied using fluorescence anisotropy measurement. The anisotropy of ClpBN labeled with FITC (FITC−ClpBN) was measured in the presence of different concentrations of thermally denatured luciferase. As shown in the open circle plot (○) of Figure 2, the change in the measured anisotropy of FITC−ClpBN increased with increasing luciferase concentration, indicating the increased binding between luciferase and the wild-type ClpBN. The changes in anisotropy values of the T7A mutant (●) and the S84A mutant (×) were significantly higher than that of wild-type ClpBN, indicating that the substrate affinity of ClpBN was enhanced by these substitutions. The cross plot (+) of Figure 2 shows that the substrate affinity of ClpBN was a little decreased by the D103A substitution. In addition to using denatured luciferase, we have also performed similar experiments studying the interaction between ClpBN mutants and denatured G6PDH, and the results were summarized in Table 1. The interaction between ClpBN and G6PDH was decreased by the E109A substitution, while it was not affected much in the case of luciferase.

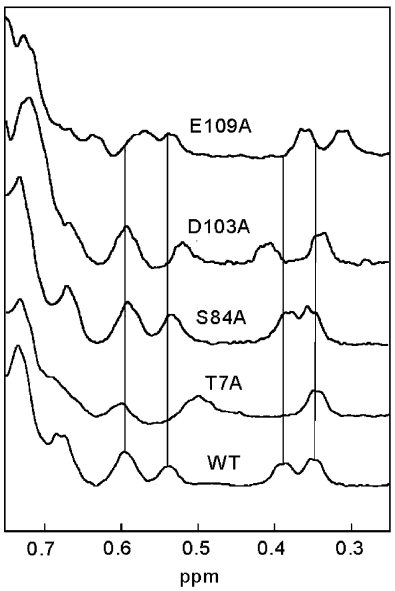

FIGURE 3: 1D-NMR spectra of ClpBN and its mutants. The spectra were taken in deuturated phosphate buffer at pH 7.5 and at 42 °C. All labile protons of ClpBN had been exchanged out. The measurements were performed at protein concentration of 0.7−1.0 mg/mL.

To gain more insight into the effect of these substitutions on the native conformation of the ClpBN, we measured the proton NMR spectra of the wild-type ClpBN and the T7A, S84A, D103A, and E109A mutants. The NMR spectra of the mutants were almost the same as that of the wild-type except the region from 0.3 to 0.7 ppm. In this region, the wild-type ClpBN possesses five NMR peaks at 0.35, 0.38, 0.54, 0.59, and 0.68 ppm, which are assigned to the alkyl groups shifted by the neighboring aromatic groups in the hydrophobic core. Therefore, the change in the conformation of the hydrophobic core of the mutants could be monitored through the chemical shift of these peaks. The five peaks observed in this region were shifted in the NMR spectrum of the T7A mutant: the peaks at 0.38, 0.54, and 0.68 ppm disappeared, while a new broad peak appeared at 0.5 ppm. This indicates that the T7A substitution induces conformational rearrangement in the hydrophobic core of ClpBN. This conformational change in the hydrophobic core may bring about positional rearrangements of other residues that are directly involved in substrate binding into positions more suitable for binding the substrate polypeptide. In addition, the NMR spectra of the D103A and E109A mutants shown in Figure 3 were slightly different from that of the wild-type, indicating these mutants might have induced subtle conformational changes in the hydrophobic core. On the other hand, the chemical shift differences of the shifted methyl signals were less than 0.02 ppm between S84A and wild-type, and those spectra were almost identical. This suggests that the increased substrate affinity of the S84A mutant could not be explained by a conformational change in the hydrophobic core.

To investigate the effect of the S84A substitution on the conformational state in detail, we monitored the thermal transition of ClpBN and its mutants by DSC measurements. As shown in Figure 4, the profile of the thermal unfolding of ClpBN is well-approximated by a two-state conformational transition with a midpoint temperature of 73.1 °C as

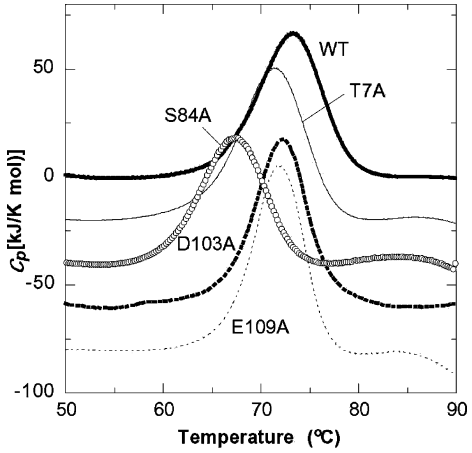

FIGURE 4: The temperature dependence of partial heat capacity of the wild-type ClpBN and its mutants. For illustrative purposes, the data sets have been offset below the wild-type ClpBN data set. The measurements were performed at protein concentration of 0.7−1.7 mg/mL in 10 mM Tris buffer at pH 7.5.

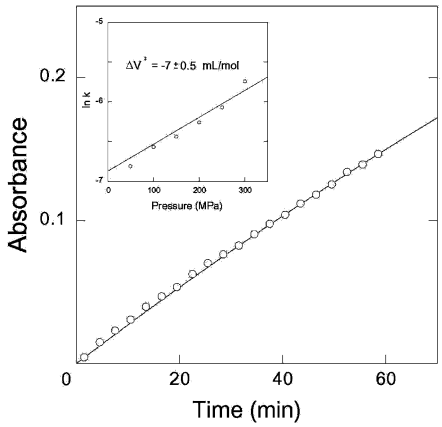

FIGURE 5: Time course of the reaction of Cys94 in the S84A mutant with NTCB at 300 MPa. Increased absorbance at 412 nm of TNB liberated by reaction of NTCB monitored by in situ measurement. The line through the data points was calculated from the best fit for the first-order kinetics. The inset shows the logarithmic plot of pressure dependence of the reaction rate constants estimated from the time course of the reaction. The $\Delta V^{\ddagger}$ value was calculated as -7 ± 0.5 mL/mol from the slope of the plot using the relationship, $\Delta V^{\ddagger} = -RT\mathrm{d} \ln k/\mathrm{d}P$.

previously reported (*9*, *14*). The same figure shows that the thermal transition temperatures of the four mutants were lower than that of wild-type ClpBN, indicating that the effect of these substitutions destabilized the native conformation. The destabilization of T7A, D103A, and E109A mutants may be explained by the conformational change monitored by NMR. While the decreases in the transition temperature of these three mutants were within 2 °C, the transition temperature of the S84A mutant was 6.1 °C lower than that of wild-type ClpBN. Nevertheless, the conformation of the S84A mutant monitored by NMR was indistinguishable from that of wild-type ClpBN. Therefore, the S84A substitution may induce loss of native interaction which is not monitored by NMR.

To further investigate the effect of these substitutions on the conformational state of ClpBN, we examined the pressure effect on the conformation of ClpBN. ClpBN possesses a single Cys94 buried inside the protein on the A5 helix (Figure 1B), and the reactivity of this residue is suppressed due to steric hindrance. When the applied pressure increases the level of conformational modulation, it will allow the reagent to react with this residue. We monitored the exposure of Cys94 through the reaction with the sulfhydryl-specific reagent NTCB measuring the absorbance of liberated TNB at 412 nm at various pressures. At atmospheric pressure, Cys94 of ClpBN was inactive to excess NTCB, indicating that this residue was not exposed to the solvent in the native conformation. The same results were obtained for the four ClpBN mutants, indicating that these substitutions did not induce conformational change that exposed Cys94. Next, we carried out measurements for ClpBN and its mutants in a high-pressure cell with two optical windows. Wild-type ClpBN was incubated with excess NTCB in the pressure range from 50 to 300 MPa, but the absorbance at 412 nm did not increase, indicating that Cys94 was not exposed to the solvent at this pressure range. The same results were obtained for the T7A, D103A, and E109A mutants. In contrast, the absorbance of TNB at 412 nm was increased when the S84A mutant was incubated with NTCB under pressure, indicating that Cys94 in the S84A mutants was exposed to the solvent at elevated pressure (Figure 5). Therefore, the conformation of the S84A mutant was sensitive to pressure perturbation, which might explain its increased polypeptide affinity.

The reaction kinetics obtained from the amount of liberated TNB could approximate to an exponential profile. We calculated the apparent rate constant (k) of the reaction between NTCB and Cys94 of the S84A mutant assuming pseudo-first-order reaction kinetics. The apparent reaction rate at elevated pressure was in the range of 0.001−0.0032 min^{-1}. The inset in Figure 5 shows the logarithmic plot of the pressure dependence of the rate constant. As shown in this figure, the rate constant gradually increased with an increase of pressure from 50 to 300 MPa. The rates of the reaction between NTCB and Cys94 of the S84A mutant were much lower than the rate of reaction between NTCB and the free cysteine (*31*), indicating that Cys94 was not completely exposed to the solvent at elevated pressure. Therefore, the enhanced reactivity was not due to global unfolding of the S84A mutant at elevated pressure. The plot of the inset in Figure 5 was approximately linear, and we calculated the apparent value of the activation volume ($\Delta V^{\ddagger}$) to be -7 ± 0.5 mL/mol using the relationship $\Delta V^{\ddagger} = -RT\mathrm{d} \ln k/\mathrm{d}P$.

We investigated the detailed mechanism of the pressure-induced increase of the reaction of Cys94 of ClpBS84A by using the local unfolding model and the penetration model, which are introduced for the transient exposure of buried residue of folded protein in H−D exchange process (*32*). In the local unfolding model, segments of secondary structure undergo local, reversible unfolding, involving the cooperative breaking of the hydrogen bonds in the segment, the disruption of the secondary structure, and movement of the segment into bulk solvent. To examine the local unfolding model for the reaction of the ClpBNS84A, we used limited proteolysis, which can monitor the dynamic difference of proteins under high pressure (*25*, *26*). The SDS−PAGE profile of the ClpBN treated with subtilisin for 1 h at 0.1 MPa showed a major band of approximate molecular weight of 15 kDa, in addition to the band of 16 kDa of the intact protein (data not shown). This indicates that the conformation of the local

region close to the N-terminus or the C-terminus is labile and susceptible to the proteolysis. The SDS−PAGE profile of the product treated with subtilisin at 300 MPa was the same as that at 0.1 MPa, and the same results were obatined for the four mutants. These results suggest that the pressure increase up to 300 MPa does not induce the local unfolding of ClpBNS84A. In addtion, the inset in Figure 5 shows that the rate constant between NTCB and Cys94 of ClpBNS84A increased with the same $\Delta V^{\ddagger}$ value from 50 to 300 MPa. If the high pressure induced unfolding of local region of Cys94, the transition curve would be observed in this plot of the pressure dependence of the rate constant. Therefore, the increased reactivity we observed in the S84A mutant was probably explained in part through the local unfolding model, and the reaction of Cys94 of ClpBNS84A under high pressure is explained well by a enhancement of fluctuation in the context of the penetration model.

DISCUSSION

We have investigated the role of several conserved amino acids thought to be important within the N-terminal domain of ClpB. Fluorescence polarization assay revealed that the D103A substitution decreased binding of luciferase and the E109A substitution decreased binding of G6PDH to an extent. On the other hand, a previous study showed the obvious decrease of substrate affinity between ClpB and G6PDH by the D103A/E109A substitution (*16*). This would be because the small reduction of the substrate affinity of each N-terminal domain is integrated in the higher oligomers of native ClpB. Interestingly, it has been reported that the binding of MDH was not affected by the D103A and E109A substitutions (*14*), suggesting that these negative charges were not essential for the binding of MDH. This interesting difference in the effect of the same substitutions on the binding of different substrates suggested that the amino acids involved in substrate binding in the ClpB N-terminal domain and their relative importance may also depend partly on the chemical properties of the substrates. In addition, this also illustrates the value of investigating changes in binding affinity using various substrates. The changes in substrate binding of the D103A and E109A mutants might also be related to subtle conformational changes in the hydrophobic core as revealed by the NMR results.

We also found that Thr7 and Ser84 have a role to play in substrate binding, as fluorescence polarization assay revealed that both the T7A and S84A substitutions increased substrate affinity with various substrates. Interestingly, further analysis suggested that the mechanisms by which they do so are quite different. For the T7A substitution, our NMR result revealed a conformational change in the hydrophobic core. The increased substrate affinity of the mutant could be due to the conformational change in the hydrophobic core, which brings about changes in other residues more directly involved in binding. On the other hand, for the S84A substitution, the NMR result did not show a conformational change in the hydrophobic core. Increased substrate binding of the S84A mutant would be explained by the unique conformational state of this mutant as revealed by pressure perturbation analysis.

The reaction kinetics of the Cys94 of the ClpBNS84A are pseudo-first-order, and the apparent reaction rate was in the range of 0.001−0.0032 min^{-1}. These values were much lower than that of the reaction between NTCB and the free cysteine (*31*). This is explained well by the fact that the reaction rate of Cys345 is limited by the slow process, which can be either local unfolding or fluctuation of the ClpBN. Since pressure-induced local unfolding was not detected by proteolysis, we interpreted the reaction of Cys94 of ClpBNS84A by the increase of fluctuation of protein in this study. In this case, the rate-limiting step of the reaction is the conformational fluctuation that exposes Cys94 to the solvent, and the $\Delta V^{\ddagger}$ value of -7 ± 0.5 mL/mol is regarded as the amplitude of the fluctuation around Cys94 of ClpBNS84A.

It has been suggested that high pressure has two opposing effects on the fluctuation of protein conformation. The dominant effect of the applied pressure is to decrease the fluctuation, but the fluctuation of several proteins is enhanced by an increase in pressure. This has been explained by the fact that the pressure rise induces a volume reduction of the protein solution by two opposing factors: a decrease in the size of the internal cavities and an increase in hydration (*29*, *30*). The pressure-induced reduction of the size of the cavities will suppress the fluctuation. On the other hand, the progressive hydrophobic hydration under high pressure will enlarge the fluctuation because the volume change of this process is negative. The observed values of protein fluctuations are regarded as arising from positive cavity expansion and negative hydration, and the negative $\Delta V^{\ddagger}$ value of the local Cys94 region of ClpBNS84A suggests a significant role of hydration in the fluctuation. The S84A mutant also had a lower transition temperature, which was 6.1 °C lower than that of wild-type. The lowered stability of the S84A mutant suggested that the S84A substitution may have reduced native contact inside that of the ClpBN molecule, which may increase the hydration when the conformational fluctuation was increased under high pressure.

In this study, we have revealed that conserved amino acids Thr7 and Ser84 both participated in maintaining the conformational integrity of the N-terminal domain. It has been reported that T7A substitution decreased chaperone activity, while S84A increased it (*15*). Hence, Thr7 may be conserved in the ClpB family because the conformational change induced by the substitution of this residue affected chaperone activity of ClpB. On the other hand, though the substitution of Ser84 seems to be harmless as it enhances chaperone activity, Ser84 may be conserved because of the reduction in the thermal transition temperature by 6.1 °C which may be harmful for the chaperone activity in vivo, especially under stress.

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