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Research Article

Elucidation of the relationship between enzyme activity and internal motion using a lysozyme stabilized by cavity-filling mutations

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Abstract. We investigated the activity and the internal motions of a stabilized mutant hen lysozyme (HEL) in which the residues M12 and L56 were mutated to L and F, respectively (LF mutant HEL). The result of the activity measurements against glycol chitin at various temperatures suggested that the temperature dependence of the activity of LF mutant HEL shifted to the high-temperature side compared with that of wild-type HEL. The detailed internal motions of LF mutant HEL in the absence and presence of a substrate analogue, $(NAG)_3$, were examined by model-free analysis at 35 °C. The results showed that the internal motions of LF mutant HEL in the presence of $(NAG)_3$ were drastically restricted compared with those in wild-type HEL. Our findings thus suggested that the mutation to the stabilized lysozyme restricted internal motions required for the enzymatic reaction.

Key words. Lysozyme; internal motion; nuclear magnetic resonance; ^{15}N relaxation; enzyme activity; cavity filling; thermal stability.

In general, proteins have various motions covering several time scale and amplitude ranges. A number of biochemical studies have observed the conformational dynamics of the active sites of enzymes during enzymatic catalyzations [1–9]. From the results of these studies proposals have come that the internal motions of proteins are important for enzyme activity, but the requirement for such motion undermines the conformational rigidity crucial protein for stabilization. Thermophilic enzymes tend to have a higher optimal temperature than mesophilic enzymes, but show less activity at lower temperature [10–14]. The reduced activity of thermophilic enzymes at lower temperature led us to the hypothesis that suitable flexibility might be required for enzyme function. The internal motions of diverse proteins have been compared using various experimental procedures, including hydrogen-deuterium (H/D) exchange [15–17], nuclear magnetic resonance (NMR) spectroscopy [18, 19] and molecular dynamics (MD) simulations [20, 21]. However, because there have been few reports on the relationship between the dynamics and the biological activity of enzymes, the contribution of the internal motions to the functions of proteins remains unclear.

Hen egg-white lysozyme (HEL) is a carbohydrate hydrolase that catalyzes the hydrolysis of the beta-1,4 glycosidic bonds of polysaccharides, such as a homopolymer of N-acetyl-D-glucosamine (chitinase activity) and an alternate co-polymer of N-acetyl-D-glucosamine and N-acetylmuramic acid, which is the major constituent of

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bacterial cell walls (biological activity) [22]. It was the first enzyme whose three-dimensional (3D) structure was elucidated using X-ray crystallography [23] and for which a detailed mechanism of action was proposed [22]. Accordingly, this enzyme has been one of the most vigorously investigated for clarification of the structure-function relationship of proteins [24–30].

Del-R14H15 HEL, in which the residues Arg14 and His15, both of which are located far from the active-site cleft, were deleted simultaneously, had higher activity against glycol chitin at low temperature, although the dissociation constant of the Del-R14H15 HEL-$(NAG)_3$ complex was similar to that of the wild-type HEL-$(NAG)_3$ complex. However, the activity of Del-R14H15 HEL at high temperature was less than that of wild-type HEL, and the temperature dependence of the activity of Del-R14H15 HEL shifted to the low-temperature side compared with that of the wild-type enzyme. Moreover, the melting temperature of Del-R14H15 HEL was lower 7 °C than that of wild-type HEL [31]. On the other hand, ^{15}N NMR relaxation analysis of Del-R14H15 HEL and wild-type HEL showed that the internal motions in several residues of Del-R14H15 HEL in the presence of $(NAG)_3$ increased more than those of wild-type HEL in the presence of $(NAG)_3$ [32]. These results suggested that variations in molecular dynamics play an important role in the function of the enzyme.

HEL has a cavity surrounded by a hydrophobic core, which consists of side chains of residues L8, M12, L17, W28, I55, L56, I88 and V92 in the alpha domain [22]. We previously achieved the stabilization of HEL through the cavity-filling mutations M12 and L56, which face each other across the cavity in the hydrophobic core without unfavorable van der Waals contacts [33]. However, the activity of the mutant HEL at 40 °C was lower than that of wild-type HEL. We hypothesized that the restriction of internal motion by the cavity-filling mutation decreased enzyme activity at low temperature. If this hypothesis is correct, the temperature dependence of the mutant HEL activity would shift to the high-temperature side and the internal motions of the mutant would be restricted. This would be of particular interest since there have been no reports of a difference in the internal motions in an enzyme causing an increase or decrease in enzymatic activity. Therefore, in this report, we examined the temperature dependence of lysozyme activity and the internal motions in the cavity-filling mutant lysozyme by means of NMR relaxation analysis.

Materials and methods

Sample preparation

LF mutant HEL where M12 and L56 are L and P, respectively was prepared according to the method of our previous work [34]. For expression of ^{15}N-labeled protein, the *Pichia pastoris* GS115 strain (Invitrogen) was transformed with the appropriate plasmid, and grown in FM22-glycerol medium which contains [^{15}N]ammonium sulfate (99% ^{15}N; Shoko Tsusho, Tokyo, Japan) as the sole nitrogen source. After incubation for 2 days at 30 °C, the cells were collected by centrifugation, suspended in the same medium containing 0.5% (v/v) methanol instead of glycerol and cultivated for 120 h with additional supplies of methanol. The protein secreted in the culture supernatant was isolated by cation-exchange chromatography on a column (4.0 × 15 cm) of CM-Toyopearl 650 M, which was eluted with a gradient of 500 ml 0.05 M phosphate buffer and 500 ml of the same buffer containing 0.5 M NaCl (pH 7) at 4 °C. The eluted lysozyme was dialyzed against distilled water and then lyophilized. Finally, we obtained about 10 mg of ^{15}N-labeled LF mutant HEL from 1 l of culture.

Activities of lysozymes

Activities of lysozymes against glycol chitin were measured in 0.1 M acetate buffer (pH 5.5) at various temperatures as described previously [31].

NMR measurements

The NMR sample was prepared to contain 1 mM protein in 90% H_2O/10% ^2H_2O (v/v), and the pH was adjusted to 3.8. For preparation of the lysozyme complex, $(NAG)_3$ was added to the protein solution to yield a slight excess of substrate relative to enzyme. Under this condition, more than 99% complex was formed.

NMR experiments were performed at 35 °C on a Varian Inova 600-MHz spectrometer equipped with a triple-resonance, pulse-field gradient probe with an actively shielded z gradient and a gradient amplifier unit. Sequential assignment of main-chain ^1H and ^{15}N resonances of LF mutant HEL were made on the basis of HSQC, ^{15}N-edited NOESY-HSQC [35], and ^{15}N-edited TOCSY-HSQC [36] spectra. Processing and analysis of NMR data and peak-picking were performed on the NMRPipe/NMRDraw package [37].

The ^{15}N longitudinal relaxation rate (R1), transverse relaxation rate (R2), and $\{^1H\}$–^{15}N heteronuclear nuclear Overhauser enhancement (NOE) were recorded for LF mutant HEL in the absence or presence of $(NAG)_3$. Pulse sequences for the measurement of the ^1H-^{15}N NOE values, and the R1 and R2 relaxation times are described elsewhere [38, 39]. The R1 values were measured using spectra recorded with seven different delay times: T = 32.94, 92.94, 172.94, 332.94, 652.94, 1292.94 and 1812.94 ms. The R2 values were measured using spectra recorded with seven different delay times: T = 4, 42, 82, 122, 202, 282 and 342 ms. The R1 and R2 values were obtained by fitting the intensities of the peak heights in each spectrum to a single exponential curve using a non-linear

least-squares fitting program, CURVEFIT, available from the web site of Palmer's group (http://cpmcnet.columbia.edu./dept/gsas/biochem/labs/palmer/software.html). Errors in R1 and R2 were estimated by Monte Carlo analysis using CURVEFIT. ^{1}H-^{15}N steady-state NOE values were obtained by recording spectra with and without ^{1}H saturation for 3.0 s, and by calculating the ratios of the intensities of the peak heights. The errors in NOE were estimated using the root-mean-square value of the background noise [40].

Relaxation data analysis

The overall rotational correlation times for backbone NH bond vectors of LF mutant HEL in the absence or presence of $(NAG)_3$ were calculated from the R2/R1 ratios using a program, R2R1_TM, available from the web site of Palmer's group (http://cpmcnet.columbia.edu./dept/gsas/biochem/labs/palmer/software.html). The values of S^2, τ_e, Rex and Sf^2 were calculated with a program, Modelfree4.0 [41], available from the same site, by applying the isotropic diffusion model with the obtained overall rotational correlation time.

Results

Temperature dependencies of lysozyme activity

We previously obtained two thermostabilized mutant HELs in which M12 and L56 were mutated to L and F, respectively (LF mutant HEL) or to F and F, respectively (FF mutant HEL) [34]. The activity of LF mutant HEL was lower than that of FF mutant HEL at 40 °C. Therefore, we examined the temperature dependence of the activity in LF mutant HEL. The activity of LF mutant HEL against glycol chitin was measured in 0.1 M acetate buffer (pH 5.5) at various temperatures. As shown in figure 1, wild-type HEL had optimum activity at 70 °C, and the activity decreased gradually above this temperature due to the denaturation. On the other hand, the activity of LF mutant HEL was less than that of wild-type HEL at low temperature and was higher than that of wild-type HEL at high temperature. Thus, the temperature dependence of LF mutant HEL activity was shifted to the high-temperature side compared with that of wild-type HEL.

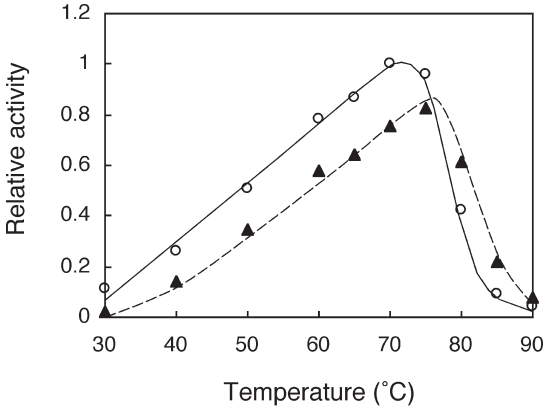

Figure 1. Temperature dependencies of activities in wild-type HEL (open circles) and LF mutant HEL (closed triangles). Activities were determined at pH 5.5 using glycol chitin as a substrate.

^{1}H-^{15}N chemical shift assignments

The backbone amide ^{1}H and ^{15}N resonances of the LF mutant in the absence or in the presence of $(NAG)_3$ were assigned with ^{15}N-edited NOESY-HSQC and ^{15}N-edited TOCSY-HSQC spectra on the basis of the published assignments for wild-type HEL [42]. There are 125 amino acid residues in LF mutant HEL, excluding the N-terminal amino acid residue and three Pro residues. The resonances of LF mutant HEL in the absence or presence of $(NAG)_3$ were observed for 110 or 83 out of 125 main-chain amide groups, respectively. The chemical shifts of resonances of LF mutant HEL in the presence of $(NAG)_3$ were broadened more than those in the absence of $(NAG)_3$. These residues were located in the active-site regions of loop A-B (Y20, N37), strand 2 (S50, D52, Y53), turn 2 (I55, F56, Q57), strand 3 (I58, N59, S60, R61), the long loop (R61, W62, W63, R73, L75, C76), 3_{10}(i) (L83), helix C (S86, S91, N93, I98), loop C-D (S100, D101, G102, N103, G104, M105, A106, A107) and helix D (W108, V109, A110, W111). The finding that the signals were broadened by the substantial conformational exchange for a large subset of residues upon binding of the substrate analogue is consistent with our previous report [33].

Relaxation analyses of lysozyme in the absence or presence of substrate analogue

We measured the relaxation parameters of LF mutant HEL in the absence or presence of $(NAG)_3$ according to our previous report [33]. These parameters were obtained by fitting the peak heights of a series of spectra recorded with different delay times, T, to single exponential curves, as described in Materials and methods. The R1, R2 and ^{1}H-^{15}N NOE values obtained are shown in figure 2. The R2/R1 values of LF mutant HEL were very similar to those of wild-type HEL. With regard to the motional anisotropy of wild-type HEL in the absence or presence of $(NAG)_3$, it was suggested that the isotropic motion observed in our previous report was valid [33]. Therefore, we adopted the isotropic diffusion models for the LF mutant HEL in the absence or presence of $(NAG)_3$ in order to perform the model-free analysis. For the isotropic motion, the overall rotational correlation time (τ_m) was calculated from R2/R1 values of residues. The calculated τ_m values were 5.010 ns and 5.166 ns for LF mutant HEL in the absence and presence of $(NAG)_3$, respectively. By applying these values, R1, R2 and NOE were fit with five models consisting of the following subsets of the ex-

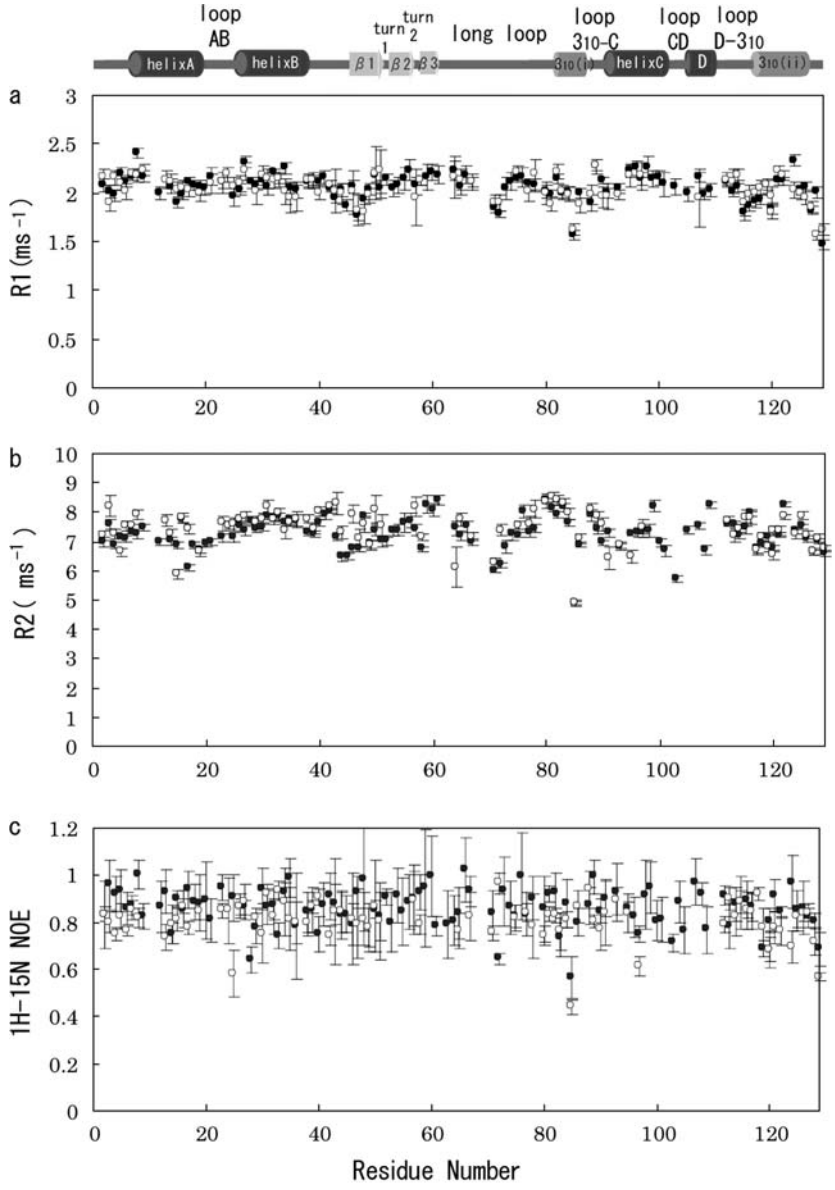

Figure 2. ^{15}N R1 (*a*), R2 (*b*) and ^{1}H-^{15}N NOE (c) values of LF mutant HEL in the absence (closed circles) or presence (open circles) of $(NAG)_3$ at pH 3.8 and 35 °C.

tended model-free parameters: (i) S^2; (ii) S^2 and τ_e; (iii) S^2 and Rex; (iv) S^2, τ_e and Rex and (v) Sf^2, Ss^2 and τ_e; where S^2 is the square of the generalized order parameter, τ_e is the effective correlation time, Rex is the chemical exchange term and Sf^2 is the square of the order parameter for the internal motion on the fast time scale. The order parameter and Rex for LF mutant HEL are shown in figure 3. For most residues, the order parameters in LF mutant HEL in the absence of $(NAG)_3$ were in the range 0.8–1.0, indicating that the motions on a fast time scale were largely restricted. The residues for which there was an increase of the order parameters in the presence of $(NAG)_3$ were located in loop A-B (G16 and L17), turn 1 (N46 and T47), the long loop (S72) and loop D-3_{10} (C115 and K116). On the other hand, in the residues that were located in the long loop (C64) and C terminus (R128), the order parameters decreased through the binding of $(NAG)_3$ (fig. 3). Rex values were observed for 19 residues for LF mutant HEL in the absence of $(NAG)_3$. Eleven Rex values observed for the free enzyme disappeared in the complex (fig. 3). These residues were located in loop A-B, the N terminal of the long loop and loop D-3_{10}.

Discussion

Usually, the limb of the high-temperature side in the curve for the temperature dependence of activity reflects

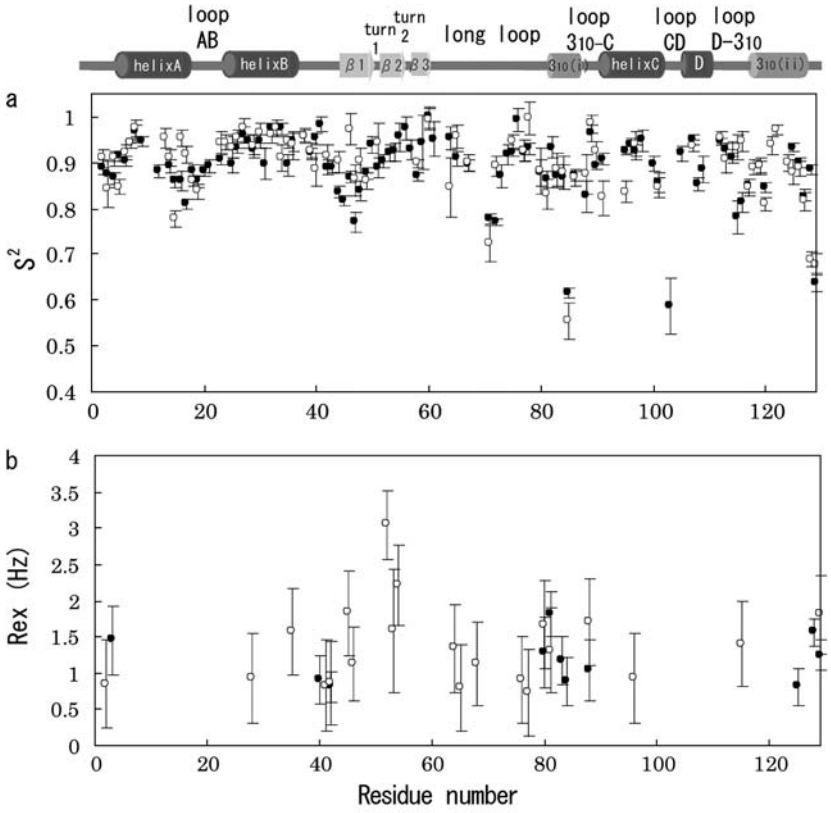

Figure 3. Order parameters and Rex values of LF mutant HEL at pH 3.8 and 35 °C. (*a*) Order parameters of LF mutant HEL in the absence (closed circles) or presence (open circles) of $(NAG)_3$. (*b*) Rex values of LF mutant HEL in the absence (closed circles) or presence (open circles) of $(NAG)_3$.

the denaturation of the protein. On the other hand, the rise in activity at the limb of the low-temperature side may reflect the attainment of the fluctuation required for the enzyme to elicit its function [32]. In this paper, we found that the temperature dependence of the activity of LF mutant HEL shifted to the high-temperature side compared with that of wild-type HEL. The characterization of the crystal structure of LF mutant HEL showed that its overall and local structures were very similar to those of wild-type HEL [34]. Since the position of the cavity is far from the active-site clefts in the lysozyme (fig. 4), the decrease in activity for LF mutant HEL did not depend on an increase in the dissociation constant [34]. Therefore, the decrease of activity at the low-temperature side in LF mutant HEL must be attributable to long-range effects, presumably changes in fluctuations. Thus, we examined the internal motions of LF mutant HEL in the absence or presence of a substrate analogue by model-free analysis. Model-free analysis, a widely accepted method for evaluating the internal motions of a molecule, combines a generalized-order parameter, such as a measure of the spatial restriction of the ^{15}N-^{1}H bond vectors (S^2), which contributes to small amplitude motions on the pico- to nanosecond time scale, and the chemical exchange in line width (Rex), which is a microenvironment change on the micro- to millisecond time scale. An increase in the order parameter (S^2) values and the disappearance of the chemical exchange in line width (Rex) values imply the restriction of internal motions of a protein. We measured the relaxation parameters of LF mutant HEL in the absence and presence of a substrate analogue, $(NAG)_3$ (fig. 2), and used these parameters to carry out the model-free analyses (fig. 3).

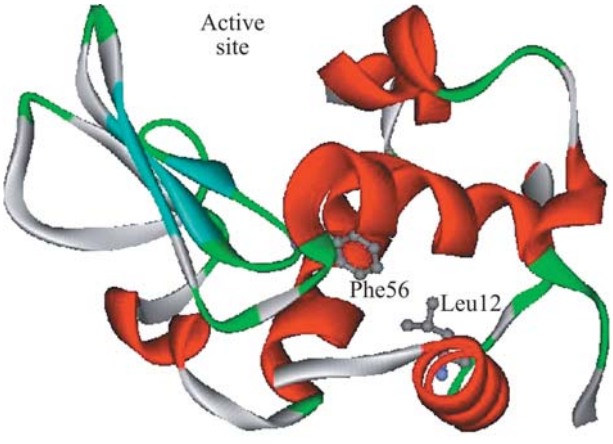

Figure 4. Structure of LF mutant HEL (1IOR). Leu 12 and Phe 56 are indicated.

To examine the effect of the cavity-filling mutations of the lysozyme in the absence of a substrate analogue, the model-free parameter of LF mutant HEL (closed circles) was compared with that of wild-type HEL (open circles) (fig. 5). As shown in figure 6, the order parameters in the LF mutant HEL did not change drastically from the delta of the order parameters, but increased slightly over the entire region. Moreover, several residues in the long loop region and turn 2 of LF mutant HEL lost Rex values compared with those of wild-type HEL (fig. 5). This suggested that the internal motions of the lysozyme were slightly restricted by the cavity-filling mutation in the absence of a substrate analogue.

In the enzyme-substrate complex, a previous study suggested that the decreases in the values of the order parameters contributed to the compensation for the entropy penalty caused by the immobilization of residues upon substrate or ligand binding [43]. The order parameter value of many residues in wild-type HEL decreased more in the presence of the substrate analogue than in its absence. This phenomenon suggested that the motion to compensate the entropy penalty was very critical to the ability of wild-type HEL to demonstrate activity. Moreover, based on the comparison between LF mutant HEL (closed circles) and wild-type HEL (open circles), the values of the order parameters of LF mutant HEL in the presence of $(NAG)_3$ were generally higher than those of wild-type HEL at 35 °C. In particular, a drastic increase in the value of the order parameters was observed in turn 1 (N46), the long loop (N65, N77) and loop D-3_{10} (C115, D119) (figs. 7, 8). This suggested that the internal motions on the pico- to microsecond time scale, motions which play a major role in compensating the entropic penalty, were restricted in the complexed LF mutant HEL.

On the other hand, the observed Rex terms in LF mutant HEL and wild-type HEL in the presence of $(NAG)_3$ are shown in figure 7. The behavior of Rex in the presence of the substrate is considered to express the motion on the catalytic action, because many enzymatic reactions occur on a micro- to milliseconds time scale [44]. The number of residues with Rex values in LF mutant HEL was drastically decreased compared with that in wild-type HEL. These residues where Rex disappeared were V2, E35, N46, C64, N65, N77 and C115, excluding residues which could not be analyzed due to the decreases in intensity by the binding of $(NAG)_3$. In particular, E35 and N46 are located at the substrate-binding cleft. We therefore suggest that the cavity-filling mutation in the hydrophobic core of

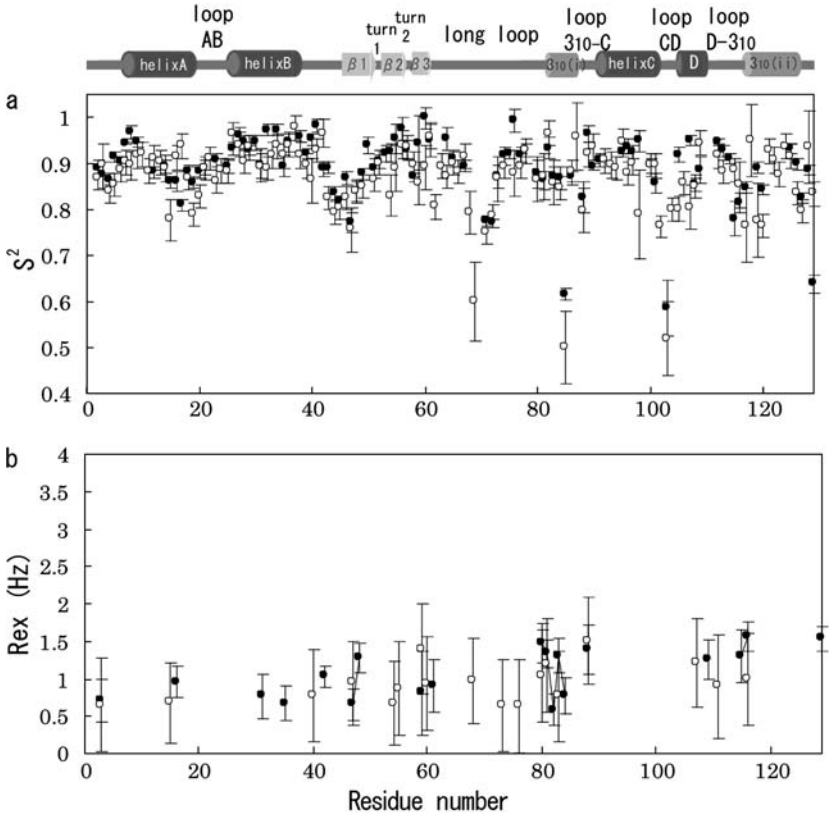

Figure 5. Order parameters and Rex values of LF mutant HEL and wild-type HEL in the absence of $(NAG)_3$ at pH 3.8 and 35 °C. The order parameters and Rex values of wild-type HEL in the absence of $(NAG)_3$ are cited from Mine et al. [32]. (*a*) Order parameters of LF mutant HEL (closed circles) and wild-type HEL (open circles). *(b)* Rex values of LF mutant HEL (closed circles) and wild-type HEL (open circles).

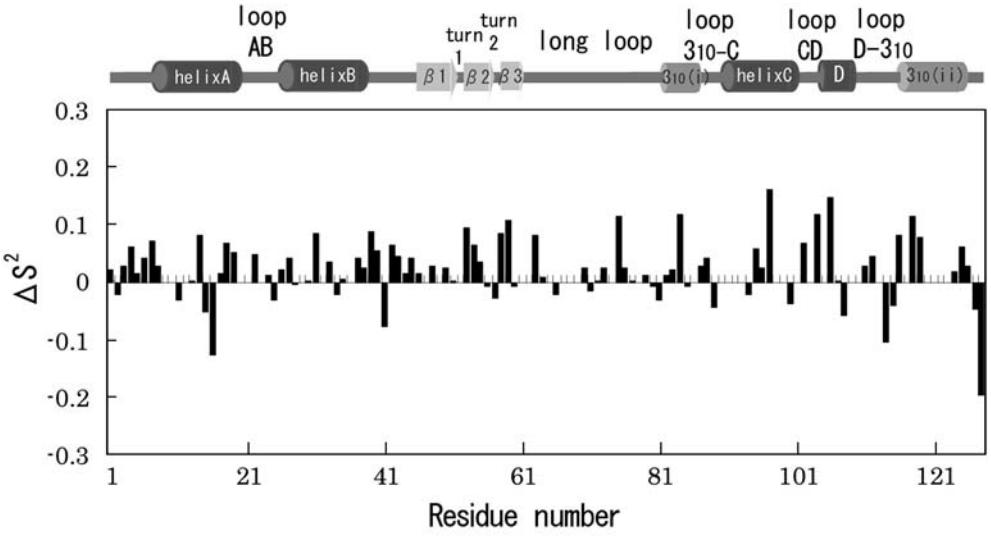

Figure 6. Plots of the differences in order parameters between LF mutant HEL and wild-type HEL in the absence of $(NAG)_3$ as a function of residue number. ΔS^2 indicates the difference order parameter that is obtained by subtracting the order parameter of wild-type HEL from that of LF mutant HEL.

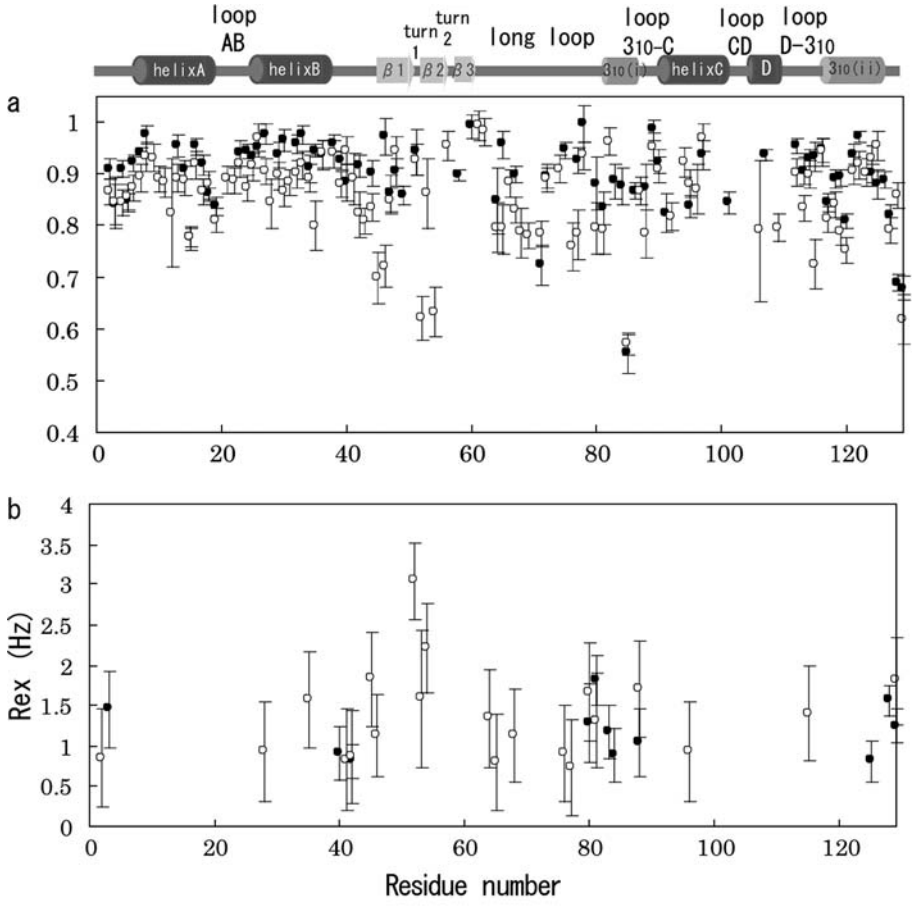

Figure 7. Order parameters and Rex values of LF mutant HEL and wild-type HEL in the presence of $(NAG)_3$ at pH 3.8 and 35 °C. The order parameters and Rex values of the wild-type HEL in the presence of $(NAG)_3$ are cited from Mine et al. [32]. (*a*) Order parameters of LF mutant HEL in the presence of $(NAG)_3$ (closed circles) and of wild-type HEL in the presence of $(NAG)_3$ (open circles). (*b*) Rex values of LF mutant HEL in the presence of $(NAG)_3$ (closed circles) and of wild-type HEL in the presence of $(NAG)_3$ (open circles).

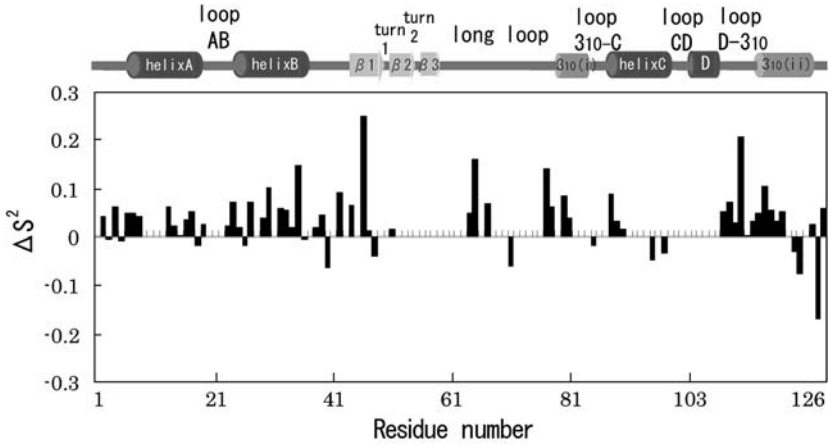

Figure 8. Plots of the differences in order parameters between LF mutant HEL and wild-type HEL in the presence of $(NAG)_3$ as a function of residue number. ΔS^2 indicates the difference order parameter that is obtained by subtracting the order parameter of wild-type HEL in the presence of $(NAG)_3$ from that of LF mutant HEL in the presence of $(NAG)_3$.

the lysozyme caused the restriction of internal motions required for enzymatic reaction at 35 °C, resulting in a decrease in lysozyme activity. The restrictions in the internal motions in LF mutant HEL were consistent with the result that LF mutant HEL showed a shift of optimum activity to the high-temperature side. Enzymes from thermophilic bacteria display little activity at room temperature. Zavodszky et al. [13] confirmed that the conformational fluctuations necessary for catalytic function were restricted at room temperature in the enzymes from a thermophilic bacterium because of the increased conformational rigidity required to stabilize the protein against heat denaturation. Moreover, this group suggested that there was a relationship between the thermal stabilities and the conformational flexibilities of 3-isopropylmalate dehydrogenases from the psychotropic bacteria *Vibrio* sp. I5, mesophilic *Escherichia coli* and *Thermus thermophilus* [18]. These results were obtained by H/D exchange. In another study, a comparison of the detailed internal motion between the enzyme from *T. thermophilus* and that from *E. coli* was made using ^{15}N nuclear magnetic spin relaxation for ribonuclease HI in the absence of substrate [20]. Although the internal motions on the picosecond to nanosecond time scale of ribonuclease HI from *T. thermophilus* were similar to those from *E. coli*, the Rex values in ribonuclease HI from *T. thermophilus* were higher than those from *E. coli* [20].

In the present study, as well as in two previous studies [32, 33], we examined the internal motions of an enzyme during both a shift to the high-temperature side and a shift to the low-temperature side for the temperature dependence of enzyme activity, and demonstrated that the elevation of activity in protein function at the low-temperature limb is related to the ability of the protein to elicit its function, and that this is intimately related to the internal motions of the protein. These findings should help to improve our understanding of the biological functions displayed by proteins at various temperatures.

We can conclude the present study as follows. We showed that the stabilization by the cavity filling in the hydrophobic core of HEL induced a shift in the temperature dependence of activity to the high-temperature side. Moreover, the model-free analysis at 35 °C, suggested that the internal motions of LF mutant HEL in the presence of $(NAG)_3$ were restricted more than those of wild-type HEL. Based on these findings and our previous results [32, 33], we conclude that the moderate internal motions are very important for the manifestation of enzymatic activity.

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1 Falzone C. J., Wright P. E. and Benkovic S. J. (1994) Dynamics of a flexible loop in dihydrofolate reductase from *Escherichia coli* and its implication for catalysis. Biochemistry **33:** 439–442
2 Epstein D. M., Benkovic S. J. and Wright P. E. (1995) Dynamics of the dihydrofolate reductase-folate complex: catalytic sites and regions known to undergo conformational change exhibit diverse dynamical features. Biochemistry **34:** 11037–11048
3 Cameron C. E. and Benkovic S. J. (1997) Evidence for a functional role of the dynamics of glycine-121 of *Escherichia coli* dihydrofolate reductase obtained from kinetic analysis of a site-directed mutant. Biochemistry **36:** 15792–15800
4 Kohen A., Cannio R., Bartolucci S. and Klinman J. P. (1999) Enzyme dynamics and hydrogen tunnelling in a thermophilic alcohol dehydrogenase. Nature **399:** 496–499
5 Osborne M. J., Schnell, J., Benkovic S. J., Dyson H. J. and Wright P. E. (2001) Backbone dynamics in dihydrofolate reductase complexes: role of loop flexibility in the catalytic mechanism. Biochemistry **40:** 9846–9859
6 Bosco D. A., Eisenmesser E. Z., Pochapsky S., Sundquist W. I. and Kern D. (2002) Catalysis of cis/trans isomerization in native HIV-1 capsid by human cyclophilin A. Proc. Natl. Acad. Sci. USA **99:** 5247–5252

7 Cole R. and Loria J. P. (2002) Evidence for flexibility in the function of ribonuclease A. Biochemistry **41:** 6072–6081
8 Eisenmesser E. Z., Bosco D. A., Akke M. and Kern, D. (2002) Enzyme dynamics during catalysis. Science **295:** 1520–1523
9 Rajagopalan P. T. R., and Benkovic S. J. (2002) Preorganization and protein dynamics in enzyme catalysis. Chem. Rec. **2:** 24–36
10 Jaenicke R. and Bohm G. (1998) The stability of proteins in extreme environments. Curr. Opin. Struct. Biol. **8:** 738–748
11 Kanaya S. and Itaya M. (1992). Expression, purification, and characterization of a recombinant ribonucleaseH from *Thermus thermophilus* HB8. J. Biol. Chem. **267:** 10184–10192
12 Wrba A., Schweiger A., Schultes V., Jaenicke R. and Zavodsky P. (1990) Extremely thermostable D-glyceraldehyde-3-phosphate dehydrogenase from the eubacterium *Thermotoga maritima*. Biochemistry **29:** 7584–7592
13 Zavodszky P., Kardos J., Svingor A. and Petsko G. A. (1998) Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. Proc. Natl Acad. Sci. USA **95:** 7406–7411
14 D'Amico S., Marx J. C., Gerday C. and Feller G. (2003) Activity-stability relationships in extremophilicenzymes. J. Biol. Chem. **278:** 7891–7896
15 Wagner G. and Wuthrich K. (1979) Correlation between the amide proton exchange rates and the denaturation temperatures in globular proteins related to the basic pancreatic trypsin inhibitor. J. Mol. Biol. **130:** 31–37
16 Wuthrich K., Wagner G., Richarz R. and Braun W. (1980) Correlations between internal mobility and stability of globular proteins. Biophys. J. **32:** 549–560
17 Svingor A., Kardos J., Hajdu I., Nemeth A. and Zavodszky P. (2001) A better enzyme to cope with cold: comparative flexibility studies on psychrotrophic, mesophilic, and thermophilic IPMDHs. J. Biol. Chem. **276:** 28121–28125
18 Hernandez G., Jenney F. E. Jr, Adams M. W. and LeMaster D. M. (2000) Millisecond time scale conformational flexibility in a hyperthermophile protein at ambient temperature. Proc. Natl Acad. Sci. USA **97:** 3166–3170
19 Butterwick J. A., Patrick Loria J., Astrof N. S., Kroenke C. D., Cole R., Rance M. et al. (2004) Multiple time scale backbone dynamics of homologous thermophilic and mesophilic ribonuclease HI enzymes. J. Mol. Biol. **339:** 855–871
20 Colombo G. and Merz K. M. (1999) Stability and activity of mesophilic subtilisin E and its thermophilic homologue: insights from molecular dynamics simulations. J. Am. Chem. Soc. **121:** 6895–6903
21 Grottesi A., Ceruso M., Colosimo A. and Di Nola A. (2002) Molecular dynamics study of a hyperthermophilic and a mesophilic rubredoxin. Proteins Struct. Funct. Genet. **46:** 287–294
22 Imoto T., Johnson L. N., North A. C. T., Philip D. C. and Rupley J. (1972) Vertebrate lysozyme. In: The Enzymes, vol. 7, 3rd edn, pp. 666–868, Boyer, P. D. (ed.) Academic Press, New York
23 Blake C. C. F., Johnson L. N., Mair G. A., North A. C. T., Philips D. C. and Sarma V. R. (1967) Crystallographic studies of the activity of hen egg-white. Proc. R. Soc. B. **167:** 378–388
24 Vocadlo D. J., Davies G. J., Laine R. and Withers S. G. (2001) Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. Nature **412:** 835–838
25 Goodnow C. C., Crosbie J., Jorgensen H., Brink R. A. and Basten A. (1989) Induction of self-tolerance in mature peripheral B lymphocytes. Nature **342:** 385–391
26 Sadegh-Nasseri S. and Germain R. N. (1991) A role for peptide in determining MHC class II structure. Nature **353:** 167–170
27 Germain R. N. and Rinker A. G. Jr (2000) Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes surface expression of occupied molecules. Nature **363:** 725–728
28 Matsumura M, Signor G. and Matthews B. W. (1989) Substantial increase of protein stability by multiple disulphide bonds. Nature **342:** 291–293
29 Radford S. E., Dobson C. M. and Evans P. A. (1992) The folding of hen lysozyme involves partially structured intermediates and multiple pathways. Nature **358:** 302–307
30 Klein-Seetharaman J., Oikawa M., Grimshaw S. B., Wirmer J., Ueda T., Imoto T. et al. (2002) Long-range interactions within a nonnative protein. Science **295:** 1719–1722
31 Imoto T., Ueda T., Tamura T., Isakari Y., Abe Y., Inoue M. et al. (1994) Lysozyme requires fluctuation of the active site for the manifestation of activity. Protein Eng. **7:** 743–748
32 Mine S., Tate S., Ueda T., Kainosho M. and Imoto T. (1999) Analysis of the relationship between enzyme activity and its internal motion using nuclear magnetic resonance. J. Mol. Biol. **286:** 1547–1565
33 Ohmura T., Ueda T., Ootsuka K., Saito M. and Imoto T. (2000). Stabilization of hen egg white lysozyme by a cavity-filling mutation. Protein Sci. **10:** 313–320
34 Mine S., Ueda T., Hashimoto Y., Tanaka Y. and Imoto T. (1999). High-level expression of uniformly 15N-labeled hen lysozyme in *Pichia pastoris* and identification of the site in hen lysozyme where phosphate ion binds using NMR measurements. FEBS Lett. **448:** 33–37
35 Fesik S. and Zuiderweg E. R. P. (1988) Heteronuclear three-dimensional NMR spectroscopy: a strategy for the simplification of homonuclear two-dimensional NMR spectra. J. Magn. Reson. **78:** 588–593
36 Marion D., Driscoll P. C., Kay L. E., Wingfield P. T., Bax A., Gronenborn A. M. et al. (1989) Overcoming the overlap problem in the assignment of 1H NMR spectra of larger proteins by use of three-dimensional heteronuclear ^{1}H-^{15}N Hartmann-Hahn-multiple quantum coherence and nuclear Overhauser-multiple quantum coherence spectroscopy: application to Interleukin 1b. Biochemistry **28:** 6150–6156
37 Delaglio F., Grzesiek S., Vuister G. W., Zhu G., Pfeifer J. and Bax A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR **6:** 277-293
38 Kay L. E., Torchia D. A. and Bax A. (1989) Backbone dynamics of proteins studied by ^{15}N inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease. Biochemistry **28:** 8972–8979
39 Kordel J., Skelton N. J., Akke M., Palmer A. G. and Chazin W. J. (1992) Backbone dynamics of calcium-loaded calbindin D9k studied by two-dimensional proton-detected ^{15}N NMR spectroscopy. Biochemistry **31:** 4856–4866
40 Farrow N. A., Muhandiram R., Singer A. U., Pascal S. M., Kay C. M. and Gish G. et al. (1994) Backbone dynamics of a free and phosphopeptide complexed Src homology 2 domain studied by 15N NMR relaxation. Biochemistry **33:** 5984–6003
41 Mandel A. M., Akke M., and Palmer A. G. (1995) Backbone dynamics of *Escherichia coli* ribonuclease HI: correlations with structure and function in an active enzyme. J. Mol. Biol. **246:** 144–163
42 Buck, M., Boyd, J., Redfield, C., Mackenzie, D. A., Jeenes, D. J., Archer, D. B. et. al. (1995) Structural determinants of protein dynamics: analysis of ^{15}N NMR relaxation measurements for main-chain and side-chain nuclei of hen egg white lysozyme. Biochemistry **34:** 4041–4055
43 Stivers J. T., Abeygunawardana C. and Mildvan A. S. (1996) ^{15}N NMR relaxation studies of free and inhibitor-bound 4-oxalocrotonate tautomerase: backbone dynamics and entropy changes of an enzyme upon inhibitor binding. Biochemistry **35:** 16036–16047
44 Fersht A. (1999) Structure and mechanism in protein science. In: *A Guide to Enzyme Catalysis and Protein Folding*, vol. 1, pp. 44–51, Freeman. New York