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Crystal Structures of K33 Mutant Hen Lysozymes with Enhanced Activities

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Using random mutagenesis, we previously obtained K33N mutant lysozyme that showed a large lytic halo on the plate coating *Micrococcus luteus*. In order to examine the effects of mutation of K33N on enzyme activity, we prepared K33N and K33A mutant lysozymes from yeast. It was found that the activities of both the mutant lysozymes were higher than those of the wild-type lysozyme based on the results of the activity measurements against *M. luteus* (lytic activity) and glycol chitin. Moreover, 3D structures of K33N and K33A mutant lysozyme were solved by X-ray crystallographic analyses. The side chain of K33 in the wild-type lysozyme hydrogen bonded with N37 involved in the substrate-binding region, and the orientation of the side chain of N37 in K33 mutant lysozymes were different in the wild-type lysozyme. These results suggest that the enhancement of activity in K33N mutant lysozyme was due to an alteration in the orientation of the side chain of N37. On the other hand, K33N lysozyme was less stable than the wild-type lysozyme. Lysozyme may sacrifice its enzyme activity to acquire the conformational stability at position 33.

Key words: enzyme activity, hen lysozyme, mutation analysis, stability, X-ray crystallographic structure.

Abbreviations: lysozyme, hen egg-white lysozyme; NAG, *N*-acetyl-D-glucosamine; K_A, association constant.

Enzyme activity has been found to be related to conformational changes, as shown in the 'lock-and-key' or 'induced-fit' hypotheses, suggesting that the conformational change-related interactions between enzyme and substrate play a role in enzyme catalysis (*1*). Additionally, it has been proposed that the internal motions of proteins are important for enzyme activity, but that the requirement of such motion undermines the conformational rigidity crucial for protein stabilization. Thermophilic enzymes tend to have higher optimal temperatures for activities than mesophilic enzymes, but show lower activities at lower temperature (*2–6*). The reduced activity of thermophilic enzymes at lower temperature led us to hypothesis that suitable flexibility might be required for enzyme function. We have previously demonstrated that the increase in internal motion required for enzymatic reaction cause an increase in activity, resulting in the destabilization of the protein (*7*, *8*). In contrast, we have also demonstrated that the intramolecular stabilization by the restriction of internal motions required for enzymatic reaction cause a decrease in activity, resulting in protein stabilization (*9*, *10*). On the other hand, enhancement of enzyme activity is one of the major challenges in protein engineering. Random mutagenesis has been utilized to enhance an enzyme activity (*11–14*). Moreover, the detailed information regarding the 3D structure of the mutant enzyme in which activity is enhanced by amino acid mutation has provided important insight into the structure–function relationship.

The X-ray crystallographic structure of hen egg-white lysozyme has been identified and thus provides information regarding the structure–function relationship of a protein (*15*). Lysozyme is carbohydrate hydrolase that catalyse the hydrolysis of β-1,4 glycosidic bonds of polysaccharides such as a homopolymer of *N*-acetyl-glucosamine (chitin) or an alternate copolymer of *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid, which are the major constituents of the *Micrococcus luteus* cell wall (*16*). Two carboxyl groups (Glu35 and Asp52) have been identified as catalytic groups (*17*, *18*). Many efforts in the elucidation of its structure (*19–22*) and function (*19*, *23–25*) have been carried out using various mutants. On the other hand, there have been few reports of X-ray crystallographic analyses of mutant lysozymes with enhanced activity.

Using random mutagenesis, we previously obtained mutant lysozymes that showed a large lytic halo on the plate coating *M. luteus* (*26*). One of these clones was identified as the K33N mutant lysozyme gene (*27*). However, the characteristics of K33N mutant lysozyme are still unclear. In this study, we performed X-ray crystallographic analysis of K33N mutant lysozyme to investigate the mechanism of activity enhancement.

MATERIALS AND METHODS

Materials—Re-crystallized (five times) hen egg-white lysozyme was donated by the QP Company (Tokyo). *Micrococcus luteus*, a substrate of lysozyme,

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was purchased from Sigma. Glycol chitin was prepared according to the literature (*28*).

Preparation of Mutant Lysozyme—K33N lysozyme was produced by yeast *Saccharomyces cerevisiae* AH22 as described in the literature (*29*). The purification of K33N lysozyme was carried out according to the previous report (*29*). Site-directed mutagenesis for K33A lysozyme was performed by PCR according to previous methods (*8*). K33A lysozyme was expressed in *Pichia pastoris* GS115 cells harbouring the recombinant plasmid constructed using a pPIC9 vector (Invitrogen), as described previously (*8*). Purification of K33A lysozyme was carried out according to the previously described method (*8*).

Lytic Activity of Lysozyme—The lytic activities of lysozymes against *M. luteus* were measured turbidometrically at 450 nm at pH 7.0 and 30°C. To a 2 ml suspension of *M. luteus* in 0.05 M phosphate buffer at pH 7.0 was added 50 µl of a lysozyme solution, and the turbidity decrease was monitored at 450 nm with a JASCO V-530 spectrophotometer with a thermostatically controlled cell holder.

GC Activity of Lysozyme—Activities of lysozymes against glycol chitin were measured in 0.1 M acetate buffer at pH 5.5 and 40°C according to the literature (*28*) with slight modification. Namely, glycol chitin in 0.1 M acetate buffer at pH 5.5 (2 mg/ml) was pre-incubated at 40°C for 10 min in a stoppered test tube with gentle shaking. To the solution was added 0.1 ml of lysozyme at several concentrations in the same buffer. The sample was mixed briefly with a vortex mixer and incubated at 40°C for 30 min. After the reaction, 2 ml of potassium ferricyanide in 0.5 M sodium carbonate (0.5 g/ml) was added and mixed briefly, and the mixture was incubated at 100°C for 10 min to complete the oxidation of reducing sugar with ferricyanide. A control solution was obtained by the same procedure except that the lysozyme solution was added after the addition of the ferricyanide solution. After cooling, the decrease in absorbance at 420 nm of the mixture compared with the control solution was determined.

Binding to the Substrate of Lysozyme—Binding constants of lysozyme to $(NAG)_4$ was measured by the fluorescence method according to the literature (*29*) with slight modification. The fluorescence spectra of lysozyme–$(NAG)_4$ complex was measured in 0.1 M acetate buffer at pH 5.5 at 37°C, using a Hitachi F-2000 fluorescence spectrophotometer.

X-ray Analysis—Crystallization was carried out using a hanging drops vapour diffusion technique by mixing 1 µl of protein solution (protein concentration at 40 mg/ml, 0.1 M sodium acetate buffer at pH 4.6) with 1 µl of reservoir solution (K33N; 0.1 M sodium acetate buffer at pH 4.6 containing 0.9 M NaCl, K33A; 0.1 M sodium acetate buffer at pH 4.6 containing 0.2 M NaCl and 20% MPD) at 20°C. The crystals were isomorphous with that of the wild-type lysozyme: they belonged to the tetragonal system, space group $P4_32_12$. X-ray diffraction data were collected using a Bruker SMART 6000 CCD detector. The statistics of data collection and processing are shown in Table 1. Refinement of the structure was carried out using the program CNS (*30*). A previously reported wild-type lysozyme structure (PDB, 1LZA) was used as the initial model. Manual fitting of the model to electron density maps was carried out with the program O (*31*).

Table 1. **Data and refinement statistics.**

Lysozyme	K33N	K33A
Crystallographic data		
Space group	$P4_32_12$	$P4_32_12$
Cell dimension (Å)		
$a = b$	78.624	78.787
c	36.814	37.082
Resolution (Å)	1.65	1.65
Completeness (%)	99.0	100.0
R_{merge} (%)[a]	6.6	5.3
Refinement		
R_{work} (%)[b]	20.7	21.8
R_{free} (%)[c]	23.8	24.9
ΔBond length (Å)	0.016	0.011
ΔBond angles ()	1.8	1.6

[a] $R_{merge} = \Sigma_h[m/(m-1)]^{1/2}\Sigma_j|<I>_h - I_{h,j}|/\Sigma_h\Sigma_j I_{h,j}$, where $<I>_h$ is the mean intensity of symmetry-equivalent reflections and m is redundancy. [b] $R_{work} = \Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$, where F_{obs} and F_{calc} are observed and calculated structure factor amplitudes. [c] R_{free} was calculated for R_{work}, using random 10% subset from all reflections.

Differential Scanning Calorimetry Measurements of K33N Lysozyme—Differential scanning calorimetry (DSC) measurements and data analyses were carried out using a MicroCal VP-DSC system equipped with a Gateway personal computer as described in the literature (*32*). The scan rate was 1.0 K/min. The sample solutions were prepared by dissolution in 0.05 M glycine buffer (pH 2.4–3.2) and the protein concentrations was 20 µM. Data analysis was performed using Origin software (MicroCal, Northampton, MA, USA).

RESULTS AND DISCUSSION

Activities of K33 Mutant Lysozymes—In order to examine the effects of mutation of K33 to N for activity, we prepared K33N and K33A mutant lysozymes from yeast. The activities of the purified wild-type lysozyme and K33 mutant lysozymes against *M. luteus* (lytic activity) and glycol chitin (GC activity) were measured (Table 2). K33N mutant lysozyme showed a large lytic halo in the screening analysis using the plate coating *M. luteus* (*27*). The lytic activity of K33N mutant lysozyme was confirmed to be higher than that of the wild-type lysozyme, in this report. Moreover, the lytic activity of K33A mutant lysozyme was also higher than that of the wild-type lysozyme. These results indicate that the enhancement of activity of K33N mutant lysozyme is not due to the effects of Asn introduced at the position 33. The electrostatic interaction between the apparent positive net charge of lysozyme and the negative one on the cell wall has been reported to be important in lytic activity (*16*). Although the apparent positive charge in the K33N or K33A mutant lysozyme was less than that in the wild-type lysozyme, the lytic activities of both mutant lysozymes increased. In addition, the activities of the K33N and K33A mutant lysozyme against glycol chitin, a neutral substrate,

Table 2. **Activities of the wild-type lysozyme and K33 mutant lysozymes against *M. luteus* and glycol chitin.**

	Lytic activity (%)	GC activity (%)
Wild-type	100	100
K33A	130	111
K33N	140	116

Table 3. **Binding of lysozyme to substrate analog, $(NAG)_4$.**

	K_A (M^{-1})
Wild-type	$1.1 \pm 0.5 \times 10^5$
K33A	$1.0 \pm 0.5 \times 10^5$
K33N	$1.0 \pm 0.5 \times 10^5$

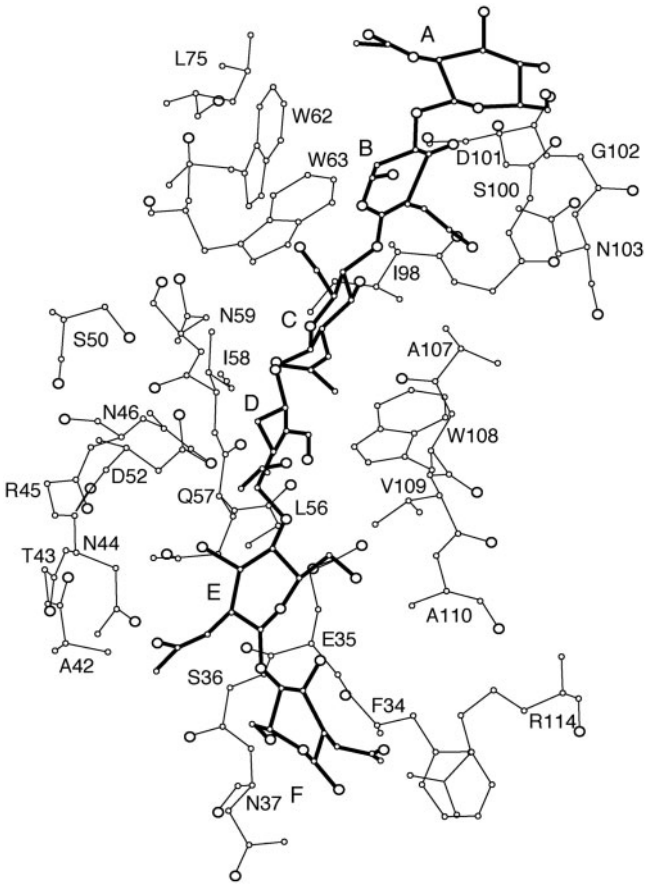

Fig. 1. **The binding model of a hexasaccharide substrate at the subsites A–F in lysozyme.** Cited from Perkins *et al.* (*15*).

were also higher than that of the wild-type lysozyme. It is therefore unreasonable to relate the enhancement of activity in the K33 mutants to the loss of a positive charge.

Bindings of the Substrate on K33 Mutant Lysozymes—The association constant (K_A) of K33N or K33A mutant lysozyme for $(NAG)_4$ was measured. As shown in Table 3, K_A of K33 mutant lysozymes was compared with that of the wild-type lysozyme. The active site of lysozyme can accommodate six saccharide units of polymeric substrate, designated subsites A, B, C, D, E and F (Fig. 1), and that

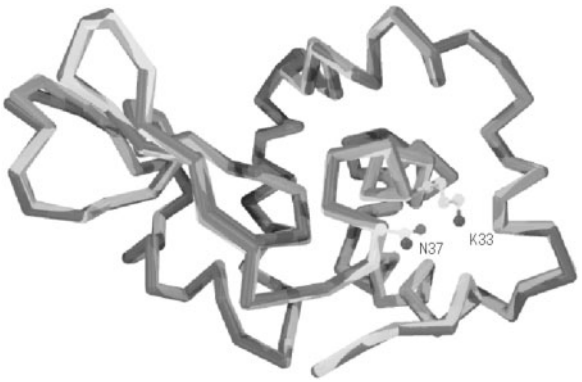

Fig. 2. **Super position of overall structures of wild-type lysozyme (black), K33N mutant lysozyme (light grey) and K33A mutant lysozyme (grey).**

only those saccharide molecules whose pyranose rings make contact with subsites D and E are catalytically hydrolysed (*15*, *17*). In the right-sided binding mode for the substrate $(NAG)_6$, the D-site saccharide residue is distorted and E- and F-site saccharide residues extended below to make contact with such residues as F34, N37 and R114 (*19*). Although glycol chitin occupies all the saccharide-binding sites (A–F subsites), $(NAG)_4$ is known to form a complex with lysozyme occupying the A–D subsites of lysozyme (*33*). It was therefore assumed that the conformations around the A–D subsites of K33 mutant lysozymes were similar to those in the wild-type lysozyme and conformations around the E–F subsites of K33 mutant lysozymes changed, thus influencing the enzyme activity.

The Crystal Structures of K33 Mutant Lysozymes—In order to examine the conformations around the E–F subsites of K33 mutant lysozyme, the 3D structures of K33N and K33A mutant lysozyme were solved by X-ray crystallographic analyses. The overall structures of K33 mutant lysozymes were similar to that of the wild-type lysozyme (Fig. 2). The local structures around position 33 of the wild-type lysozyme and K33 mutant lysozymes are shown in Fig. 3. While K33 is not in direct contact with the substrate, the side chain of K33 interacts with N37, which involves substrate binding. In the case of the wild-type lysozyme, the Nζ2 atom of K33 was found to form a hydrogen bond with the Oδ1 atom of N37, the distance between both atoms being 3.0 Å. On the other hand, the hydrogen bonds in K33 mutant lysozymes disappeared, but the distance between the carbonyl oxygen of residue 33 and the Nδ2 atom of N37 became close (2.6 Å), indicating formation a hydrogen bond between these atoms in the K33 mutant lysozymes. Moreover, the orientations of the side chain of N37 in the wild-type and K33 mutant lysozymes were different. Namely, the side chains of N37 in K33 mutant lysozymes have a reversed orientation compared with that of N37 at the active-site cleft in the wild-type lysozyme. In N37G mutant lysozyme, both lytic and glycol chitin activities increased due to the decrease in the energy level of the transition state in the catalytic reaction of lysozyme (*19*). Additionally, K_d of N37G mutant lysozyme to $(NAG)_3$ and the affinity for

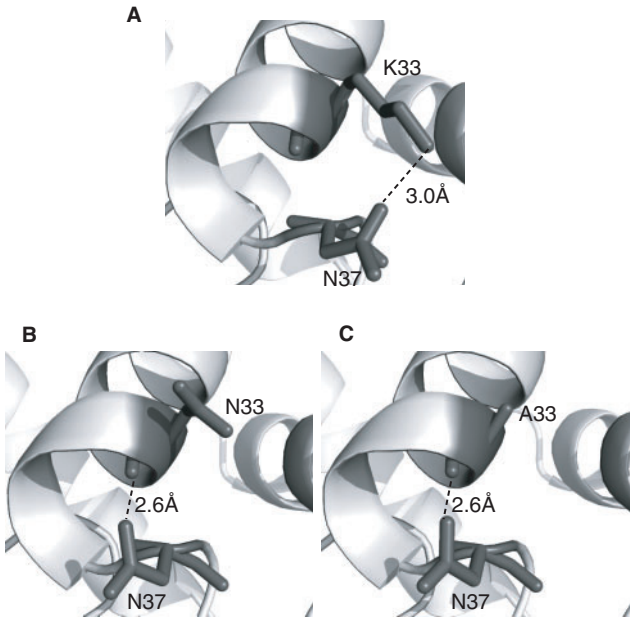

Fig. 3. **Structure around the residue 33 in (A) wild-type lysozome, (B) K33N mutant lysozyme and (C) K33A mutant lysozyme.**

immobilized chitin were not altered relative to those of the wild-type lysozyme (*19*). This biological property was similar to that in K33N mutant lysozyme as shown in Table 3. Since the orientation of the side chain of N37 in K33A lysozyme was identical to that of K33N mutant lysozyme, it appears that the enhancement of activity of K33N mutant lysozyme is due to the varying orientation of the side chain of N37 against the orientation of that in the wild-type lysozyme. The orientation of N37 in K33N mutant lysozyme might be a favourable one for the productive binding to the substrate. Alternatively, the favourable flexibility for enzyme activity might be acquired by the alteration of the orientation of the side chain of N37.

Importance of K33 in the Wild-type Lysozyme—The side chain of N37 in the wild-type lysozyme was oriented toward K33 and this orientation was unfavourable for enzyme activity as discussed above. It is likely that K33 controls the enzyme activity through the side chain of N37. K33 is located at the C-terminus of the helix 25–35 in lysozyme. The α-helix has a dipole moment with the positive pole at the N-terminus and the negative one at the C-terminus (*34*). Moreover, the basic amino acid residue at position 33 was highly conserved in c-type lysozymes. In order to examine the stability of K33N mutant lysozyme with enhanced activity, the analysis of thermal stability using DSC was performed under various acidic conditions according to our previous paper (*9*). K33N mutant lysozyme was found to be less stable by 3.14 kJ/mol than that of the wild-type lysozyme (data not shown). This result indicates that K33 contributed to the stability of the lysozyme. Active-site mutations can dramatically increase stability, while obviously sacrificing activity (*7*, *35–41*). Yutani *et al*. (*42*, *43*) have demonstrated that 19 mutants of the α-subunit of tryptophan synthase that increase protein stability are accompanied by decreases in their enzymatic activities. These relationships with activity and stability are termed activity–stability trade-offs (*41*). It is therefore suggested that lysozyme sacrifices the enzymatic activity to acquire the conformational stability at position 33.

A mutation showing a minor and indirect effect on enzyme activity such as K33N would be challenged only in random mutagenesis. Furthermore, the 3D structure analysis of such a mutant enzyme could provide more detailed information. The present observation would provide further insight into our understanding of the relationship between the function and structure of an enzyme.

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CONFLICT OF INTEREST

None declared.

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