Conformational changes in mutant lysozymes detected with monoclonal antibody

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Abstract **A monoclonal antibody (mAb) against hen egg white lysozyme (HEWL) with the exquisitely sensitive specificity to native conformation was prepared to detect the conformational changes in mutant lysozymes constructed by genetic modification in a yeast expression system. The binding of mAb with lysozyme was decreased both by denaturation with heat and guanidine-HCl, corresponding to the denaturation curves of lysozyme. These results demonstrate that mAb is a powerful probe to monitor the conformational changes in the lysozyme molecule. By using this probe, the conformational change of various mutant lysozymes was detected. A good correlation was observed between the binding with mAb and the ΔG (Gibbs free energy change), reflecting the conformational stability of wild-type and seven mutant lysozymes. This result suggests that a monoclonal antibody with the specificity for native conformation can be used as a powerful probe of protein conformation.**

Key words: Mutant lysozymes; Anti-lysozyme mAb

1. Introduction

The advent of the monoclonal antibody technology has enabled the detection of local conformational changes using the specificity of a given epitope. Kaminogawa et al. reported that anti-β-lactoglobulin mAbs could monitor local conformational changes and detect conformational differences between renatured and native forms [1,2]. Kitajima et al. reported that the anti-human aldolase A mAbs were highly sensitive to the amino acid substitution of the epitope region and thermal treatment of aldolase A molecule, thereby detecting conformational change in the aldolase A molecule [3]. We have investigated the conformational changes in mutant lysozymes secreted in yeast using genetic engineering [4,5]. The wild and mutant lysozymes are correctly processed in the expression system as well as in chicken egg and secreted in their soluble forms into the yeast medium [4–6]. Therefore, it became possible to investigate the conformational changes in various mutant lysozymes. The available conformational analysis, such as circular dichroism (CD) and fluorometric spectra, are not necessarily sensitive to the conformational changes in mutant lysozymes. There are no differences in CD spectra between wild-type and mutant lysozymes having considerably different conformational stability [4]. Therefore, mAb is expected as a powerful probe of the subtle conformational changes of mutant lysozymes. It is well known that the mAbs does not necessarily recognize a native state of the antigen. This is because the proteins may be denatured to some extent by the adjuvant used for immunization, some proteins may be processed (changed into peptides) in animal before they give rise to immuno response, and protein may result in surface denaturation during the immunization and the enzyme-linked immunosorbent assay (ELISA). Despite these difficulties, the mAb recognized a native state of lysozyme conformation was successfully obtained, probably because lysozyme kept stable or rigid structure during the immunization and ELISA. Thus, the conformational analysis of mutant lysozyme was possible to carry out by using monoclonal antibody as a probe.

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2. Materials and Methods

2.1. Preparation and purification of mAb

Seven-week-old male BALB/c mice were immunized intraperitoneally with 50 μg lysozyme in complete Freund's adjuvant (500 μl/mouse). After three weeks, they were boosted with 250 μg antigens in 500 μl of phosphate-buffered saline (PBS(−)). The preparation of hybridoma was carried out as follows. Cells were fused by the general method of Köhler and Milstein [7]. Spleen cells of immunized BALB/c mice were harvested and hybridized with NS-1 mouse myeloma cells, using 50% polyethylene-glycol (PEG 4000, Merck). Hybridized cells were seeded in 96-well culture plates (5×10^5 cells/well) and were fed for two weeks after hybridization in HAT medium and then the medium was exchanged to HT medium. The supernatants from the growing culture were tested to detect antibody activity by an enzyme-linked immunosorbent assay (ELISA) with lysozyme as the coated antigen. Antibody-producing hybridoma for lysozyme were cloned twice by limiting dilution with ddY mouse thymocytes as feeders. The cloned antibody-producing hybridoma were grown by mass culture in NS-1 medium. To purify γ-globulin fraction from the culture medium, the culture medium was adjusted to 40–50% saturation by addition of saturated ammonium sulfate, and allowed to stand over-night at 4°C. After centrifugation at 6000–8000 rpm for 30 min, the supernatant was discarded, and the precipitate was dissolved in 30 ml of water, dialyzed against distilled water and freeze-dried.

2.2. Denaturation of lysozyme by heat and guanidine-HCl treatments

Lysozyme was prepared from chicken egg white by the direct crystallization method in 5% NaCl at pH 9.5. The crystallization was repeated five time to completely purify. The lysozyme solution (0.1mg/ml) in 50 mM sodium carbonate buffer (pH 9.5) was heated at a heating speed of 1°C/min. When it reached a given temperature, the solution was cooled in the water rapidly and then used for the experiments. Lysozyme solutions dissolved in a given molarity (0 to 6 M) of guanidine-HCl were incubated at room temperature for 3 h. These solutions were diluted 10 times and immediately used as antigen solutions of denatured lysozymes.

2.3. Lytic activity of lysozymes

Lysozyme activity was measured by the lytic action against *M. lysodeikticus*. To 2.4 ml of the suspensions of *M. lysodeikticus* cells ($OD_{450} = 0.7$) in 0.1 M acetate buffer (pH 6.0) was added 0.1 ml of lysozyme solution ($OD_{280} = 0.05$). The initial decrease in absorbance at 450 nm of the mixture was measured at 20°C for 1 min.

2.4. Construction of mutant lysozyme cDNAs

Seven mutant lysozymes, N103D (Asn103 to Asp), P70N (Pro70 to Asn), K13D (Lys13 to Asp), C94A(Cys94 to Ala), C76/94A (Cys76,94

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to Ala), G49N (Gly49 to Asn) and M105T (Met105 to Thr) were constructed by the site-directed mutagenesis, as described previously [4,5]. The yeast expression plasmid, pYG-100, was supplied by K. Matsubara, University of Osaka. The recombinant plasmid (pKK-1) contains 16 bp of the 5′-noncoding region, 440 bp of the coding region, and about 120 bp of the 3′-noncoding region of hen pre-lysozyme cDNA in the same orientation as *lac*Z in pUC18. The conversion of amino acids was carried out by site-directed mutagenesis with bacteriophage vector M13mp19. The *Eco*RI/*Hin*dIII fragment of the pKK-1 plasmid containing almost full-length cDNA encoding HEWL was subcloned into the *Eco*RI/*Hin*dIII site of bacteriophage vector M13mp19. The mutant HEWL cDNAs were constructed in the recombinant M13mp19 vector by the Amersham oligonucleotide-directed mutagenesis system (version 2). The DNA sequence of each mutant was confirmed by the dideoxy sequencing method. The mutant HEW prelysozyme cDNA fragment was isolated by double digestion of M13mp19 with *Eco*RI/*Sal*I. The cDNA fragments were ligated to pYG-100, which had been digested with *Sal*I. The non-ligated *Eco*RI site in cDNA fragments and *Sal*I site in PYG-100 were blunt-ended by the blunt-end kit and self-ligated. The recombinant plasmid was propagated in *E. coli* JM 107 (*rec*A) in LB medium (1% Bacto trypton, 5% yeast extract, 0.5% NaCl). Whether the cDNAs were inserted downstream of the GPD promoter in the correct orientation or not was identified by a digestion analysis with restriction endonucleases, *Hin*dIII and *Bam*HI.

2.5. Preparation of mutant lysozymes

The expression plasmid of HEWL was introduced in *S. cerevisiae* AH22 (*a, Leu2, His4, Cir*$^+$) according to the lithium acetate procedure. Leu$^+$ transformants were selected in yeast minimum medium plates supplemented with histidine (20 μg/ml) at 30°C. Cultivation of the Leu$^+$ transformants was then carried out. *S. cerevisiae* AH22 (*a, Leu2, His4, Cir*$^+$) carrying the lysozyme expression plasmid was inoculated into 3 ml of yeast minimum medium supplemented with histidine (20 μg/ml) in a 15 cm test tube and incubated with vigorous shaking for 2 days at 30°C. This seed culture (2 ml) was subcultured into 100 ml of the same medium in a 500 ml Sakaguchi flask with shaking for another 2 days at 30°C. A second seed culture (20 ml) was inoculated with 1 liter of yeast minimum medium in a 3 liters Sakaguchi flask and incubated with vigorous shaking for 2 days at 30°C. Mutant lysozymes secreted in the growth medium were purified in two steps by cation-exchange chromatography on CM-Toyopearl. The growth medium of *S. cerevisiae* AH22 (*a, Leu2, His4, Cir*$^+$) carrying the lysozyme expression plasmid cultivated for 2 days was centrifuged at 5000 rpm for 20 min at 4°C and diluted with deionized water at least 2 times. The solution was directly applied to a CM-Toyopearl 650M opened column (12 × 90 mm) in 50 mM Tris-HCl buffer (pH 7.5), and the column was washed with the buffer until the washing solution was free from proteins. The adsorbed lysozyme was eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. The protein solution was diluted with deionized water at least 5 times and again applied to the regenerated CM-Toyopearl 650M opened column (12 × 90 mm). The purified mutant lysozymes were rechromatographed and eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM Tris-HCl buffer at pH 7.5.

2.6. Determination of ΔG

The Gibbs free energy change (*ΔG*) of unfolding was determined from the denaturation curves which were drawn by following the changes in the ellipticity at 222 nm during heating according to the method of Yutani et al. [8]. The temperature was controlled during all the measurements by circulating water in the cell holder from a thermostated bath with a heating rate of 1°C/min from 30 to 82°C. By using the data of thermal denaturation curves, the apparent fraction of unfolding was represented as a function of temperature to show clearly the denaturation curves. Since the thermal denaturation of mutant lysozymes at pH 3 were completely reversible, we can calculate the equilibrium constant between the native and the denatured forms. The Gibbs free energy change (*ΔG*) of unfolding of mutant lysozymes was calculated from various thermodynamic parameters estimated from the denaturation curves as described in a previous paper [4].

2.7. Solid phase ELISA

The wells of microtiter plates were seeded with 100 μl of the antigens (100 μg/ml) in 50 mM sodium carbonate buffer (pH9.5) and coated with 1% Bovine serum albumin in PBS(−). The plates were rinsed four times with PBS(−) containing 0.05% Tween 20 (PBS(−) Tween), and 100 μl of mAbs (100 μg/ml) in PBS(−) were added to each well. After the plates were washed with PBS(−)Tween, 100 ml of diluted goat anti-mouse antibody conjugated with peroxidase (10^3 times) was added to each well. After washing with PBS(−)Tween, 100 ml of substrate (0.04% *O*-phenylenediamine and 0.02% H_2O_2 in 0.05 M citrate-0.1 M phosphate buffer at pH 5.0) was added. The reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4, and the absorbance of each well was measured at 490 nm on a microplate reader (Bio-Rad Model 450).

2.8. Competitive ELISA

Competitive ELISA was done in the same way as the standard ELISA described above, except that the mAbs solutions contained different amounts (1–1000 μg/ml) of antigen as a competitor together with a constant amount of each mAb and incubated at 37°C for 2 hours before seeding to the plate. B/B0 is the ratio of the absorbance (492 nm) of ELISA in the presence of various concentrations of a competitive antigen to that in the absence of the competitive antigen.

3. Results and discussion

3.1. Preparation and characterization of monoclonal antibodies

Two stable clones with specificity for lysozyme were obtained from the fusion cells between splenocytes of lysozyme immunized BALB/c mice and NS-1cells. Each mAb produced by hybridoma clone was named mAb 1E6 and mAb 4G5. Isotypic analysis showed that 1E6 and 4G5 belong to IgM and IgG1, respectively. Since the binding affinity of IgG to antigen is stronger than IgM, mAb 4G5 was used for the experiment of ELISA.

3.2. Binding of mAb 4G5 with denatured lysozyme

The binding properties of heat-denatured lysozyme were investigated. In solid phase ELISA, native lysozyme showed the binding affinity with mAb, while denatured lysozyme did not show any binding with mAb. Similarly the binding with denatured lysozyme was investigated in competitive ELISA (Fig. 1). The B/B0 of denatured lysozyme is constant at increasing con-

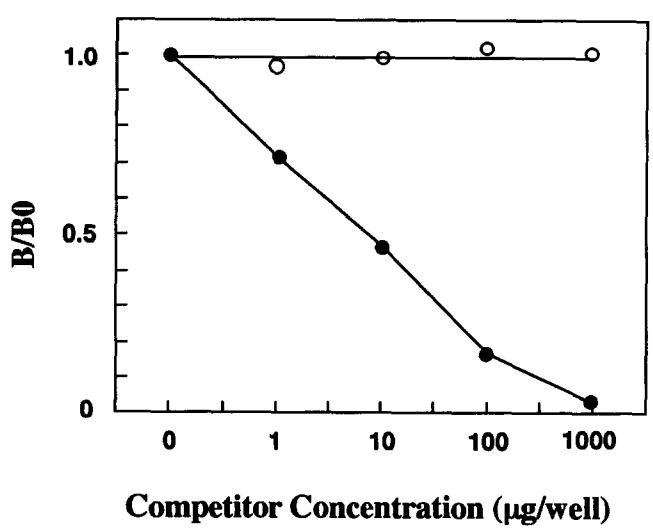

Fig. 1. Binding curves of mAb to heat-denatured lysozyme (○) and native lysozyme (●). Lysozyme was heated at 90°C for 5 min in 0.1 M sodium carbonate buffer (pH 9.5). B/B_0 is the ratio of the absorbance (492 nm) at the last step of ELISA in the presence of various concentrations of the competitive antigen to the absorbance in the absence of the competitive antigen.

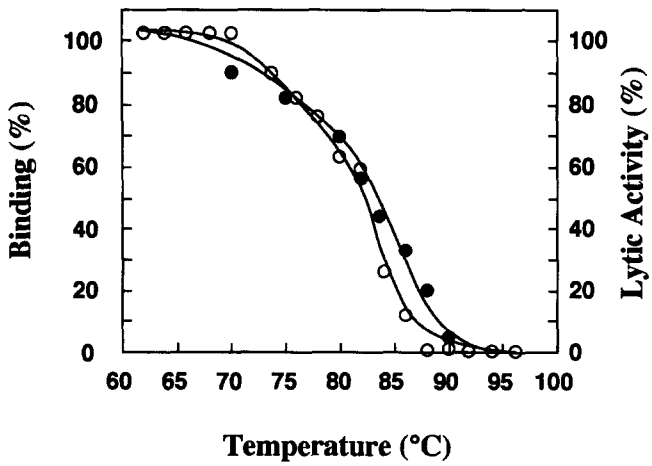

Fig. 2. Denaturation curves detected by binding with mAb (●) and enzymatic activity (○) of heated lysozymes. Lysozyme solution (0.1 mg/ml) in 0.1 M sodium carbonate buffer (pH 9.5) was heated upto 96°C at a heating speed of 1°C/min. When it reached a given temperature, the solution was immediately cooled and then used for the ELISA and the measurement of lytic activity.

centrations of competitor, while that of native protein is decreased with the increase in antigen concentration, indicating that no binding was observed similarly in the denatured protein. To elucidate more closely whether the subtle structural changes during heating can be followed or not, the change in the binding of heat-denatured lysozyme with mAb 4G5 was compared to that of the lytic activity during heating. As shown in Fig. 2, the binding affinity of 4G5 to lysozyme was reduced with the increase in the heating temperature. The curve of binding affinity was almost consistent with that of enzymatic activity during heating. This result suggest that mAb 4G5 can recognize exactly the conformational changes in lysozyme induced by thermal treatment. To further confirm that mAb 4G5 can detect the conformational changes in lysozyme, the binding of mAb with guanidine HCl-treated protein was investigated

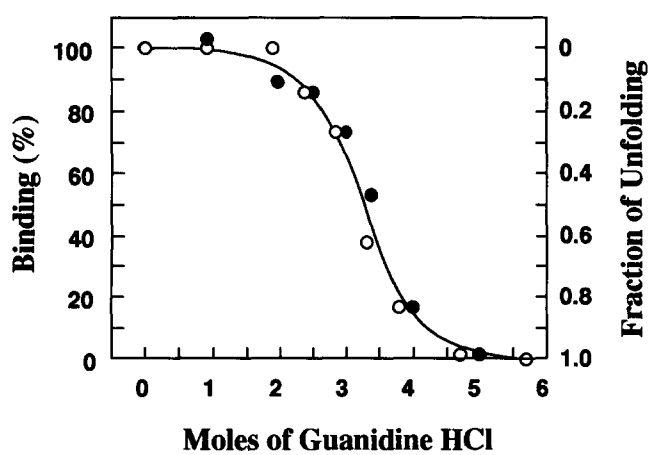

Fig. 3. Denaturation curves detected by binding with mAb (●) and unfolding fraction of guanidine HCl-treated lysozyme (○). Lysozyme solutions (0.1 mg/ml) in a given molarity (0 to 6 M) of guanidine HCl were incubated at room temperature for 3 h and then 10 times dilutions were used for the ELISA. The unfolding fractions were determined from the changes in the ellipticity at 222 nm during incubation at a given mole concentration of guanidine HCl.

(Fig. 3). The binding affinity of mAb 4G5 to denatured lysozyme was decreased with the increase in guanidine HCl concentration. The denaturation curve calculated from the decrease in α-helix was well consistent with that of the binding affinity with mAb. These results reveal that mAb4G5 can detect the conformational changes induced by heat or guanidine HCl. Hence it appears that this mAb can be used as a probe detecting conformational changes of lysozyme.

3.3. Binding of mAb4G5 with various mutant lysozymes

By using the mAb sensitive to conformational changes in lysozyme, the conformational changes of seven mutant lysozymes were investigated. As reported previously [4,5], the wild-type and mutant lysozymes had the same N-terminal lysine as hen egg white lysozyme, suggesting correct processing in yeast, and showed the same CD spectra and lytic activity as those of hen egg white lysozyme. The wild-type lysozyme secreted in yeast showed a slightly lower binding affinity than native enzyme in hen egg white. However, there may be no significant defference between them in the range of experimental variation. The standard deviations of five replicates were between 2.5 and 7.5%. The single amino acid substitutions for the deamidation, the cleavage of disulfide bond and the destabilization of α-helix were carried out to construct unstable lysozymes. The binding of deamidated mutants (N103D) is almost the same as that of wild type lysozyme. On the other hand, the mutant (K13D) destabilized the dipole in the helix 5–15, and the mutants cleaved the disulfide bond between 74 and 94 cysteine (C94A, C94/76A) were greatly decreased the reactivity with mAb. The structural changes in these mutants were not detected with lytic activity and CD spectra [4,5]. On the other hand, big differences were observed by the binding with mAb sensitive to native structure of lysozyme, as shown in Fig. 4.

3.4. Correlation between the binding with mAb and ΔG

In order to evaluate the relationships between the binding of mAb and the conformational stability, the Gibbs free energy change (ΔG) of unfolding in various mutant lysozymes was

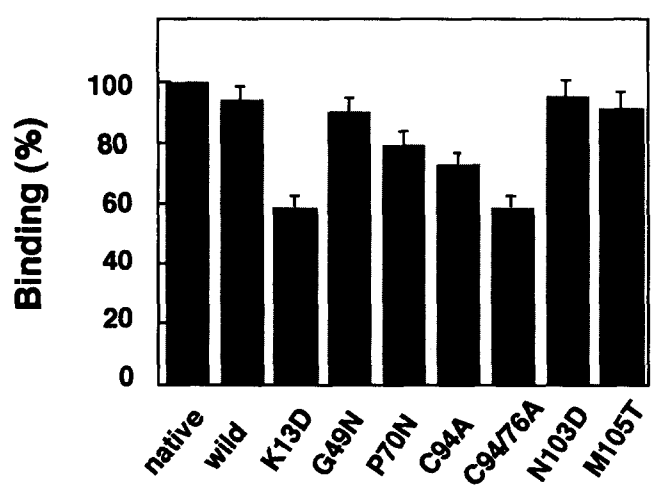

Fig. 4. Binding of mAb with various mutant lysozymes. Binding of mAb with mutant lysozymes was represented as the percentages of the absorbance of mutant lysozymes to that of native lysozyme in solid phase ELISA. The standard deviations of five replicates were between 2.5 and 7.0% in all samples.

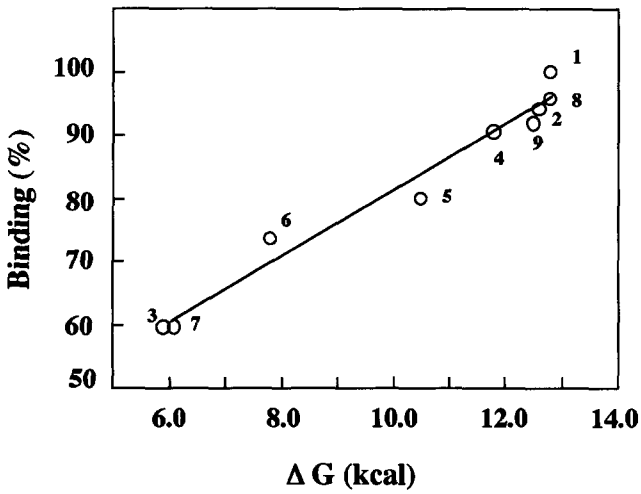

Fig. 5. Relationship between Gibbs free energy of unfolding (ΔG) and binding with mAb of various mutant lysozymes. 1, native; 2, wild-type; 3, K13D; 4, G49N; 5, P70N; 6, C94A; 7, C94/76A; 8, N103D; 9, M105T.

calculated from various thermodynamic parameters estimated from the denaturation curves. A good correlation between the binding with mAb and ΔG was observed in mutant lysozymes, as shown in Fig. 5. This result suggests that the binding of a monoclonal antibody against native lysozyme reflects the subtle conformational changes. It is apparent that the loss of the binding affinity with mAb is attributed to the changes in the polypeptide folding rather than to localized changes around the site of the amino acid substitutions, because a good correlation was observed between the loss of the binding affinity with mAb and the drop in ΔG of unfolding in mutants substituted amino acids at different site.

The further construction of unstable mutant lysozymes is desirable for evaluating the relationship between the binding affinity and the conformational stability. However, it is difficult to obtain unstable proteins because they are destroyed by the quality control in host cells due to unstable conformation. It was reported that human lysozyme was converted to unstable protein without loss of lytic activity by deleting the SS bond between 77 and 95, although the same attempt was unsuccessful for three other SS bonds [9]. Thus, hen egg white lysozyme was also converted to unstable form by deleting the coresponding SS bond between 76 and 94. It is reasonable that the deletion of one SS bond of four SS bonds in lysozyme causes unstable conformation. On the other hand, it is interesting that another unstable mutant can be obtained by substitution of Lys-13 with Asp. It is well known that the negatively charged and the positively charged amino acids are incorporated, respectively, at the N- and C-terminal helical ends to stabilize the α-helix dipole. Nicholson et al. reported that the introduction of Asp to N-terminal region of helix in T4 lysozyme enhanced the conformational stability [10]. It is probable that the stability of the helix region along positions 5 to 15 may be kept by the positive charge in Lys 13. The substitution of Lys with Asp must cause dramatic destabilization for the 5–15 helix. The destabilization of helix region, one of the longest α-helix in lysozyme, may cause the partial distortion of the compact conformation.

In conclusion, the monoclonal antibody recognized the conformation of native lysozyme is a powerful tool to monitor the conformational changes in lysozyme. The conformational changes in mutant lysozymes were detected by using mAb 4G5, although significant differences in the lytic activity and the CD spectrum could not detect between wild-type and mutant lysozymes. Hence monoclonal antibody can be used as a molecular probe sensitive to the conformational changes.

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