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Fluctuations in Free or Substrate-Complexed Lysozyme and a Mutant of It Detected on X-Ray Crystallography and Comparison with Those Detected on NMR[1]

Tadahiro Ohmura, Hiroyuki Motoshima, Tadashi Ueda, and Taiji Imoto[2]

Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582

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A mutant lysozyme in which Arg14 and His15 were deleted together exhibited higher activity toward glycol chitin than the wild-type lysozyme. Moreover, the mutant lysozyme, which is less stable than the wild-type lysozyme by 7°C, showed a shift of temperature dependence of activity to the low temperature side compared with the wild-type lysozyme [*Protein Eng.* 7, 743–748 (1994)]. In the free enzyme, the internal motion of the mutant lysozyme was similar to that of the wild-type. The internal motions of the wild-type and mutant lysozymes in the enzyme-substrate complex increased more than those in the free enzymes. Moreover, the increased internal motions of the substrate-complexed mutant lysozyme were greater than those of the substrate-complexed wild-type lysozyme in several residues [*J. Mol. Biol.* 286, 1547–1565 (1999)]. The structure of the mutant lysozyme was very similar to that of the wild-type lysozyme. Both structures were also alike in the complex of the trimer of *N*-acetyl-D-glucosamine. The mobility from B-factors agreed to some degree with that from order parameters in the regions showing great mobility of the protein, but this was not the case in the regions showing fast motion. However, we came to the same conclusion that the increased activity of the mutant lysozyme is due to the increase in the fluctuation of the lysozyme molecule. B-factor and order parameter do not always exhibit harmony because the time-scale of the analysis of mobility is different. However, they are not incompatible but complementary for detecting precise protein motions.

Key words: B-factor, lysozyme, mobility, order parameter, X-ray crystallography.

It is well accepted that proteins are not rigid entities but are rather flexible and dynamic in solution. It is considered that this motion of a protein is related to protein functions such as enzyme activity and recognition of antigens. The motion of a protein molecule can be experimentally analyzed by means of H-D exchange, ^{15}N NMR relaxation analysis or B-factors on X-ray crystallography. B-factors have been analyzed for several crystal structures under various conditions (*1–4*). Moreover, protein mobility was inferred from the change in conformation on binding to an antigen or substrate (*5*). These reports raised the idea that the structure of a protein becomes rigid on the formation of a complex. On the other hand, from the results of comparison of X-ray and NMR results, it is suggested that some protein mobilities in X-ray crystal structures are lost due to contact of molecules in crystals (*6*). Although B-factors have been employed to analyze protein mobility, they were not correlated with order parameters, which were obtained by ^{15}N NMR relaxation analysis (*7*). This would be attributable to the difference in time-scale between X-ray and NMR, and to the difference in the contact of molecules in crystals and solution.

Hen egg white lysozyme (HEL) is becoming more and more widely used in studies because it can be easily obtained. HEL was the first enzyme for which a detailed reaction mechanism was advocated. Moreover, since its crystallization can be comparatively easy, it was the first enzyme for which perfect X-ray crystallography was performed (*8*). Therefore, HEL has attracted attention as an effective model protein with which to explore various protein functions. A mutant lysozyme in which Arg14 and His15, which are located far from the active-site cleft, were deleted together exhibited higher activity toward glycol chitin than the wild-type lysozyme. This mutant lysozyme, which is less stable by 7°C, showed a shift of temperature dependence of activity to the low temperature side compared with the wild-type lysozyme (*9*). We concluded from the results as to H-D exchange rates of indole nitrogens that the structure of the mutant lysozyme was more flexible than that of the wild-type lysozyme. Recently, it was elucidated that there is no difference in internal motion between the wild-type and mutant lysozymes by analysis of the order parameters of hen lysozyme (*10*). On analysis of the order parameters of the trimer of *N*-acetyl-D-glucosamine [$(NAG)_3$]–complexed hen lysozyme, it was found that the internal motions of some residues in the complexed lysozyme increased more than those in the free

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[2] To whom correspondence should be addressed. Tel: +81-92-642-6662, Fax: +81-92-642-6667, E-mail: imoto@phar.kyushu-u.ac.jp

Abbreviations: $(NAG)_3$, trimer of *N*-acetyl-D-glucosamine; RMSD, root-mean square deviation; RMSF, root-mean square fluctuation.

lysozyme. This phenomenon was more remarkable for the mutant lysozyme (*10*). These findings contradicted the finding that complexed proteins are more rigid than free proteins on X-ray crystallography. Thus, we performed X-ray crystallographic analyses of free and complexed lysozymes at 1.75 Å resolution, and discussed the differences in the results as to mobilities obtained from B-factors and order parameters.

MATERIALS AND METHODS

Materials—CM-Toyopearl 650M, a cation-exchange resin for the purification of secreted hen lysozymes, was obtained from Tosoh (Tokyo). $(NAG)_3$ was purchased from Seikagaku Kogyo.

Preparation and Purification of Lysozymes Secreted by Yeast—The wild-type lysozyme was obtained as described previously (*11*). The mutant lysozyme gene was obtained by site-directed mutagenesis to delete Arg14 and His15 using the method of Kunkel *et al.* (*12*). The mutant lysozyme was obtained as described previously (*9*).

X-Ray Analysis—Protein crystals were obtained by vapor diffusion using the hanging-drop method at 23°C. The well buffer contained 0.9 M NaCl, 0.1 M Na-acetate, pH 4.7. For the free protein, hanging drops were made by mixing 2 μl of the well buffer with an equal volume of a 20 mg/ml protein solution in 0.1 M Na-acetate, pH 4.7. For the $(NAG)_3$-complexed protein, the well buffer contained $(NAG)_3$ at 1.2-fold the concentration of protein used. X-ray diffraction data for all proteins were collected at 23°C using an automated oscillation camera system, R-AXCIS IIC (RIGAKU), equipped with an Imaging Plate detector, on a Cu rotating anode generator operated at 40 keV and 120 mA. Structures were refined with the program X-PLOR (*13*), and models were built with the program TURBO-FRODO (*14*). Coordinates have been deposited in the Protein Data Bank, the PDB entry codes of all lysozymes being presented in Table I.

TABLE I. **Data collection statistics and refinement parameters.**

Lysozyme	Wild-type		Mutant	
	Free	Complex	Free	Complex
Crystallographic data				
Space group	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$
Cell dimensions (Å)				
$a = b =$	79.21	78.81	79.72	79.23
$c =$	37.97	38.27	37.40	37.92
Resolution (Å)	1.75	1.75	1.76	1.76
Unique reflections ($F \geq 1\sigma$ [F])	11,948	12,026	10,820	11,765
Completeness (%)	93.2	95.0	86.2	94.0
R-merge[a] (%)	3.69	5.35	4.83	4.43
Refinement				
R-factor[b] (%)	16.8	16.8	18.0	16.6
Δbond length (Å)	0.009	0.009	0.009	0.010
Δbond angle (°)	1.448	1.472	1.525	1.546
PDB entry code	1RFP[c]	1UIH	1UIA	1UIB

[a] $R\text{-merge} = (\sum (I - \langle I \rangle) / \sum I)$. [b] $R\text{-factor} = (\sum (F_o - F_c) / \sum F_o)$. [c] Motoshima, H., Mine, S., Masumoto, K., Abe, Y., Iwashita, H., Hashimoto, Y., Chijiiwa, Y., Ueda, T., and Imoto, T. (1997) Analysis of the stabilization of hen egg white lysozyme by helix macrodipole and charged side chain interaction. *J. Biochem.* **121**, 1076–1081.

RESULTS AND DISCUSSION

X-Ray Crystal Structure Analysis—To perform detailed analyses of B-factors, we carried out the crystallization of the free and $(NAG)_3$-complexed wild-type and mutant lysozymes under the same conditions (see "MATERIALS AND METHODS"). All protein crystals in space group $P4_32_12$ were obtained by vapor diffusion using the hanging-drop method at 23°C. X-ray diffraction data for all proteins were collected at 23°C. The structures of all proteins were refined well. R-merges were about 6% and R-factors fell about 17% (Table I). For the free proteins, the crystal structure of the mutant lysozyme was similar to that of the wild-type lysozyme (Fig. 1). The root-mean square deviation (RMSD) of the main-chain between them was 0.15 Å. For the complexed proteins, the structures were also similar to that of the free wild-type lysozyme (Fig. 1). The main-chain RMSDs of the structures of the complexed wild-type lysozyme and complexed mutant lysozyme against free wild-type lysozyme were 0.11 Å and 0.13 Å, respectively. $(NAG)_3$ had got into the same binding site in the mutant lysozyme as in the wild-type lysozyme (Fig. 2). Thus, a clear difference causing the changes in activity was not seen on comparison of the two crystal structures. Thus, it is thought in this case that the dynamic motion is more important for the activity change.

Comparison between Root-Mean Square Fluctuation Calculated from B-Factors and Order Parameters—The following were discussed by comparison of the root-mean square fluctuations (RMSF) calculated from the B-factors and order parameters obtained by ^{15}N relaxation analysis (*10*). RMSF was calculated using the relationship $\langle \Delta r \rangle = (3B/8\pi^2)^{1/2}$ (*15*).

For the wild-type lysozyme, lower order parameter-enhanced fluctuations were observed for loop A-B (residues 16–23), strand 1 (residues 41–45), the long loop (residues 65–76), in a short loop connecting the C- and D-helices (residues 101–104), loop D (residues 116–119), the C-terminal residues, and residue 85. In the crystal structure, the peak-top residues of RMSF plots were observed for Asp18 (loop A-B), Thr47 (β-sheet turn), Trp62 (long loop), Ser71 (long loop), Ser85 (310-helix), Asp101 (loop C-D), and C-terminal

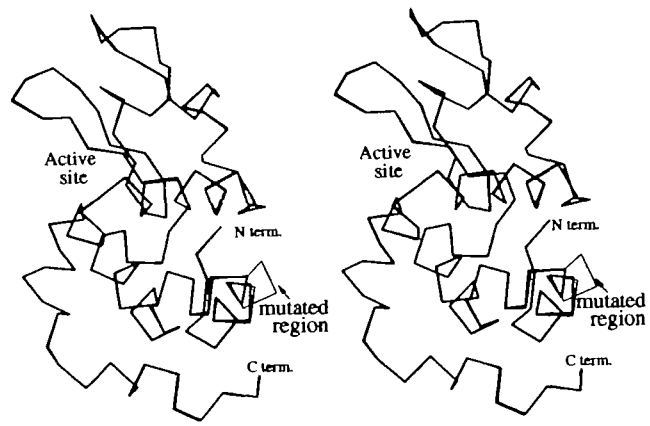

Fig. 1. **Structure comparison of the wild-type and mutant lysozymes.** Stereo-view of superpositioning of the wild-type (thin line) and mutant (thick line).

residues (Fig. 3A). Thus, although there were the differences in the degrees, it would be stated that the results for B-factors and order parameters agreed for some residues showing great mobility.

On comparison between the free and complexed wild-type lysozyme, the order parameters of some residues in the complexed one were found to be lower than those in the free one. This shows that the internal motion in the complexed wild-type lysozyme increased more than that in the free wild-type lysozyme. On the other hand, the RMSF values of the substrate-binding site in the complexed wild-type lysozyme were slightly lower than those in the free wild-type lysozyme (Fig. 3A). This result was similar to that in a previous X-ray crystallographic report (*16*). However, it was different from the results for order parameters.

RMSFs in the complexed mutant lysozyme were generally lower than those in the free mutant lysozyme (Fig. 3B). This showed that the overall structure in the complexed mutant lysozyme was more rigid than that in the free mutant lysozyme. Since, on order parameter analysis, the residues showing greater mobility were found to be increased more in the complexed mutant lysozyme than in the free mutant lysozyme. Thus, the result as to RMSFs did not agree with those obtained on order parameter analysis.

RMSFs in the complexed wild-type lysozyme were similar to those in the complexed mutant lysozyme (Fig. 3C). On order parameter analysis, the degrees of increased mobility for some residues were found to be increased more in the complexed mutant lysozyme than in the complexed wild-type lysozyme. Thus, these results are not consistent.

On comparison of RMSFs in the free wild-type and mutant lysozymes, it was shown that the mutant lysozyme was more flexible than the wild-type one (Fig. 3D). This finding coincided with the finding that the H-D exchange rate in the mutant lysozyme increased more than that in the wild-type lysozyme at Trp63 (*9*). Trp63 is located in the active-site cleft of lysozyme. However, the order parameters for the free wild-type lysozyme were similar to those for the free mutant lysozyme.

Great mobility in the wild-type lysozyme was observed in the regions around the active-site cleft (Fig. 3A). This structural flexibility of these regions may be important for the enzymatic activity. The increased flexibility would favor the

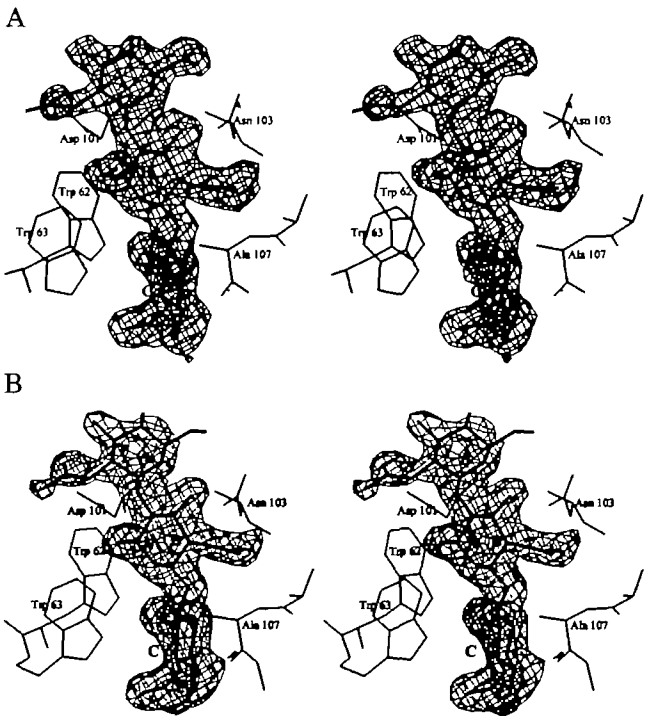

Fig. 2. **Stereo-view of the electron-density maps of the active-site in the $(NAG)_3$ complexed (A) wild-type and (B) mutant lysozymes.** A $2F_o$–F_c map showing the $(NAG)_3$ molecule at 1.76 Å with the final model is presented for comparison.

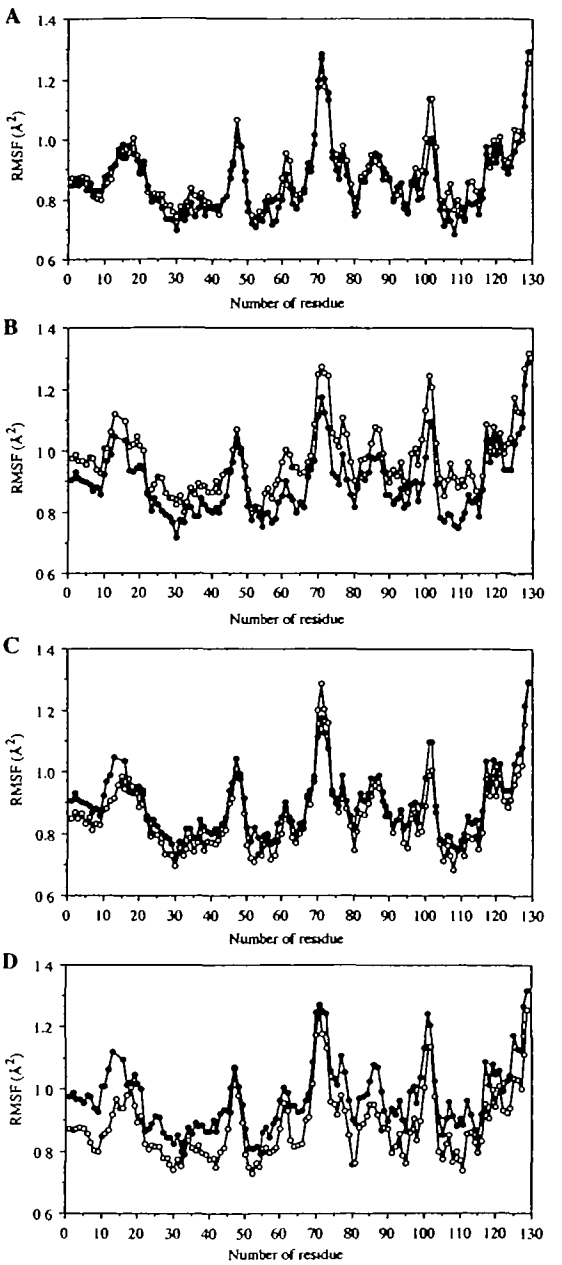

Fig. 3. **Plots of RMSF for the main-chain (N, Cα, C, O) of each residue.** A: Free (open circles) and complexed (closed circles) wild-type lysozyme. B: Free (open circles) and complexed (closed circles) mutant lysozyme. C: Complexed wild-type (open circles) and mutant (closed circles) lysozymes. D: Free wild-type (open circles) and mutant (closed circles) lysozymes.

easier accommodation of a substrate in the active-site. Therefore, we concluded that the increased activity of the mutant lysozyme is due to the increase in these motions. This idea coincided with that in the previous paper (*9*).

On analysis of order parameters, the difference in molecular mobility between the free and complexed enzymes was compared for the wild-type and mutant lysozymes. It was found that the mobility in the complexed form increased more in the mutant lysozyme than in the wild-type lysozyme. Thus, analysis of order parameters led to the same idea (*10*).

CONCLUSION

The crystallization and X-ray measurements were carried out under the same conditions in order to allow reliable comparison of RMSFs. However, the motions obtained from RMSFs were largely inconsistent with the results as to order parameters. These inconsistencies would be caused by the fact that the time-scale or amplitude of mobility reflected by B-factors was larger than that reflected by order parameters. This would be supported by the suggestion in a previous report that order parameters reflect internal motion on the picosecond to nanosecond time-scale, and B-factors reflect both internal motions on a small time-scale and static disorders on a large time-scale (*17*). The wild-type human lysozyme showed a similar phenomenon to the hen lysozyme on the analysis of order parameters (*18*). Thus, the results of analysis of order parameters would be general as to the mobility of proteins on the picosecond to nanosecond time-scale. The internal motions on the picosecond to nanosecond time-scale would originally be of a small amplitude. It would be impossible with B-factors, which could not be used to distinguish the mobility in each time-scale range, to recognize the mobility on such a small time-scale. The slow motion between two domains (*i.e.* hinge-bending motion) was reflected by a large time-scale movement and had a large amplitude. As mentioned above, the increased activity of the mutant lysozyme was explained on comparison of B-factors between the free and complexed proteins. Therefore, it would be reasonable to state that B-factors showed the mobility on a large time-scale and/or the mobility with a large amplitude. B-factors should be used to explore such mobility. B-factors are not entirely out of harmony with order parameters but they are complementary for detecting the detailed protein mobility.

Finally, to discuss the relationship between protein mobility and protein function in detail, it would be necessary to analyze the direction of mobility. By means of order parameters, it is difficult to analyze the direction of mobility and the motion of side-chains. Although B-factors could decide the scale of the motion of side-chains, it is also difficult to analyze the direction. To solve these problems, molecular dynamics and normal mode analysis based on X-ray crystal structures should be applied. It would increase the confidence as to the results obtained with these methods to collect the results of comparison of B-factors and order parameters.

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