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Situation of Monomethoxypolyethylene Glycol Covalently Attached to Lysozyme

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We selectively introduced monomethoxypolyethylene glycol (mPEG) 5000, 2000, and 550 into Asp119 in lysozyme. To examine how the mPEGs were present on the surface of the modified lysozyme, the activities, binding abilities to the Fab fragment of anti-lysozyme monoclonal antibody, net charges and nuclear magnetic resonance (NMR) spectra of mPEG lysozymes were examined. With the increase in molecular weight of mPEG, the activities and binding abilities to the Fab of mPEG lysozyme decreased. However, introduced mPEG5000 did not cause complete inhibition of the activities and binding abilities to the Fab, while the maximum length of mPEG5000 was so great that it largely covered the surface of the lysozyme molecule. Analyses of the net charges and NMR suggested that the introduced mPEG preferentially assumed a folded conformation on the surface rather than spread all over the surface. Based on the structure of mPEG lysozyme, the mechanism of the reduced immunogenicity of mPEG lysozyme was discussed.

Key words: immunogenicity, lysozyme, monomethoxypolyethylene glycol, NMR, structure of mPEG.

Specific regulation of the production of antibody by the chemical modification of an antigen with polymers has been tried for the therapy of allergy or cancer (*1-6*). In particular, polyethylene glycol is often used in the modification of antigens. For example, the modification of asparaginase or adenosinedeaminase with monomethoxypolyethylene glycol (mPEG) allowed continuous administration with the help of the depressed production of their specific antibodies (*5, 6*). However, the mechanism by which the production of the specific antibody is depressed by the modification with mPEG remains unknown, despite several studies (*7-9*).

Recently, we demonstrated that the reduced immunogenicity of mPEG-modified lysozyme was due to blockade of intracellular antigen processing by mPEG (*10*). This may be closely related to the unique physical properties of mPEG proteins. The physical properties of proteins modified with mPEG have been discussed in terms of how mPEG participated in protease digestion and what conformation it assumed (*11, 12*). Unfortunately, in these cases, because mPEG was not introduced into the proteins selectively, the information on the conformation of mPEG in modified proteins lacked a molecular basis. To define in exactly what conformation mPEG is present in modified proteins, selective modification of the proteins is essential.

Lysozyme is a small protein that is abundant in nature and, therefore, widely used in immunochemistry. Many selective chemical modifications of lysozyme have been possible following the elucidation of its structure by X-ray crystallography (*13-17*). In this paper, we selectively modified lysozyme with mPEGs of various molecular weights and examined what conformation mPEG took in the modified lysozyme.

MATERIALS AND METHODS

Material—Five times recrystallized lysozyme was donated by QP. Bio-gel P-4 and Bio-Rex 70 were purchased from Bio-Rad Lab. CM-Toyopearl 650 M and TSKgel CM-5PW (7.5×75 mm) columns for ion-exchange HPLC were purchased from Tosoh (Tokyo). Wakosil 5C18-200 (4.6×250 mm), a column for RP-HPLC, was obtained from Wako Pure Chemical (Osaka). Monomethoxypolyethylene glycol 5000, 2000, and 550 were the products of Aldrich. TPCK-trypsin was the product of Worthington. DCC, bromoacetic acid, and DTT were purchased from Nacalai Tesque (Kyoto). mAb37-7 was prepared as before (*18*). Carboxymethylated His15 (*14*), 6-, 127-*S* carboxymethylated lysozyme (*19*), diacetylated (*20*), and Asn119 lysozyme (*16*) were prepared according to the respective literature sources. Glycol chitin was synthesized by the method of Yamada and Imoto (*21*). Other reagents used were the highest quality commercially available.

Preparation of Monomethoxypolyethylene Glycol Bromoacetate 5000, 2000, and 550—In 30 ml of benzene, 5, 2, and 0.55 g of monomethoxypolyethylene glycol 5000, 2000, and 550 were dissolved, respectively. After addition of 0.16 g of bromoacetic acid, each solution was cooled in an ice bath and 0.23 g of DCC was added in limited amounts. The mixtures were stirred for 1 day in the dark, then the solvent was removed under vacuum. Each residue was

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Abbreviations: DCC, *N,N'*-dicyclohexyl carbodiimide; DTT, dithiothreitol; NMR, nuclear magnetic resonance; mAb, monoclonal antibody; mPEG, monomethoxypolyethylene glycol; Asp119-PDE lysozyme, a lysozyme derivative in which the β-carboxylic acid of Asp119 is modified with (2-pyridyl)-dithioethylamine; RP-HPLC, reversed phase-high performance liquid chromatography; TPCK-trypsin, L-1-(*p*-tosylamino)-2-phenylethyl chloromethyl ketone treated trypsin.

redissolved in 50 ml of water and washed five times with 50 ml portions of diethyl ether. Each aqueous layer was extracted with five 50 ml portions of chloroform. Chloroform was removed under vacuum and each residue was dried exhaustively.

Preparation of (2-Pyridyl)-Dithioethylamide Asp119 Lysozyme—(2-Pyridyl)-dithioethylamide Asp119 lysozyme (Asp119-PDE lysozyme) was prepared according to the literature (*15, 16*). Briefly, 200 mg of lysozyme was dissolved in 40 ml of 1 M imidazole-HCl buffer at pH 5 containing 100 mg of (2-pyridyl)-dithioethylamine and 20 mg of $(NAG)_3$. Then 64 mg of EDC was added in limited amounts and the solution was stirred for 12 h at room temperature. The reaction mixture was dialyzed against distilled water, then against 0.02 M borate buffer, pH 10, at 4°C for 12 h, and again against distilled water exhaustively. The dialysate was applied to an ion-exchange chromatographic column, Bio-Rex 70 (3×40 cm), and eluted with a gradient of 1,000 ml of 0.1 M phosphate buffer, pH 7, and 1,000 ml of 0.4 M phosphate buffer, pH 7. The protein fraction eluted after the unreacted lysozyme was collected. After dilution, this protein fraction was rechromatographed on an ion-exchange chromatographic column of CM Toyopearl 650 M (1.5×120 cm). Elution with a gradient of 1,000 ml of 0.1 M phosphate buffer, pH 7, and 1,000 ml of 0.4 M phosphate buffer, pH 7, yielded two major protein fractions. The earlier peak was collected, precipitated by adding $(NH_4)_2SO_4$, collected by centrifugation, dialyzed against distilled water, and lyophilized. The yield was 15%.

Selective Modification of Asp119 in Lysozyme with mPEGs of Various Molecular Weight—Asp119-PDE lysozyme (5 mg) was dissolved in 0.05 M acetate buffer of pH 4.5 containing 1 mM EDTA. Then 0.5 mg of DTT was added and the solution was stirred for 20 min at room temperature in order to produce a liberated thiol at Asp119. The reaction mixture was chromatographed on Bio-gel P-4 (1×40 cm) equilibrated with the reaction buffer, and the protein fraction which passed freely through the gel was collected. To this was added 100, 40, or 10 mg of $BrCH_2COO$-mPEG 5000, 2000, or 550, respectively, followed by 1 M phosphate buffer, pH 7, to increase the pH to 6.5. After stirring for 1 h at room temperature, the solution was applied directly to an ion-exchange HPLC column of CM Toyopearl 650 S (6×600 mm) equilibrated with 0.05 M phosphate buffer of pH 7. The column was eluted with a gradient of 200 ml of 0.05 M phosphate buffer, pH 7, and 200 ml of the same buffer containing 1 M NaCl at a flow rate of 2 ml/min. The effluent was monitored by measuring the absorbance at 280 nm. The material in each major peak eluted was collected and exhaustively dialyzed against distilled water.

RP-HPLC of the Tryptic Peptides Derived from Lysozyme—Preparation of the tryptic peptides derived from native and mPEG lysozymes and RP-HPLC of the peptides were performed by the method of Ueda *et al.* (*17*).

Measurement of Two-Dimensional ^1H-NMR—^1H-NMR spectra were recorded with a VARIAN Unity plus 600 MHz NMR spectrometer. The probe temperature was calibrated using ethylene glycol. Dioxane was employed as the internal standard (3.743 ppm). The pH values were the pH meter readings without adjustment for isotope effects (*22*). Phase-sensitive double quantum filtered COSY (*23*) and phase-sensitive NOESY (*24*) experiments were carried out at 600 MHz using standard procedures. Typically, 32 transients were recorded for each of 512 increments. NOESY spectra were acquired with mixing times of 150 ms. A digital resolution of 2.4 Hz/point in both dimensions was used for the COSY and NOESY.

Evaluation of the Binding Energy of Lysozyme with Immobilized Fab Fragment of mAb37-7—Association constants (K_{ass}) of native and mPEG lysozymes with the immobilized Fab fragment of mAb37-7 at pH 7 and 4°C were evaluated according to the literature (*18*). The binding energy (ΔG) was calculated using the following equation,

$$\Delta G = -RT\ln K_{ass} \quad (1)$$

where R and T are the gas constant and the absolute temperature in degrees Kelvin.

Activity of Lysozymes against Glycol Chitin—Activities of native and mPEG lysozymes against glycol chitin were measured in 0.1 M acetate buffer of pH 5.5 according to the literature (*21*).

Ion-Exchange HPLC of Lysozyme—Samples of 50 μg of the lysozyme were applied to a column of TSK-gel CM-5PW (7.5 mm×75 mm), which had been equilibrated with 0.05 M phosphate buffer containing 0.1 M NaCl at pH 7. The column was isocratically eluted with the same buffer at a flow rate of 0.5 ml/min.

Evaluation of Stability of Lysozymes Determined by GdnHCl Denaturation—The unfolding transitions of lysozymes induced by GdnHCl were analyzed by observing changes in the tryptophyl fluorescence (emission at 360 nm excited at 280 nm) as a function of the denaturant concentration at pH 5.5 and 35°C according to the literature (*25*). Briefly, the equilibrium constant between the folded state (N) and the fully unfolded state (D), $K_D = D/N$, and the free energy change of unfolding, $\Delta G_D = -RT\ln K_D$, at a given concentration of GdnHCl were calculated from each unfolding curve. To obtain $\Delta G_D(H_2O)$, ΔG_D was plotted against GdnHCl concentration, and a least-squares analysis was used to fit the data to the equation,

$$\Delta G_D = \Delta G_D(H_2O) - m[\text{GdnHCl}] \quad (2)$$

where $\Delta G_D(H_2O)$ is the value of ΔG_D in the absence of GdnHCl, and m is a measure of the dependence of ΔG_D on GdnHCl concentration. The protein concentration was 0.9 μM.

RESULTS

Identification of Selective Modification—To determine the location of the modified site, samples modified with $BrCH_2COO$-mPEG 5000, 2000, or 550 were each reduced, alkylated, and digested with TPCK-trypsin. The elution patterns on RP-HPLC of tryptic peptides from the lysozymes modified with $BrCH_2COO$-mPEG 5000, 2000, and 550 are shown in Fig. 1, C, D, and E, respectively. For comparison, those from native and Asp119-PDE lysozyme are shown in Fig. 1, A and B, respectively. Peaks a and b in Fig. 1A have been assigned to peptides *S*-carboxymethylated Cys115-Arg125 and Gly117-Arg125 from their amino acid composition, respectively (*17*). Peaks c and d in Fig. 1B, which contain Asp119, are slightly shifted from the elution positions of peak a and b in Fig. 1A. Since only carboxylic acid was modified under the modification condi-

tions, the carboxylic acid in Asp119 was suggested to be amidated by (2-pyridyl)-dithioethylamine and to be converted to $CONHCH_2CH_2SCH_2COOH$ by the reduction and alkylation (*17*). In Fig. 1, C-E, peaks c and d, which contain Asp119, disappeared and the no new peaks appeared. From this result, it was concluded that the mPEGs were introduced only into Asp119 in the lysozyme and did not react with the other carboxylic acids.

NMR Analysis of mPEG5000 Modified Lysozyme—To define the location of mPEG5000, the largest mPEG used, on the lysozyme molecule, ^1H-^1H COSY spectra of native and mPEG5000 lysozymes were measured. Their fingerprint regions are shown in Fig. 2, A and B. Several cross-peaks in the fingerprint region of mPEG5000 lysozyme could not be detected due to heavy broadening. The assignment of the detectable signals in the ^1H-^1H COSY spectrum of mPEG5000 lysozyme was carried out based on ^1H-^1H COSY and ^1H-^1H NOESY spectra according to the literature (*26*) (Table I). The chemical shifts for most of the detectable protons were almost identical to those of native lysozyme. This strongly suggested that mPEG5000 did not cause any structural change of the lysozyme molecule.

In comparison with the ^1H-^1H COSY spectrum of native lysozyme, that of mPEG5000 lysozyme showed considerable broadening of the cross-peaks. The amino acid residues involved in these broadenings may have several conformations. Namely, it is possible that these amino acid residues may have interacted with the mPEG5000 moiety. Therefore, we picked up cross-peaks that were not broadened in the ^1H-^1H COSY spectrum of mPEG5000 lysozyme relative to those of native lysozyme. The residues corresponding to the unchanged cross-peaks are marked on the α-carbon tracing of lysozyme (Fig. 3). Many of the marks were separated from the site of introduction of mPEG5000. Because unaffected atoms are not always buried in the molecule, the remote sites were suggested to be less affected by the introduced mPEG5000 molecule. However, we were unable to detect cross-peaks of mPEG5000 and lysozyme on NOESY spectra. This may be due to the high mobility of the mPEG molecule. On the other hand, in order to examine the effect of disappearance of negative charge, COSY analysis of Asn119 lysozyme, in which Asp119 is amidated, was carried out. There was little difference in the spectrum between native and Asn119 lysozyme, indicating that the negative charge at Asp119 did not cause structural change of the lysozyme molecule (data not shown).

Interactions of mPEG Lysozymes with Immobilized Fab Fragment of mAb37-7—We have previously reported that the binding ability of anti-lysozyme mAb 37-7 to lysozyme is reduced by carboxymethylation of the His15 residue, while other modifications such as mutation of His15 to Gly, mononitration of Tyr23, mutation of Asn43 to Asp, conversion of the aspartylglycyl sequence at Asp101Gly102 to an isoaspartylglycyl one, and deletion of Leu129, have no such effect. Thus, we concluded that electrostatic repulsion resulting from the introduction of a carboxyl group at His15 disturbed the association between mAb 37-7 and lysozyme, and that this mAb recognized the prominently exposed region (hills and ridges) around His15 of lysozyme, while His15 itself was not directly involved in the binding (*18*). The arrowhead in Fig. 3 indicates the position of His15. The distance between CαH of His15 and CαH of Asp119 was 22.5 Å based on X-ray crystallography of lysozyme (*27*); therefore, the antibody recognized a region apart from the site of introduction of mPEG. To investigate how much the introduced mPEG disturbs association between the lysozyme molecule and anti-lysozyme mAb37-7, the binding energy between the three mPEG lysozymes and the immobilized Fab fragment of mAb37-7 was evaluated according to our previous method (*18*). The results are shown in Table II and reveal that the binding energy decreased with the increase in molecular weight of the introduced mPEG. This suggests that the introduced PEG molecule partially hindered the association between the immobilized Fab fragment of mAb37-7 and the lysozyme molecule.

Activities of mPEG Lysozyme against Glycol Chitin—The arrow in Fig. 3 indicates the position of the active site cleft. The active site cleft is also separated from the introduced site of mPEG. The activities of mPEG lysozymes against glycol chitin are also shown in Table II. A similar dependency to that of the association of the immobilized Fab fragment of mAb37-7 and the mPEG lysozymes was observed: the activities of mPEG lysozymes decreased with the increase in molecular weight of the introduced mPEG. This result suggests that the introduced PEG molecule partially disturbed the interaction of glycol chitin with the active site cleft.

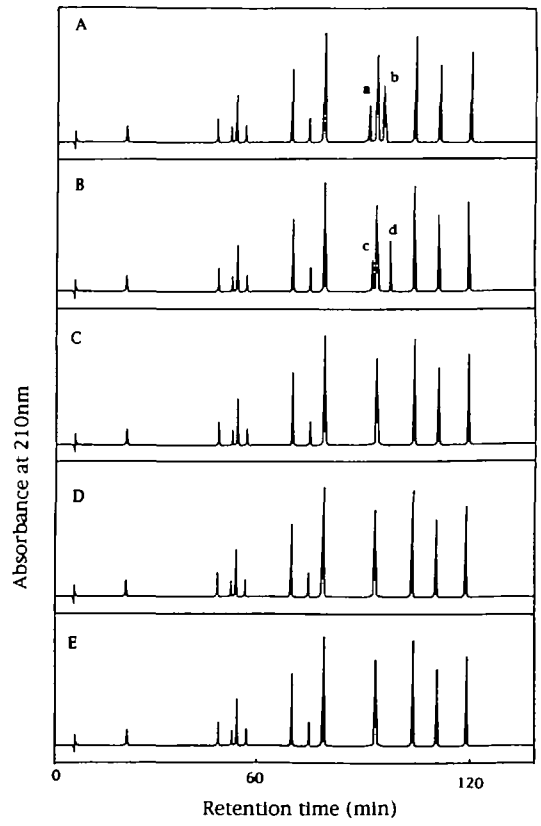

Fig. 1. **RP-HPLC of tryptic peptides derived from tryptic digestion of reduced and *S*-carboxymethylated Asp119-PDE lysozyme and its modified lysozymes with mPEG on Wakosil 5C18-200 (4.6×250 mm).** The column was eluted with a gradient of 40 ml of 1% acetonitrile and 40 ml of 40% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min. A: from native lysozyme, B: from Asp119-PDE lysozyme, C: from lysozyme modified with mPEG5000, D: from lysozyme modified with mPEG2000, E: from lysozyme modified with mPEG550.

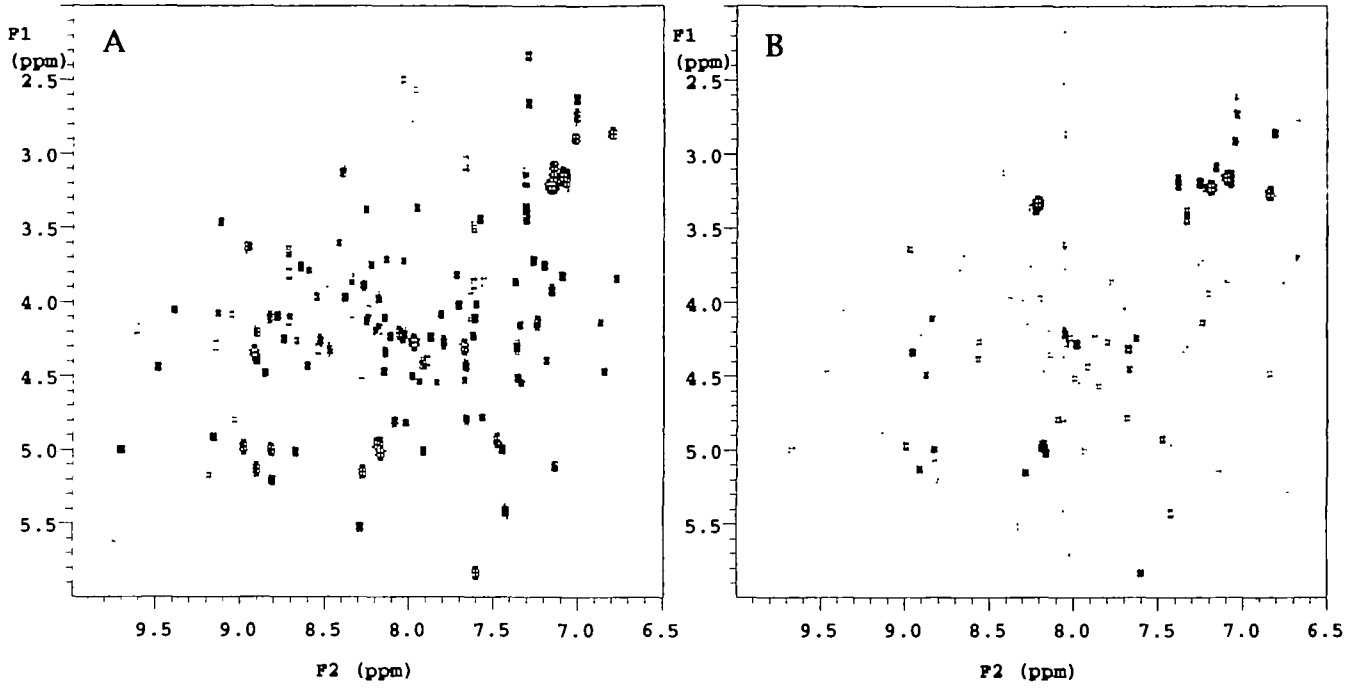

Fig. 2. **The phase-sensitive ^1H-^1H COSY spectra in the fingerprint region of native (A) and mPEG5000 (B) lysozyme.**

TABLE I. **Chemical shifts of the proton resonances in native and mPEG5000 lysozyme at pH 3.8 and 35°C.**[a]

Residues	Native lysozyme		mPEG5000 lysozyme		Residues	Native lysozyme		mPEG5000 lysozyme	
	CH	NH	CH	NH		CH	NH	CH	NH
2	4.93	8.91	4.90	8.90	64	5.78	7.53	5.76	7.51
3	4.17	8.82	4.14	8.82	65	5.47	8.22	5.08	8.19
8	3.72	8.57	3.70	8.57	66	4.95	9.61	4.92	9.58
10	3.93	8.11	3.90	8.11	74	3.71	8.15	3.70	8.15
11	4.24	7.72	4.17	7.78	76	4.40	9.41	4.38	9.37
12	3.41	9.05	3.37	9.02	77	4.18	8.03	4.14	7.96
17	3.88	7.09	3.87	7.11	78	4.95	8.75	4.92	8.73
19	3.92	8.47	3.92	8.29	82	4.19	7.55	4.17	7.54
20	4.17	8.03	4.16	7.98	83	4.19	7.80	4.17	7.78
21	3.59	8.88	3.57	8.87	84	5.06	7.06	5.07	7.05
28	3.71	7.12	3.67	7.16	85	4.43	6.77	4.42	6.75
29	3.39	7.51	3.36	7.50	86	4.22	8.45	4.21	8.48
34	4.25	7.27	4.25	7.25	87	4.91	8.09	4.92	8.09
35	4.39	8.52	4.31	8.47	92	3.09	8.32	3.06	8.31
36	4.51	7.87	4.49	7.86	94	4.96	7.84	4.93	7.84
37	4.42	8.07	4.41	8.07	97	4.09	7.16	4.06	7.15
38	3.82	7.29	3.80	7.28	100	4.39	7.58	4.39	7.58
39	5.37	7.35	5.36	7.33	101	4.75	8.02	4.73	8.00
41	4.38	7.84	4.37	7.82	105	3.80	7.01	3.78	7.00
43	5.10	8.21	5.08	8.19	109	3.58	8.86	3.57	8.88
44	4.98	8.08	4.95	8.07	113	4.46	7.91	4.45	7.90
45	4.43	8.80	4.42	8.78	114	4.26	7.59	4.25	7.58
46	5.09	8.84	5.06	8.82	115	4.47	7.29	4.47	7.29
47	4.06	8.75	4.04	8.75	118	4.72	7.49	4.73	7.59
48	4.51	7.76	4.48	7.79	119	4.97	8.61	5.02	8.73
51	4.84	9.07	4.83	9.05	120	4.30	8.07	4.28	8.04
52	5.16	8.73	5.14	8.72	127	4.89	7.40	4.86	7.38
61	4.04	8.72	4.01	8.71	128	4.29	8.85	4.27	8.86
62	4.36	7.14	4.36	7.10	129	4.24	7.90	4.21	7.89
63	4.94	7.35	4.91	7.34					

[a] In ppm.

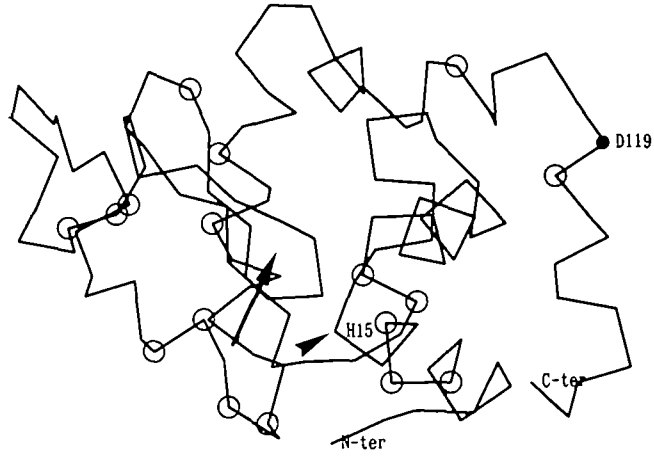

Fig. 3. **α-Carbon tracing of native lysozyme.** Alpha-carbons of the residues in mPEG5000 lysozyme whose protons did not show the change in peak intensity from those of native lysozyme are marked with open circles. Asp119 is marked with a filled circle. The arrow and arrowhead indicate the active site cleft and the position of His15, respectively.

Evaluation of Net Charges of mPEG Lysozyme by Ion-Exchange Chromatography—To examine how much the polyethylene glycol molecule covered the charged groups on the lysozyme molecule, the net charges of mPEG lysozymes were evaluated by comparison of their retention times on ion-exchange chromatography with those of native and modified lysozymes whose net charges were known. The elution patterns are shown in Fig. 4. Under the elution condition (pH 7), the net charge of native lysozyme is +7 (Fig. 4A). That of carboxymethylated His15 lysozyme would be +6 (Fig. 4D) and those of 6-, 127-*S* carboxymeth-

TABLE II. **The binding energy between lysozymes and the Fab fragment of mAb37-7 and the activity of lysozyme against glycol chitin.**

Lysozyme	Binding energy[a] (kcal/mol)	Activity[b] (%)
Native	−3.8	100
mPEG500	−3.3	90
mPEG2000	−3.1	75
mPEG5000	−2.4	38

[a]pH 7.5 and 4°C. [b]pH 5.5 and 40°C.

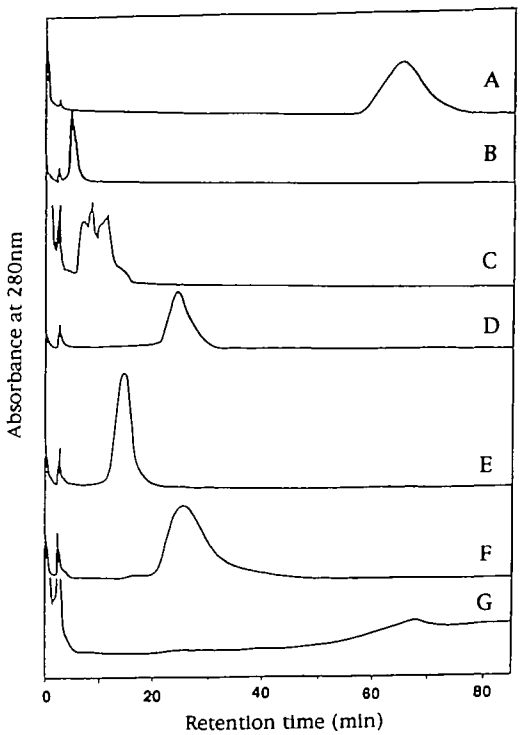

Fig. 4. **Ion-exchange chromatography of native and modified lysozymes.** A: native lysozyme, B: 6-,127-*S* carboxymethylated lysozyme, C: diacetylated lysozyme, D: 15 carboxymethylated lysozyme, E: mPEG5000 lysozyme, F: mPEG2000 lysozyme, G: mPEG550 lysozyme. The column (TSK-gel CM-5PW, 7.5×75 mm) was isocratically eluted with 0.05 M phosphate buffer, pH 7, containing 0.1 M NaCl at a flow rate of 1.0 ml/min.

ylated lysozyme and diacetylated lysozyme could be regarded as +5 (Fig. 4, B and C). mPEG5000 lysozyme (Fig. 4E) eluted between carboxymethylated His15 lysozyme and 6-, 127-*S* carboxymethylated lysozyme or diacetylated lysozyme. mPEG2000 and mPEG550 lysozyme had the same retention time as carboxymethylated His15 lysozyme and native lysozyme, respectively (Fig. 4, F and G). Because one negative charge at Asp119 has disappeared due to the modification, the apparent positive net charge of 2-3, 2, and 1 in the mPEG5000, 2000, and 550 lysozymes, respectively, was regarded as having decreased. Thus, the apparent positive net charge of the lysozyme molecule also decreased with the increase in molecular weight of the introduced mPEG.

Stabilities of mPEG Lysozymes—To examine the effect on the stability of lysozyme of modification with mPEGs of various lengths, $\Delta G_D(H_2O)$ of native, Asn119 and mPEG lysozymes at pH 5.5 and 35°C were determined. Under the conditions employed, the reversibility of unfolding in each experiment was good, indicating that aggregation did not occur. Table III lists $\Delta G_D(H_2O)$ of native, Asn119 and PEG modified lysozyme. The stabilities of mPEG lysozymes were smaller than those of native and Asn119 lysozyme and decreased with the decrease in molecular weights of the mPEG molecule.

TABLE III. **Stabilization energy of native, Asn119, and mPEG lysozymes in aqueous solution at pH 5.5 and 35°C.**

Lysozyme	$\Delta G_D(H_2O)$ (kcal/mol)
Native	10.1
Asn119	10.0
mPEG550	8.0
mPEG2000	8.3
mPEG5000	9.0

DISCUSSION

Hen lysozyme contains a single histidine residue, which can be alkylated with haloacetic acids or their derivatives at pH 5-5.5 (*13, 14*). When lysozyme was allowed to react with a large excess of $BrCH_2COO$-mPEG at pH 5.5 and 40°C for 1 day according to the above literature, however, the major reactive site was the ε-amino group of the Lys residue, and His15 scarcely reacted with $BrCH_2COO$-mPEG (data not shown). His15 is relatively buried in the molecule (*27*), and its interaction with $BrCH_2COO$-mPEG is hindered by the bulkiness of the reagent. Thus, we tried to introduce mPEG into selectively modified Asp119. Based on the disappearance of tryptic peptides c and d in Fig. 1B, which contain Asp119, the results in Fig. 1, C-E, indicated that mPEG was selectively introduced into Asp119. Why the selective modification of mPEG occurs at Asp119 may be because Asp119 is highly exposed to the solvent (*27*), and the thiol group ($pK_a = 7$-8) somewhat dissociates and rapidly reacts with the haloacetic acid group under these reaction conditions.

In this report, we showed that the three-dimensional conformation of mPEG lysozyme was similar to that of native lysozyme by analyzing their NMR spectra (Fig. 2 and Table I). On the other hand, the amidation of a carboxyl groups of Asp119 resulted in loss of a negative charge, but again the three-dimensional conformation was similar to that of unmodified lysozyme. Therefore, introduction of mPEG into Asp119 in lysozyme did not cause drastic structural change of the lysozyme molecule.

Polyethylene glycol is a straight-chain molecule, and the polymer consists of $-CH_2CH_2O-$ units. Because the unit is at most 3.8 Å in length, the maximum length of mPEG5000, 2000, and 550 were estimated to be 430, 170, and 45 Å, respectively. If a lysozyme molecule is a sphere of 30 Å diameter, the maximum length of mPEG5000, 2000, and 550 would be 15, 6, and 1.5 times longer than the diameter of lysozyme (Fig. 5). Therefore, it was supposed that the mPEG molecules introduced into Asp119 might spread over the lysozyme surface, resulting in highly decreased lysozyme activity and binding activity of anti-lysozyme mAb 37-7. However, the mPEG molecules did not abolish the enzymatic activities and the antigen-antibody interactions (Table II). The distances of the active site cleft and the His15 residue from the introduced site (Asp119) were 20-30 Å according to X-ray crystallography (*27*). This

distance is shorter than the length of mPEG550, the shortest in mPEGs used in this study. The results thus suggested that the introduced mPEG was confined to the region of the site of introduction. This idea was supported by NMR analysis, which showed that many of the atoms unaffected by mPEG5000 were present in regions separated from this site (Fig. 3).

The apparent positive net charge decreased with the increase in molecular weight of the introduced mPEG (Fig. 4). The decreased charge was 1, 2, and 2-3 for mPEG550, mPEG2000, and mPEG5000, respectively. To analyze this finding, charged atoms are plotted in Fig. 6 centered on Asp119, based on X-ray crystallography (*27*). Considering that the disappearance of the positive charge around Asp119 caused a decrease in the positive net charge of mPEG lysozymes, we concluded that Arg125 in mPEG550 lysozyme, Arg125 and Arg114 in mPEG2000 lysozyme, and Arg125, Arg114, Arg5, Lys33, and Asp18 in mPEG5000 lysozyme were covered by the respective mPEGs. On this basis, the possible range where each mPEG may be present is surrounded by a dotted line in Fig. 6. The fact that the dotted circles are much smaller than the chain length is consistent with the idea that the introduced mPEG may prefer a folded conformation on the surface of lysozyme molecule centered at Asp119. However, the conformation of mPEG may be flexible rather than rigid, because it affected to some extent regions, such as the active site, remote from the introduced site.

T cells play a critical role in humoral immune response. For example, B cells require T-cell-help in an early stage of immune reactions to produce antibodies towards protein antigens. Recognition of protein antigens by T cells requires their endocytosis and intracellular proteolysis by antigen-presenting cells to produce immunogenic peptides that are bound and presented by MHC class II molecules (*28*). Thus, digestion of protein antigens by intracellular proteases is a key step in T cell activation. Protease digestion is considered to proceed *via* the unfolded state of the protein. This kind of irreversible process can be described by the following general scheme:

$$N \rightleftharpoons D \xrightarrow{P} \text{peptide} \qquad (3)$$

where N and D are the folded and the unfolded states and P is the protease (*29*). As was shown in Table III, mPEG lysozymes were less stable than native lysozyme. Since Asn119 lysozyme has similar stability to native lysozyme, the introduction of mPEG into the lysozyme must have caused the decrease in lysozyme stability. This result indicated that the introduction of mPEGs into Asp119 did not reduce T cell activation capacity of lysozymes. On

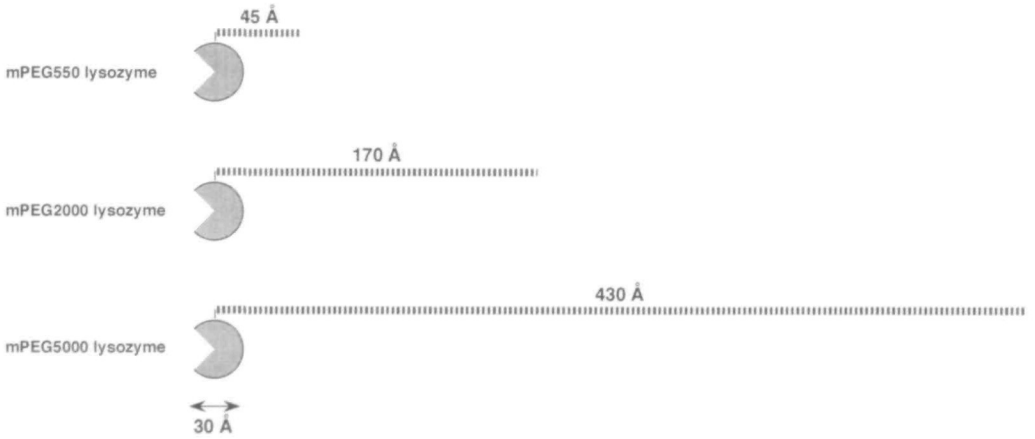

Fig. 5. The relationship between the diameter of lysozyme and the maximum length of various mPEGs.

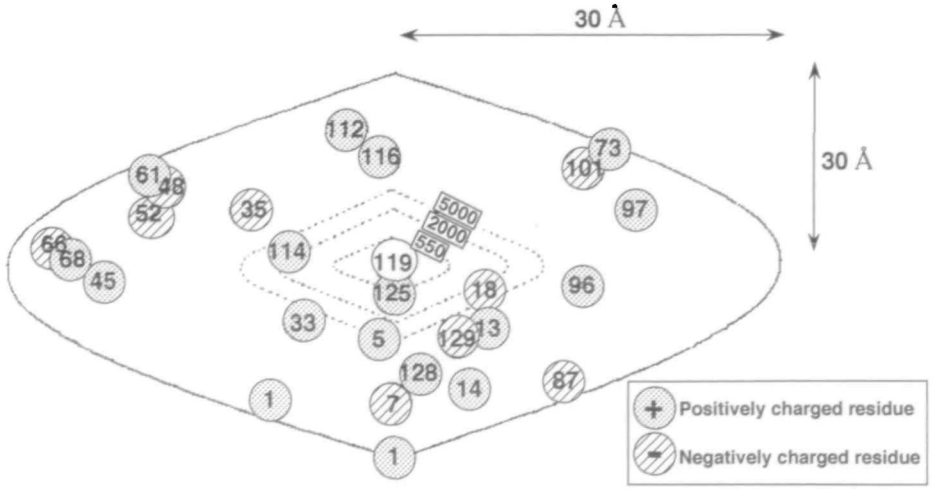

Fig. 6. Plots of charged atoms in lysozyme centered at β-carbon of Asp119. Positive and negative charges are expressed by filled circles with dots and slashes, respectively. The possible ranges of occupation by mPEGs of various molecular weights are indicated by dotted circles.

comparing the T cell response of unmodified lysozyme and mPEG5000 lysozyme, no difference was observed in the proliferative responses and the IL-2 production activities of lymph node T cells from HEL immunized mice (unpublished data). However, we have demonstrated that a mPEG5000 lysozyme in which almost all seven amino groups were modified was not susceptible to protease digestion, resulting in reduction of T cell response (*10*). These results demonstrated that low introduction of mPEG into protein antigens was not effective in avoiding T cell activation.

On the other hand, B cells recognize antigenic epitopes on the surface of protein antigens with their membrane immunogloblin receptor. Hence, if mPEG molecules are introduced into B cell epitopes, the antigenicity may be nullified by inhibiting an antigen-antibody interaction. As was shown in Fig. 6, the mPEG5000 molecule covered at most 500 Å^2 of lysozyme surface. Thus, in order to reduce B cell responses, it is reasonable to consider that a lysozyme may require the introduction of a substantial amount of mPEG5000 in order for it to cover the whole surface. We measured the reactivity of anti-HEL sera to mPEG5000 lysozyme with ELISA, it had the same potency as unmodified lysozyme had. However, a mPEG5000 lysozyme in which almost all seven amino groups were modified had a very low reactivity to anti-HEL sera (unpublished data). In the case of uricase and asparaginase, the complete disappearance of their antigenicities required the introduction of a large amount of mPEG5000 into the proteins, to the extent of 44 and 79% of the amino groups, respectively (*30, 31*). Thus, our result not only confirmed the idea that the ability to mask epitopes could theoretically be expanded by introducing additional functional sites for mPEG addition (*32*), it also gave a rough value for the expansion of the mPEG molecule on the antigen surface. We also showed that the thiol group in a protein rapidly reacted with $BrCH_2COO$-mPEG molecules under physiological conditions. Therefore, we may replace an amino acid residue in a protein with Cys, which will selectively react with $BrCH_2COO$-mPEG molecule, by genetic engineering in order to reduce the immunogenicity of the protein. The information on how far the mPEG molecule expands will be helpful in determining the position and the number of amino acids that need to be replace in order to reduce the immunogenicity of a protein.

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