J. Biochem. 119, 157-161 (1996)

Stabilization of Lysozyme by Introducing *N*-Glycosylation Signal Sequence

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Received for publication, September 20, 1995

We designed mutant lysozymes with *N*-glycosylation signal sequences (Asn48-Gly49-Thr-50 and Asn87-Ile88-Thr89) by substituting Asp to Asn at positions 48 and 87. When these mutant lysozymes were expressed by using yeast (*Saccharomyces cerevisiae*) in Burkholder minimum medium, *N*-glycosylation occurred in both lysozymes. The mutant lysozyme with the oligosaccharide at Asn87 showed a similar character to a reported polymannosyl lysozyme [Nakamura, Takasaki, Kobayashi, and Kato (1993) *J. Biol. Chem.* 268, 12706-12712; Kato, Takasaki, and Ban (1994) *FEBS Lett.* 355, 76-80]. As judged from the thermodynamic stabilities of the lysozymes obtained by the guanidine hydrochloride denaturation method, the oligosaccharide-bearing mutant lysozymes were more stable by 0.4-1.6 kcal/mol than the corresponding unglycosylated lysozymes. Therefore, it is suggested that the introduction of an *N*-glycosylation signal sequence into a protein is an effective means to increase the stability of the protein.

Key words: *N*-glycosylation signal, lysozyme, stabilization.

If an enzyme protein is made more stable, it can remain intact at higher temperature where it will exhibit a higher activity (*1*). Moreover, as the stabilized protein tends to retain the native folded structure, it becomes resistant against chemical reactions (*2*). Therefore, such stabilization of a protein could be of value in medical or industrial applications.

A protein is usually in an equilibrium between the folded (N) and unfolded (D) states (N⇌D, $K = [N]/[D]$, where K is the equilibrium constant for reversible denaturation). Under physiological conditions, the equilibrium greatly favors the folded state ($K \ll 1$). For the thermodynamic stabilization of a protein, it is necessary to increase the energy gap between the folded and unfolded states. For this purpose, the addition of additives such as glycerol (*3*), glucose (*4*), and sarcosine (*5*) to the protein solution is known to be effective. Moreover, the interaction between a protein and additives may increase with an increase in effective concentration of the additives, especially if the additives are covalently attached to the protein.

Marshall demonstrated that asparagine-linked (*N*-linked) glycosylation occurred at Asn-X-Ser/Thr sequences in a protein (*6*). Nakamura *et al.* have succeeded in glycosylation of lysozyme by introduction of Asn-X-Ser/Thr sequences and showed that the glycosylated lysozyme had remarkable heat stability (*7*). However, when we evaluate the thermal stability of a protein based on its residual activity, an irreversible process should be included as follows,

$$N \rightleftharpoons D \xrightarrow{X} P \qquad (1)$$

where P is the irreversibly denatured product and X is a chemical reaction, aggregation, *etc.* If the oligosaccharide depresses only the irreversible reactions, the modified lysozyme may not be thermodynamically more stable. Thus, it is of interest to examine whether modification with oligosaccharide thermodynamically stabilizes lysozyme or not. Glycosylation of lysozyme occurred at only two sites (Asn19 and Asn49) among Asn residues at positions 19, 49, 67, 70, and 103 when the appropriate sequences Asn-X-Ser/Thr were introduced (*7, 8*).

We examined whether glycosylation increased the thermodynamic stability and whether glycosylation occurred at Asn-X-Ser/Thr sequences at other sites in lysozyme. In this paper, we show that glycosylation also occurred at the sequences Asn48-Gly49-Ser50 and Asn87-Ile88-Thr89 in lysozyme, and that the thermodynamic stability of the lysozyme was increased by the glycosylation.

MATERIALS AND METHODS

Materials—Restriction enzymes, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were purchased from either Takara Shuzo (Kyoto) or New England Biolabs (Beverly). DNA sequencing kits (Sequenase) were purchased from Amersham Japan (Tokyo), and CM-Toyopearl 650M, a cation-exchange resin for purification of secreted hen lysozymes, was obtained from Tosoh (Tokyo). Columns of Wakopak 5C18 column (mesh

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Abbreviations: CM Cys, carboxymethylated cysteine; GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; Asn48 lysozyme, a mutant lysozyme in which Asp48 is mutated to Asn; Asn48-HM lysozyme, a mutant lysozyme in which Asn48 is modified with a sugar chain of high mannose type; Asn87 lysozyme, a mutant lysozyme in which Asp87 is mutated to Asn; Asn87-HM lysozyme, a mutant lysozyme in which Asn87 is modified with a sugar chain of high mannose type; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

200) and CM-Cellulofine C-500 P-9 were obtained from Wako Pure Chemicals Institute (Osaka) and Chisso (Tokyo), respectively. Glycol chitin was prepared according to the literature (*9*).

Mutant Lysozymes—Mutant lysozymes were prepared as described before (*10*). The structures of the mutagenic primers used for site-directed mutagenesis to replace Asp48 with Asn48 and Asp87 with Asn87 were 5′-GTAAC-ACCAACGGGAGTAC-3′ and 5′-CGCTGTTATGTTTGA-GCTCAG-3′, respectively. The mutations in the lysozyme gene were confirmed using a DNA sequence analyzer.

Purification and Identification of Lysozymes Secreted from Yeast—Each yeast (*Saccharomyces cerevisiae* AH22) transformant was cultivated in Burkholder minimum medium (*11*) at 30°C for 125 h to express and secrete each lysozyme from yeast under routine conditions as described before (*10*). As for cultivation of Asn87 lysozyme in the presence of tunicamycin, after the AH22 transformant had been cultivated at 30°C for 50 h, tunicamycin (2 mg/liter) was added to the culture medium and cultivation was continued in Burkholder minimum medium (*11*) at 30°C for 75 h. Lysozyme secreted in the culture medium was isolated by cation-exchange chromatography on a column (3.5×27 cm) of CM-Toyopearl 650 M with a gradient formed from 1,000 ml of 0.05 M phosphate buffer at pH 7 and 1,000 ml of the same buffer containing 0.5 M NaCl at 4°C. Lysozyme elution was monitored by measuring the absorbance of effluents at 280 nm. The eluted lysozyme was dialyzed against distilled water. The dialyzate was adsorbed and chromatographed on a column (9×100 mm) of CM-Cellulofine C-500 P-9 in an HPLC system using a gradient formed from 50 ml of 0.05 M acetate buffer and 50 ml of the same buffer containing 1 M NaCl (pH 5) at a flow rate of 1.0 ml/min. The eluted lysozyme was dialyzed against water and then lyophilized.

Analytical Methods—Determination of DNA sequences was carried out using an Applied Biosystems Model 373A DNA sequencer. Amino acid analyses were performed on a Hitachi L-8500 amino acid analyzer after hydrolysis of samples in azeotropic 6 N HCl vapor containing 1% phenol under vacuum at 150°C for 60 min. The NH_2-terminal sequences of peptide samples were determined with an Applied Biosystems Model 473/120A gas-phase protein sequencer. Digestion of reduced and *S*-carboxymethylated lysozyme with TPCK-trypsin and separation of the resulting peptides on RP-HPLC were accomplished as described by Ueda *et al.* (*12*).

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out according to the previous report (*7*).

Unfolding Equilibrium—Unfolding equilibria of lysozymes with GdnHCl were followed in a 0.1 M acetate buffer at pH 5.5 and 35°C by measuring fluorescence at 360 nm (excited at 280 nm). The protein concentration was 0.9 μM.

Enzymatic Activity—The activities of lysozymes against glycol chitin were determined in 0.1 M sodium acetate at pH 5.5 and 40°C as described previously (*9*).

RESULTS

Expression and Secretion of Asn48 Lysozyme and Its Derivative—Asp48 is located in the β-sheet in lysozyme and the following sequence is Gly-Ser (*13*). Therefore, we introduced a glycosylation signal sequence by the replacement of Asp48 with Asn. Lysozyme secreted from transfected yeast in the culture medium under the routine conditions (*10*) was purified by cation-exchange chromatography. Two major peaks were eluted (Fig. 1A). Figure 1B shows the SDS-polyacrylamide gel electrophoretic patterns of the wild type and the mutated enzymes in fractions 1-1 and 1-2. The SDS-PAGE analysis revealed that the derivative in fraction 1-2 was the same size as the wild-type lysozyme, whereas the derivative in fraction 1-1 had a larger molecular size (panel A). The SDS-PAGE patterns stained with carbohydrate-specific reagent (periodic acid/Schiff reagent) showed a single stained band (panel B). This result suggested that the enzyme in fraction 1-1 was glycosylated with a small oligosaccharide chain in the *N*-linked form. From the amino acid composition after acid hydrolysis, the amino acid compositions of the enzyme in fraction 1-1 and 1-2 were identical to that of wild type, and the enzyme in fraction 1-1 contained 2 residues of glucosamine per molecule. Among asparagine-linked sugar chains, only the high-mannose type contains 2 residues of *N*-acetylglucosamine per one signal sequence. Therefore, it was suggested that a sugar chain of high mannose type was linked to the enzyme in fraction 1-1.

In order to confirm the modified site, the enzyme in fraction 1-1 was reduced, *S*-alkylated and digested with TPCK-trypsin (see "MATERIALS AND METHODS"). The tryptic peptides derived from the enzyme in fraction 1-1 on RP-HPLC are shown in Fig. 2B. For comparison, those

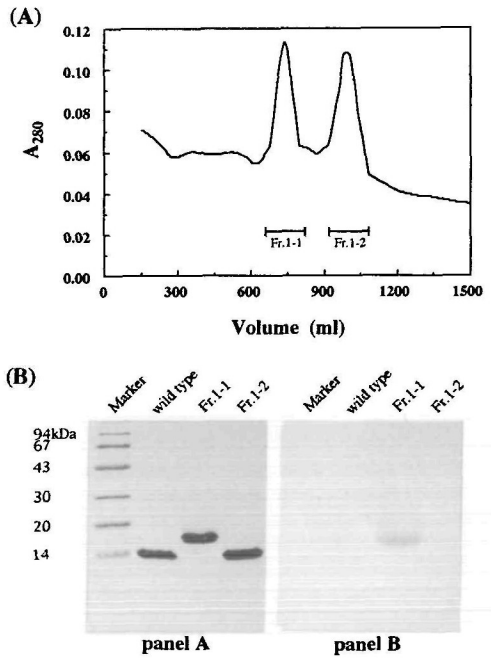

Fig. 1. **(A) Ion-exchange chromatography of Asn48 lysozyme secreted from yeast on CM-Toyopearl (35×270 mm).** The column was eluted with a gradient formed from 1,000 ml of 0.05 M phosphate buffer and 1,000 ml of the same buffer containing 0.5 M NaCl at pH 7 and 4°C. **(B) Electrophoretic patterns of wild-type and mutant lysozymes secreted from *S. cerevisiae*.** The gel sheets were stained for proteins and carbohydrates with Coomassie Brilliant Blue (panel A) and periodic acid-Fuchsin (panel B), respectively.

from the wild type are shown in Fig. 2A [the assignment of the peptides was carried out previously (*12*)]. In Fig. 2B, peptide a (Asn46-Thr-Asn-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg61) disappeared and a new peptide c appeared. The NH_2-terminal amino acid sequence of peptide c was determined to be Asn-Thr, but the next amino acid was not detected. Thus, it was concluded that the modification of the sugar chain had occurred at Asn48, since peptide a was the only tryptic peptide that has the Asn-Thr sequence at the N-terminus. From the above results, we concluded that the enzyme in fraction 1-1 was the derivative in which Asn48 was modified with a sugar chain of high mannose type (Asn48-HM lysozyme). Moreover, the enzyme in fraction 1-2 was concluded to be Asn48 lysozyme in which Asp48 was mutated to Asn (Asn48 lysozyme). The yields of Asn48-HM lysozyme and Asn48 lysozyme were 0.07 and 0.07 mg/liter, respectively. These mutant lysozymes were further purified by HPLC using a column of CM-Cellulofine C-500 P-9, a cation-exchanger, at pH 5 before the examination of their properties.

Expression and Secretion of Asn87 Lysozyme and Its Derivative—Asp87 is located in the α-helix in lysozyme and the following sequences are Ile-Thr (*13*). Therefore, we introduced a glycosylation signal sequence by the replacement of Asp87 with Asn. Firstly, lysozyme secreted from yeast in the culture medium under the routine conditions (*10*) was purified by cation-exchange chromatography. Two major broadened peaks were eluted (Fig. 3A). Figure 3B shows the SDS-polyacrylamide gel electrophoretic patterns of the wild type and the enzymes in fractions 2-1, 2-2, and 2-3. The SDS-PAGE analysis revealed that these enzymes were larger than the wild-type lysozyme, especially, that in fraction 2-1, and 2-2 consisted of a very large protein (panel A). On the other hand, all bands except for the wild type were stained with carbohydrate-specific reagent (periodic acid/Schiff reagent) (panel B). This result suggested that the enzymes in these fractions were *N*-glycosylated with a small oligosaccharide or with a large polysaccharide.

Since we could not obtain enough Asn87 lysozyme to examine as reference protein, the AH22 transformant was cultivated in the presence of tunicamycin, which is known to depress *N*-glycosylation (*14*). Lysozyme secreted into the culture medium under these conditions was purified by cation-exchange chromatography. Three peaks were eluted (Fig. 4A). Figure 4B shows the SDS-polyacrylamide gel electrophoretic patterns of the wild type and the enzymes in fractions 3-1, 3-2, and 3-3. The SDS-PAGE analysis revealed that the enzyme in fraction 3-3 was the same size as the wild-type lysozyme, whereas the enzyme in fraction 3-1 was larger (panel A). The protein in fraction 3-3 was not stained with carbohydrate-specific reagent (periodic acid/Schiff reagent) (panel B), suggesting that the enzyme in fraction 3-3 was not glycosylated.

From the amino acid composition after acid hydrolysis, the amino acid composition of the enzyme in fraction 2-2 and 2-3 were identical to that of wild type, and the enzyme in fraction 2-2 contained 2 mol glucosamine per molecule. Therefore, it was considered that a sugar chain of high mannose type was linked to the enzyme in fraction 2-2. In order to confirm the modified site, the enzyme in fraction 2-2 was reduced, *S*-alkylated and digested with TPCK-trypsin (see "MATERIALS AND METHODS"). The RP-HPLC pattern of the tryptic peptides is shown in Fig. 2C. In Fig.

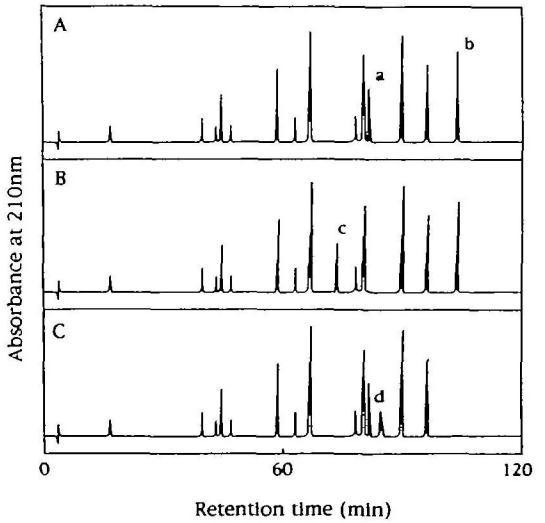

Fig. 2. **Reversed-phase HPLC of tryptic peptides obtained from reduced and *S*-carboxymethylated lysozymes on a column (4×250 mm) of Wakopak 5C18.** The column was eluted with a gradient formed from 50 ml of 1% acetonitrile and 50 ml of 50% acetonitrile, both containing 0.1% concentrated HCl at a flow rate of 0.6 ml/min. (A), wild type; (B), the derivative in fraction 1-1 in Fig. 1A; (C), the derivative in fraction 2-2 in Fig. 3A.

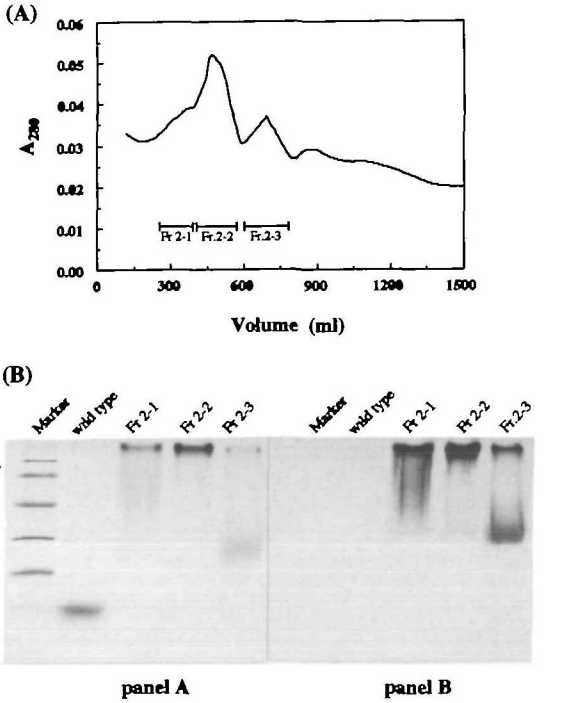

Fig. 3. **(A) Ion-exchange chromatography of Asn87 lysozyme secreted from yeast on CM-Toyopearl (35×270 mm).** The column was eluted with a gradient formed from 1,000 ml of 0.05 M phosphate buffer and 1,000 ml of the same buffer containing 0.5 M NaCl at pH 7 and 4°C. **(B) Electrophoretic patterns of wild-type and mutant lysozymes secreted from *S. cerevisiae*.** The gel sheets were stained for proteins and carbohydrates with Coomassie Brilliant Blue (panel A) and periodic acid-Fuchsin (panel B), respectively.

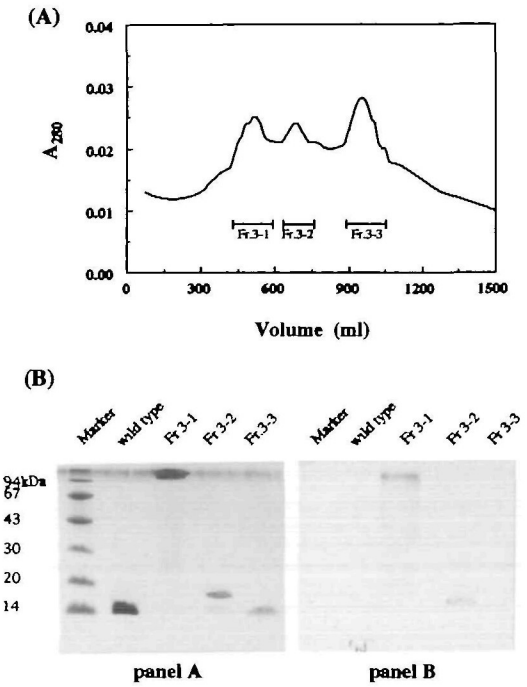

Fig. 4. **(A) Ion-exchange chromatography of Asn87 lysozyme secreted from yeast in the presence of tunicamycin on CM-Toyopearl (35×270 mm).** The column was eluted with a gradient formed from 1,000 ml of 0.05 M phosphate buffer and 1,000 ml of the same buffer containing 0.5 M NaCl at pH 7 and 4°C. **(B) Electrophoretic patterns of wild-type and mutant lysozymes secreted from *S. cerevisiae*.** The gel sheets were stained for proteins and carbohydrates with Coomassie Brilliant Blue (panel A) and periodic acid-Fuchsin (panel B), respectively.

2C, peptide b (Asn74-Leu-CMCys-Asn-Ile-Pro-CMCys-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-CMCys-Ala-Lys96) disappeared and a new broad peak of peptide d appeared. The NH_2-terminal amino acid sequence of peptide d was determined to be Asn74-Leu-CMCys-Asn-Ile-Pro-CMCys-Ser-Ala-Leu-Leu-Ser-Ser86, but the next amino acid was not detected. It was concluded that the modification with a sugar chain had occurred at Asn87. Thus, the enzyme in fraction 2-2 was a lysozyme derivative in which Asn87 was modified with a sugar chain of high mannose type (Asn87-HM lysozyme). Moreover, the enzyme in fraction 3-3 was concluded to be Asn87 lysozyme in which Asp87 had been mutated to Asn (Asn87 lysozyme). The yields of Asn87-HM lysozyme and Asn87 lysozyme were 0.03 and 0.01 mg/liter, respectively. These mutant lysozymes were further purified by HPLC using a column of CM-Cellulofine C-500 P-9, a cation-exchanger, at pH 5 before the examination of their properties.

Conformational Stabilities of Wild Type and Mutated Lysozymes Determined by GdnHCl Denaturation—The unfolding transitions of wild type, Asn48, Asn48-HM, Asn87, and Asn87-HM lysozymes induced by GdnHCl were analyzed by observing changes in the tryptophyl fluorescence (emission at 360 nm and excitation at 280 nm) as a function of the denaturant concentration at pH 5.5 and 35°C. Since lysozyme is fully unfolded in the concentrated GdnHCl (*15*), the difference in tryptophyl fluorescence was attributed to the transition between the folded state (N) and the fully unfolded state (D). Therefore, the GdnHCl-induced denaturation curves were analyzed by fitting the data to the equilibration process N⇌D (*16*). The equilibrium constant between the folded and the fully unfolded states, $K_D = D/N$, 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. In all cases, ΔG_D was found to vary linearly with GdnHCl concentration, and a least-squares analysis was used to fit the data to the equation,

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

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 (*16*). At the midpoint of GdnHCl denaturation, $C_{1/2} = \Delta G_D(H_2O)/m$, because $\Delta G_D = 0$ at $C_{1/2}$.

TABLE I. **Thermodynamic parameters characterizing the GdnHCl denaturation of wild type and mutant lysozymes at pH 5.5 and 35°C.**

Lysozyme	$\Delta G_D(H_2O)$ (kcal/mol)	m (kcal/mol/M)	$C_{1/2}$ (M)
Wild	10.1±0.10	2.8	3.62
Asn48	9.4±0.13	2.8	3.36
Asn48-HM	9.8±0.12	3.0	3.26
Asn87	9.1±0.14	2.8	3.24
Asn87-HM	10.7±0.12	3.1	3.45

In Table I, the values of $C_{1/2}$ and $\Delta G_D(H_2O)$ as well as m are summarized. The stabilities of Asn48-HM and Asn87-HM lysozyme were higher than those of Asn48 and Asn87 lysozyme, respectively. Because each value of $C_{1/2}$ obtained by increasing GdnHCl concentration (N→D) was identical to that obtained by decreasing GdnHCl concentration (D→N), the denaturation of the lysozyme may be reversible.

Enzymatic Activity of Glycosylated Lysozymes—Lysozyme is a carbohydrate hydrolase that catalyzes the hydrolysis of β-1,4 glycosidic bonds of polysaccharides such as *N*-acetylglucosamine homopolymer (chitin) (*13*). In order to examine the effect of the introduction of the sugar chain on the activity of lysozyme, lysozyme activity against glycol chitin was measured. The enzymatic activities of Asn48-HM and Asn87-HM lysozyme against glycol chitin were 30 and 100% of that of the wild type lysozyme, respectively.

DISCUSSION

Mutant lysozymes with the sequences Asn19-Tyr20-Thr21 and Asn49-Ser50-Thr51 were reported to be expressed in large polymannose chain ($Man_{3\sim10}GlcNAc_2$)-linked forms in the yeast *S. cerevisiae* (*7, 8*). In the present paper, a mutant lysozyme with the sequence Asn87-Ile88-Thr89 was expressed as a large oligosaccharide chain form. In SDS-PAGE, a stained broad band appeared near the boundary between the stacking and separating gels. The behavior on SDS-PAGE was similar to the previous results (*7, 8*). Moreover, two residues of glucosamine per molecule of lysozyme were detected in the sample after acid hydrolysis of the glycosylated lysozyme. Therefore, the oligosaccharide linked to Asn87 in the mutant lysozyme was suggested to be a polymannosyl chain, as in the previous reports (*7, 8*). On the other hand, a mutant lysozyme with the sequence Asn48-Gly49-Ser50 carried a small oligosaccharide chain as judged from its behavior on SDS-PAGE.

Moreover, two residues of glucosamine per molecule were detected in the sample after acid hydrolysis of the glycosylated lysozyme. Therefore, a small oligosaccharide was suggested to be linked to Asn48 in the mutant lysozyme, as also found in the previous report (*7*).

To investigate the glycosylation sequences, Asn-X-Ser/Thr, we mutated Asp48 and Asp87 to Asn. In general, electrostatic interaction contributes to the stability of a protein, and these modifications may affect the stability under the conditions of measurement (pH 5.5) because the pK_a values of Asp48 and Asp87 in the presence of 400 mM NaCl were 3.2 and 2.7, respectively, and both pK_as are lower than that of the aspartic residue (4.0) (*17*). Therefore, we prepared reference samples devoid of oligosaccharide, Asn48 and Asn87 lysozymes. Asn48 lysozyme was easily obtained, but Asn87 lysozyme devoid of oligosaccharide could not be readily obtained under the routine conditions (*10*). However, it was obtained in the presence of tunicamycin, an inhibitor of glycosylation. Therefore, the addition of tunicamycin to the culture may be applicable to obtain mutant protein without bearing a sugar chain in the mutant protein with *N*-glycosylation signal sequence.

A comparison of the stability of lysozymes bearing oligosaccharide with that of the Asn lysozymes showed that the thermodynamic stability was clearly increased. Asn87-HM lysozyme was more stable than wild type lysozyme, although the electrostatic interaction derived from aspartic acid had been eliminated. The stabilization energy owing to glycosylation at position 48 or 87 was 0.4 or 1.6 kcal/mol, respectively. Judging from the deviation, the difference in stabilization energy between Asn48 and Asn48-HM lysozyme was found to be significant. Since the stabilization energy of a native protein in aqueous solution is at most 5-15 kcal/mol, glycosylation appears to be an effective method of stabilization. The stabilization of a protein by oligosaccharide has been explained in terms of "preferential hydration" (which states that waters are compressed on a protein surface with the help of reagents) (*18*), which might affect on unfolded state of a protein more than the folded state, because hydrophobic residues are more exposed to the solvent in the unfolded state. The value of m increased with increase of the content of oligosaccharide (Table I). Therefore, the interaction of GdnHCl with the unfolded structure in lysozyme with oligosaccharide might be favored by preferential hydration owing to the addition of oligosaccharide.

Asn-HM lysozyme showed full activity of wild type lysozyme against glycol chitin. Asp87 is located at the N-terminus of the helix 88-98 apart from the active site (*13*). Therefore, the introduced sugar chain may not affect the glycol chitin activity of lysozyme. On the other hand, Asp48 is located in the β-sheet in the active site cleft (*13*), and the activity of Asn48-HM lysozyme against glycol chitin was 30% of that of the wild type lysozyme. Chitin occupies the whole saccharide binding site (subsites A-F) of lysozyme, especially the left side of binding site involved in equilibrium binding to the substrate as for the lower subsites (subsites D-F) (*19*). Since Asp48 is also located near the left side binding site of saccharide binding one, the sugar chain may hinder the access of glycol chitin to the binding site at lower subsite. However, the activity of mutant lysozyme with a polymannosyl group at position 49 against glycol chitin was 91% of that of wild type lysozyme (*7*). As positions 48 and 49 are both located in the β-sheet region, the side chains of neighboring residues should be oriented in opposite directions. Indeed, X-ray crystallographic structure analysis indicated that the side chain of Asp48 is oriented to the active site cleft (*13*). Therefore, in mutant lysozyme with a polymannosyl group at position 49, the sugar chain may be oriented away from the active site cleft.

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