Site-Specific *O*-Glycosylation of Cell Adhesive Lysozyme in Yeast

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ABSTRACT: The cell adhesive protein RGD8 has been constructed using a yeast expression system by inserting eight amino acid residues (TGRGDSPA) between Val74 and Asn75 of human lysozyme [Yamada et al. (1993) *J. Biol. Chem. 268*, 10588–10592]. Purified RGD8 from yeast culture supernatant was found to contain glycosylated variants, in addition to the unglycosylated form. Peptide mapping analyses suggested that the glycosylation occurred at the inserted Thr residue in the RGD8 molecule. Electrospray ionization mass spectrometric analysis demonstrated the presence of four or five hexose residues in the glycosylated variants. Only mannose was detected in the sugar analysis of the oligosaccharide mixture obtained by mild alkaline treatment of the variants, and the structures of these carbohydrate chains were identified as Manα1–3Manα1–2Manα1–2Manα and Manα1–3Manα1–3Manα1–2Manα1–2Manα by ^{1}H-NMR spectroscopy. No other glycosylation was found, although the RGD8 molecule possesses a total of 13 Thr and Ser residues. In addition, no *O*-glycosylation was observed when the RGD8 protein was expressed in mouse L-cells. Thus, this *O*-glycosylation looks specific for yeast and the site of the Thr residue. The *O*-glycosylated variants of RGD8 exhibited a high level of adhesion activity to baby hamster kidney cells, which was almost comparable to that of the unglycosylated form.

The *N*-glycosylation of proteins has been studied extensively in various eukaryotes (Elbein, 1981; Kornfeld & Kornfeld, 1985; Tanner & Lehle, 1987), while the *O*-glycosylation of proteins has been investigated in detail only in mammalian cells (Beyer et al., 1981; Roth, 1984). The *O*-glycosylation seems to proceed differently in mammals, plants, and fungi (Beyer et al., 1981; Roth, 1984; Tanner & Lehle, 1987). For *Saccharomyces* yeasts, as well as for fungi, *O*-glycosylation is initiated in the ER lumen with the transfer of a mannose residue from Dol-*P*-Man to Ser or Thr in the nascent protein (Orlean et al., 1991). The *O*-glycosylated yeast proteins include bulk cell wall mannoprotein, a cell-surface agglutinin, and chitinase (Orlean et al., 1991). However, the specific sequences to initiate the *O*-glycosylation are not known.

The Arg-Gly-Asp (RGD)[1] sequence is well-known as a site in cell adhesive proteins such as fibronectin (Pierschbacher & Ruoslahti, 1984), vitronectin (Suzuki et al., 1985), and fibrinogen (Watt et al., 1979) for binding to their receptors, the integrins (Hynes, 1987; Hemler, 1991). To determine the functional conformation of this cell adhesion signal, we constructed mutant proteins by inserting the RGD-containing sequence of human fibronectin between Val74 and Asn75 of human lysozyme, using a yeast expression system (Yamada et al., 1993a). We previously reported the structural examination of these mutant lysozymes by X-ray crystallographic and two-dimensional NMR techniques, and discussed the functional conformation of the RGD sequence (Yamada et al., 1993b). During this study, we have found that RGD8, a mutant lysozyme containing the extra Thr-Gly-Arg-Gly-Asp-Ser-Pro-Ala sequence at the insertion site, is resolved into two peaks by cation-exchange HPLC.

Here we report that the RGD8 mutant contains yeast-specific *O*-glycosylated variants, in addition to the unglycosylated form. The structural elements affecting the site specificity of the yeast *O*-glycosylation are discussed.

MATERIALS AND METHODS

Expression and Purification of RGD8. RGD8 was expressed in yeast, and purified by HPLC equipped with a cation-exchange column (Asahipak ES-502C, Asahikasei Co. Ltd., Japan) and a hydroxyapatite column (TAPS-020810, Tonen K. K., Japan), as described previously (Yoshimura et al., 1987).

Pepsin Digestion. Purified RGD8-I or -II (each 0.3 mg) was dissolved in 0.3 mL of 0.05 N HCl and digested with 1 μg of pepsin (Sigma) at 37 °C for 8 h. The resultant peptides were separated by reverse-phase HPLC equipped with a Shodex ODSpak F-411/S column (Showa-dencoh K. K., Japan) (Taniyama et al., 1990).

Thermolysin Digestion of the Peptic Peptides. The peptic peptides P-I and -II were freeze-dried. Each peptide was dissolved in 0.5 mL of 2 mM $CaCl_2$/10 mM Tris-HCl, pH 7.8, and digested with 2 μg of thermolysin (Seikagaku-kogyo K. K., Japan) at 50 °C for 15 h. The resultant peptides were also separated by reverse-phase HPLC, as described above.

Analysis of N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence was determined using an Applied Biosystems Model 477A sequencer equipped with a 120A on-line PTH-amino acid analyzer.

Analysis of Amino Acid Composition. The amino acid composition was determined on a 24-h hydrolysate with 6 N HCl at 110 °C in the presence of 4% thioglycolic acid. Amino acid analysis using ninhydrin was performed on a Hitachi Model 835 amino acid analyzer.

Electrospray Ionization (ESI) Mass Spectrometry. The ESI (Whitehouse et al., 1985) mass spectrum was measured

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[1] Abbreviations: RGD, Arg-Gly-Asp; ESI, electrospray ionization; BHK, baby hamster kidney.

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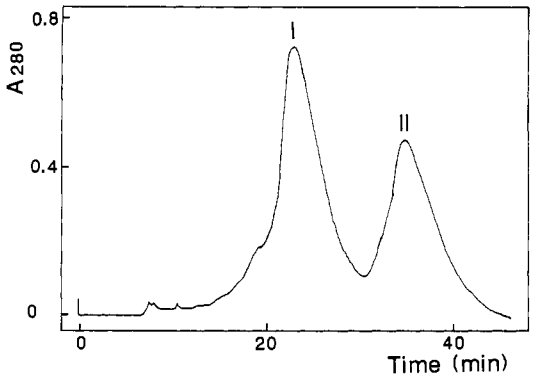

FIGURE 1: Elution pattern of RGD8 on cation-exchange HPLC. The preparation containing the RGD8 protein (5 mg) was applied to an Asahipak ES-502C column (0.76 × 10 cm) equilibrated with 90 mM Na_2SO_4/50 mM sodium phosphate buffer (pH 6.5). Elution was performed with the same solution at a flow rate of 1 mL/min.

with a JEOL JMS-HX110/110A double-focusing mass spectrometer equipped with an ESI ion source (Analytica of Branford, CT). Purified RGD8-I or -II (10 μg of each) was dissolved in 50 μL of H_2O/acetic acid/acetonitrile (5:1:4) and infused into the ion source at a flow rate of 1 μL/min.

Isolation of O-Linked Carbohydrate Chains. The *O*-linked carbohydrate chains were released by alkaline treatment (Nakajima & Ballou, 1974). RGD8-I (18 mg) was dissolved in 18 mL of 0.1 N NaOH and incubated for 18 h at 21 °C. After neutralization with 2 M acetic acid and lyophilization, the released oligosaccharides were separated from the protein by gel filtration on a column (1.5 × 45 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad), using water as an eluent with a flow rate of 10 mL/h. For carbohydrate detection, an aliquot of each fraction was subjected to phenol/sulfuric acid reaction. The carbohydrate-positive fractions were pooled and desalted on a column (1.5 × 4 cm) containing equal layers of AG1 X-2 (acetate form, Bio-Rad) and AG 50W (H^+-form, Bio-Rad) with water as an eluent.

Monosaccharide Analysis. An aliquot of the carbohydrate sample was subjected to hydrolysis by incubating with 2 N TFA for 6 h at 100 °C, and analyzed by anion-exchange HPLC equipped with a TSK gel Sugar AXG column (TOSOH, Japan), as described previously (Mikami & Ishida, 1983).

NMR Spectroscopy. The carbohydrate sample was repeatedly exchanged in D_2O. 500-MHz ^{1}H-NMR spectra were recorded in D_2O at 28 °C on a JEOL JNM-GSX500 spectrometer. Chemical shifts (δ) were read relative to an internal acetone standard (δ = 2.225 ppm) (Vliegenthart et al., 1983).

Expression of Human Lysozymes in Mouse L-Cells. Native lysozyme, RGD4, and RGD8 were secreted using a mouse L-cell expression system (Omura et al., 1992). The construction of each expression plasmid was performed as described previously (Omura et al., 1992).

Measurement of Cell Adhesion Activity. Cell adhesion activity was determined using BHK cells as described previously (Maeda et al., 1989).

RESULTS

The RGD8 mutant was resolved into two peaks, I and II (Figure 1), when it was subjected to cation-exchange HPLC, a step for the purification of human lysozyme from yeast culture supernatant (Taniyama et al., 1990). The final preparations from peaks I and II were obtained by the following hydroxyapatite HPLC, and designated as RGD8-I and -II, respectively. The N-terminal amino acid sequences of both RGD8-I and -II were identified as Lys-Val-Phe-Glu-Arg- by the automated Edman degradation method. The amino acid analysis showed that RGD8-I and -II had the same amino acid composition, which was in good accordance with the theoretical values of RGD8 (data not shown). In addition, quantitative analysis of the free thiol group by the DTNB method (Ellman, 1959) showed that all eight Cys residues in RGD8-I and -II were linked via four disulfide bridges.

To gain information on the structural differences between RGD8-I and -II, they were subjected to pepsin digestion (Taniyama et al., 1990). As shown in Figure 2, the peptide fragments had almost the same retention times on reverse-phase HPLC, except for the fragments designated as P-I and -II, respectively. P-I and -II were further digested by thermolysin, because no difference was detected between them by protein–chemical analyses. In both cases, thermolysin digestion yielded the fragment containing Th1(-I or -II) and Th3, and that containing Th2(-I or -II) and Th4 (Figure 3). The results suggested that RGD8-I and -II possessed the same

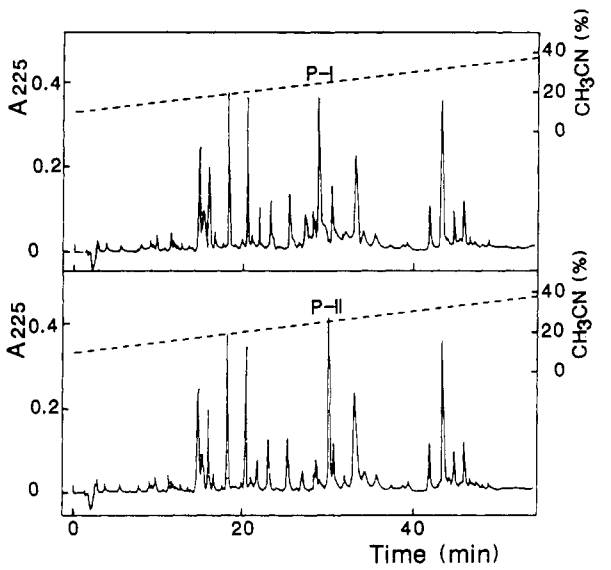

FIGURE 2: Elution patterns of pepsin digests of RGD8-I (top) and -II (bottom) on reverse-phase HPLC. The pepsin digests of RGD8-I and -II (each 0.15 mg) were applied to a Shodex ODSpak F-411/S column (0.46 × 10 cm). Elution was performed with a linear gradient of acetonitrile concentration in the presence of 0.1% TFA at a flow rate of 0.8 mL/min. By amino acid analysis and N-terminal sequence analysis, both P-I and -II were identified as the fragments consisting of the two sequences Gln58–Leu84 and Val93–Ala108.

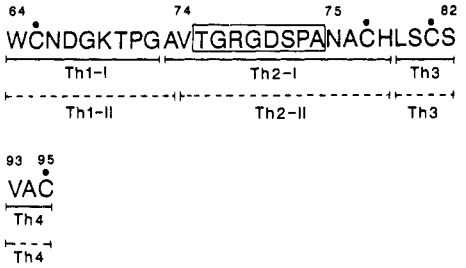

FIGURE 3: Thermolysin digestion of P-I and -II. The solid and dashed lines correspond to the thermolytic peptide fragments of P-I and -II, respectively. Each fragment was identified by amino acid analysis and N-terminal sequence analysis. The boxed residues indicate the amino acid sequence inserted into human lysozyme. Dots show the positions of Cys residues.

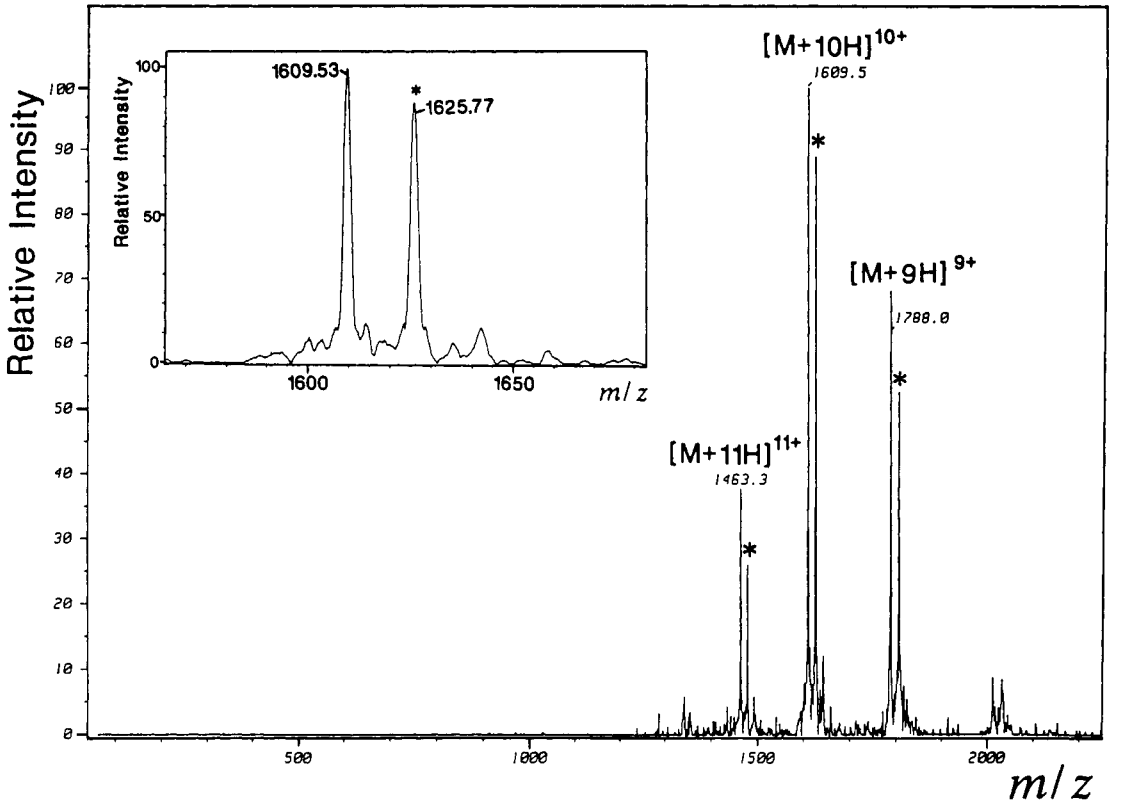

FIGURE 4: Electrospray mass spectrum of RGD8-I. From each m/z value of the multiply charged ion signals ($[M + nH]^{n+}$), the mass of RGD8-I was calculated. The inset shows the expanded region of the $[M + 10H]^{10+}$ ion signals. The signals marked by asterisks arise from the five hexose residue-attached RGD8 molecule.

mode of disulfide linkages, namely, Cys65–Cys81 and Cys77–Cys95, as was the case for native human lysozyme (Taniyama et al., 1990). More interestingly, we found that the cleavage site between Th1(-I or -II) and Th2(-I or -II) was different in the two cases (Figure 3), and that the Thr residue was not detected on the sequencer at the third position of Th2-I from RGD8-I. The Thr residue was also not detectable in the trypsin-digested fragment of P-I, TPGAVTGR (data not shown). These results strongly suggest that the Thr residue is modified in the RGD8-I molecule, which is consistent with the fact that RGD4 and RGD6 (Yamada et al., 1993b) without the inserted Thr residue contained only one molecular species (data not shown).

Electrospray mass spectrometric analysis was carried out to examine what kind of modification could occur at the Thr residue of RGD8-I. As shown in Figure 4, the RGD8-I molecule produced multiply charged ion signals ($[M + 9H]^{9+}$, $[M + 10H]^{10+}$, and $[M + 11H]^{11+}$), each separating into two major ion peaks. These two ion peaks gave calculated average molecular weights of 16 084.4 ± 1.2 and 16 248.3 ± 1.7, which were in good agreement with the theoretical molecular weights of the 4 and 5 hexose residue-attached RGD8 molecules (16 082.9 and 16 245.0, respectively). On the other hand, no modification on the RGD8-II molecule was demonstrated by electrospray mass spectrometry (data not shown).

To examine the carbohydrate structure in detail, all carbohydrate chains of RGD8-I were released by 0.1 N NaOH treatment, and separated from the protein by gel filtration. In the sugar analysis of the sample thus obtained, only mannose was detected (data not shown). Furthermore, the oligosaccharide mixture was subjected to NMR analysis. The anomeric region of the ^{1}H-NMR spectrum and the assignment of each H-1 signal are shown in Figure 5. Comparison with the ^{1}H-NMR spectra of the reference saccharides (Hård et al., 1989) allowed the structures of these carbohydrate chains to be identified as Manα1–3Manα1–2Manα1–2Manα and Manα1–3Manα1–3Manα1–2Manα1–2Manα (Figure 5).

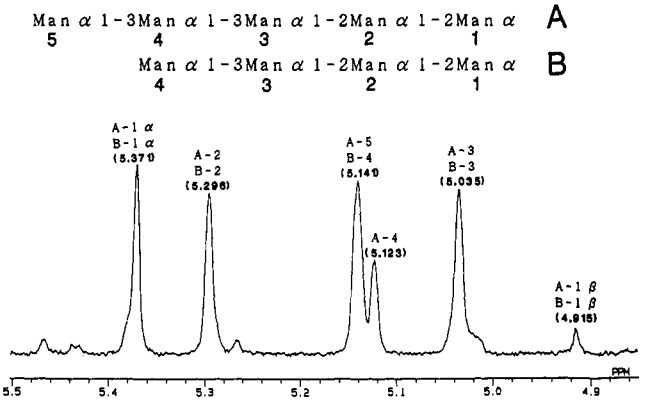

FIGURE 5: Anomeric region of the ^{1}H-NMR spectrum of the oligosaccharide mixture derived from RGD8-I. The assignment of each signal was performed on the basis of comparison with the spectra of the reference saccharides (Hård et al., 1989). Chemical shifts (δ) of each H-1 signal are given in parentheses.

As mentioned above, the RGD8 protein expressed in yeast could be O-glycosylated at the inserted Thr residue. We next examined whether this O-glycosylation was yeast-specific or not by expressing RGD8, as well as RGD4 and native lysozyme, in mouse L-cells (Omura et al., 1992). As shown in Figure 6, the RGD8 protein (lane 3) expressed in L-cells migrated as a single molecular species with a mass corresponding to that of the unglycosylated form (lane 6, lower band) expressed in yeast, as were the cases for RGD4 and native lysozyme. This result shows that the inserted Thr residue in the RGD8 molecule is not recognized by the O-glycosylation machinery (Beyer et al., 1981; Roth, 1984) of mammals.

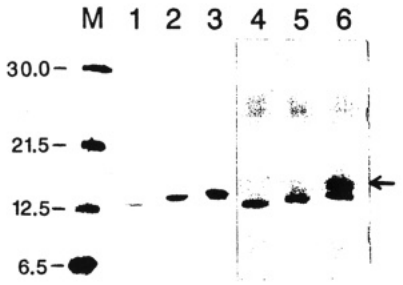

FIGURE 6: Expression of human lysozymes in mouse L-cells. Native lysozyme, RGD4, and RGD8 were expressed in mouse L-cells as described previously (Omura et al., 1992). Each L-cell transfectant was labeled in methionine-free medium containing 300 μCi/mL [^{35}S]-methionine. The labeled product thus obtained was immunoprecipitated with rabbit anti-(human lysozyme) antibody, and adsorbed to protein A–Sepharose CL-4B. The immunoprecipitate was mixed with 2 μg of the unlabeled counterpart derived from yeast, and subjected to 14% SDS–PAGE. After electrophoresis, the gel was stained (lanes 4–6) and exposed to an Imaging Plate (lanes 1–3). Lanes 1 and 4, native lysozyme; lanes 2 and 5, RGD4; lanes 3 and 6, RGD8; lane M, molecular size markers (values in kilodaltons on the left). The arrow refers to the *O*-glycosylated variants of RGD8.

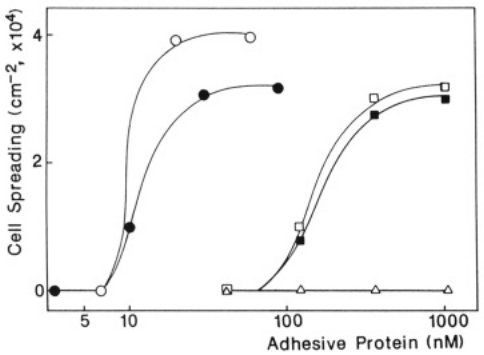

FIGURE 7: Quantitative cell spreading assay on substrates coated with each adhesive protein. The plastic substrates were coated with different concentrations of fibronectin (○), vitronectin (●), native lysozyme (△), *O*-glycosylated variants (■), and the unglycosylated form (□) of RGD8. BHK cells were incubated on the substrates for 60 min in a CO_2 incubator. The extent of cell spreading was expressed as the number of cells adhered to a unit surface area (cm^2).

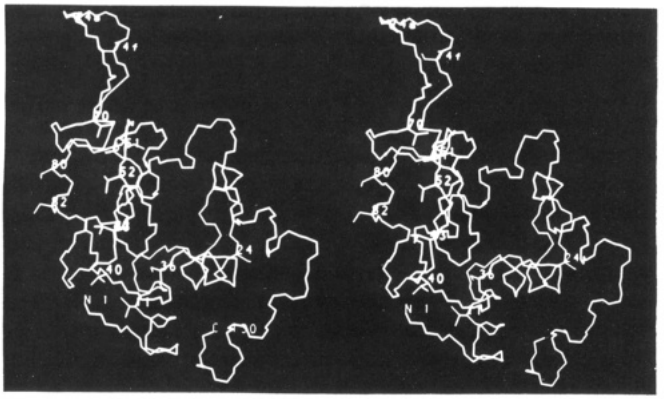

FIGURE 8: Refined structure of RGD8 by X-ray crystallography. The side chains of the Ser and Thr residues are shown in the backbone structure of the RGD8 molecule. Each α-carbon atom of the Ser and Thr residues is labeled.

The cell adhesion activity of RGD8 was measured using BHK cells. As shown in Figure 7, the *O*-glycosylated variants of RGD8, as well as the unglycosylated form, exhibited a high level of cell adhesion activity, nearly equal to 10% of human vitronectin activity. The results suggest that the oligosaccharide moiety has little effect on the functional conformation of the RGD sequence, in spite of the location near the RGD-containing region of the RGD8 molecule.

DISCUSSION

We previously expressed human lysozyme using a yeast secretion system, and confirmed that the recombinant lysozyme possesses the same structure as the native enzyme (Yoshimura et al., 1987; Inaka et al., 1991). More recently, when we constructed the RGD8 mutant by inserting the TGRGDSPA sequence derived from the cell binding region of human fibronectin between Val74 and Asn75 of human lysozyme (Yamada et al., 1993b), we found that the RGD8 protein contained molecular species other than the normal form. We have identified these species as *O*-glycosylated variants containing a linear carbohydrate chain of four or five mannose residues, which is attached to the inserted Thr residue of the RGD8 molecule. Similar examples were reported in the cases of human insulin-like growth factor I (Gellerfors et al., 1989) and human parathyroid hormone (Olstad et al., 1992) expressed in yeast. In both cases, the *O*-glycosylation sites as well as the carbohydrate structures were successfully determined, and the site specificity of yeast *O*-glycosylation was discussed only in terms of the amino acid sequences containing Ser or Thr. Using purified yeast mannosyltransferase and various acceptor peptides, Strahl-Bolsinger and Tanner (1991) suggested that acidic amino acids next to Ser or Thr strongly inhibited acceptor activity, as did Gly and Pro residues as N-terminal and C-terminal neighbors, respectively. In fact, the glycosylated Ser or Thr residues in the three recombinant proteins (Gellerfors et al., 1989; Olstad et al., 1992; Yamada et al., 1993b) expressed in yeast possess no such inhibitory neighboring residues. It is also certain that no specific sequences to initiate yeast *O*-glycosylation can be found among these proteins.

In the case of RGD8, no residues other than the inserted Thr were *O*-glycosylated, although the RGD8 molecule contained a total of 13 Ser and Thr residues. The choice of the glycosylation site could not be simply explained by the aforementioned glycosylation inhibitory rule (Strahl-Bolsinger & Tanner, 1991) for the amino acid sequence surrounding the Ser or Thr residue. Here we should pay attention to the effect of the secondary structure of the region containing Ser or Thr on *O*-glycosylation. It is conceivable that the secondary structure, prior to the *O*-glycosylation, is attained as soon as each component peptide part is synthesized (Freedman, 1992). On the basis of the refined structure of RGD8 by X-ray crystallography (Yamada et al., 1993b) (Figure 8), we speculate that particular secondary structures, such as α-helix, 3_{10}-helix, and β-sheet, sterically prevent *O*-glycosylation. Taking these viewpoints into account, it is reasonable that the Ser and Thr residues other than Ser51, Ser61, and Thr74a (inserted) in the RGD8 molecule are not glycosylated (Table 1). In addition, in the cases of Ser51 and Ser61, the *O*-glycosylation might occur on them in the loop region. However, such molecular species of RGD8 would fail to be folded correctly, because the side chains of these two Ser residues are destined to be buried inside the protein molecule (Figure 8). The structures of each Ser and Thr residue in the RGD8 molecule are summarized in Table 1.

To test this speculation, we have constructed a mutant protein by inserting the NTRGDS sequence between Val74 and Asn75 of human lysozyme. The inserted sequence contains the Thr residue surrounded by the same N-terminal and C-terminal neighbors as in the case of Thr40 and Ser61. Nevertheless, this mutant lysozyme is found as variants glycosylated at the inserted Thr residue in addition to the unglycosylated form (data not shown). Thus, it seems likely that various residues neighboring the Thr residue allow

Table 1: Structures of Each Ser and Thr Residue in the RGD8 Molecule

	primary structure[a]	secondary structure[b]	tertiary structure[b,c]
Thr11	-Arg-Thr-Leu-	α-helix	outside
Ser24	-Ile-Ser-Leu-	α-helix	outside
Ser36	-Glu-Ser-Gly-	α-helix	inside
Thr40	-Asn-Thr-Arg-	β-sheet	inside
Thr43	-Ala-Thr-Asn-	β-sheet	outside
Ser51	-Arg-Ser-Thr-	loop	inside
Thr52	-Ser-Thr-Asp-	loop	inside
Ser61	-Asn-Ser-Arg-	loop	inside
Thr70	-Lys-Thr-Pro-	loop	inside
Thr74a(inserted)	-Val-Thr-Gly-	loop	outside
Ser74f(inserted)	-Asp-Ser-Pro-	loop	outside
Ser80	-Leu-Ser-Cys-	3_{10}-helix	outside
Ser82	-Cys-Ser-Ala-	3_{10}-helix	outside

[a] The glycosylation inhibitory amino acid residues (Strahl-Bolsinger & Tanner, 1991) are underlined. [b] Determined on the basis of the refined structure by X-ray crystallography (Yamada et al., 1993b). [c] Defined as whether each side chain is inside or outside the protein molecule.

O-glycosylation when the Thr is located in the solvent-exposed loop region of the lysozyme molecule (Inaka et al., 1991). We have also tried to construct mutant proteins by inserting the NTRGDS sequence in an α-helix region (between Arg14 and Leu15) or in a β-sheet region (between Arg41 and Ala42) of human lysozyme. Such mutant lysozymes, however, failed to be folded correctly (data not shown).

The glycosylated variants of RGD8 possess the extra carbohydrate chain in the vicinity of the functional RGD sequence. Nevertheless, they exhibited a high level of cell adhesion activity, almost comparable to that of the unglycosylated form. The linear chain of carbohydrate must have little effect on the accessibility of the RGD sequence to the integrin receptors. In addition, the effect of the carbohydrate chain might be decreased under conditions where the RGD region is solvent-exposed and lies on a conformationally flexible loop (Inaka et al., 1991; Yamada et al., 1993b).

In the present investigation, we have found yeast-specific O-glycosylated variants of the mutant human lysozyme RGD8 and have discussed the structural elements affecting the site specificity of yeast O-glycosylation. This information may be helpful in understanding the O-glycosylation mechanism of yeast.

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