Site-Dependent Effect of O-Glycosylation on the Conformation and Biological Activity of Calcitonin†

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ABSTRACT: We synthesized seven O-glycosylated calcitonin derivatives, each with a single GalNAc residue attached to either Ser or Thr, and studied their three-dimensional structure and biological activity to examine site-dependent effects of O-glycosylation. The CD spectra in an aqueous trifluoroethanol solution showed that the GalNAc attachment at Thr6 or Thr21 reduced the helical content of calcitonin, indicating that the O-glycosylated residue functions as a stronger helix breaker than the original amino acid residue. Only the GalNAc attachment at Ser2 or Thr21 retained the hypocalcemic activity of calcitonin. This result corresponded well to that of the calcitonin−receptor binding assay. The GalNAc attachment other than Ser2 or Thr21 perturbed the interaction with the receptor, resulting in the loss of the hypocalcemic activity. The biodistribution did not change much among the seven derivatives, but some site dependency could also be observed. Thus, we can conclude that the O-glycosylation affects both the conformation and biological activity in a site-dependent manner.

Accumulated incidents show that the carbohydrate portions of glycoproteins and glycopeptides play important roles in many biological phenomena (*1*−*3*). The evidence, however, is fragmental, and the specific function of each carbohydrate still awaits detailed description. We have been using eel calcitonin (CT)[1] as a model peptide to study the three-dimensional structure and the biological activity of the glycosylated derivatives with chemically pure and structurally defined samples, because full exploration of the potential function of carbohydrates is difficult using only naturally derived glycoconjugates. CT is a 32-amino acid peptide hormone with hypocalcemic activity, and some of the derivatives are currently used as therapeutic drugs for hypercalcemia, Paget's disease, and osteoporosis (*4*).

We have found that the N-glycosylation at Asn3 of CT did not change the three-dimensional structure of the peptide backbone but affected the biological activity depending on the carbohydrate structure (*5*). The change in the biological activity mainly arose from the biodistribution modified by the glycosylation. To extend our knowledge about the function of carbohydrates in glycopeptides, we should examine the positional effect of glycosylation as a next step.

Unfortunately, CT has only one Asn residue in the sequence. We cannot introduce any other N-glycosylation in a natural way, unless we change the amino acid sequence. On the other hand, there are three Ser and four Thr residues to which GalNAc can be attached. Because of the recent advances in the synthesis of O-glycosylated peptides (*6*, *7*) and the scattered Ser and Thr residues along the CT sequence, we thought that O-glycosylation would be a good tool for investigating the positional effect of glycosylation on the structure and activity of glycopeptides.

We synthesized seven GalNAc-attached CT derivatives and studied their CD spectra, hypocalcemic activity, receptor binding activity, and biodistribution. The effect of O-glycosylation was indeed site-dependent in every respect.

EXPERIMENTAL PROCEDURES

Other than the sample preparation, experimental details will be described elsewhere, and the description of each experiment is kept to a minimum.

Sample Preparation. The CT−GalNAc derivatives were synthesized according to the method reported by Teshima et al. (*8*, *9*). Starting from the methylbenzhydrylamine resin

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; ODS, octadecylsilyl; MALDI-TOFMS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TFE, trifluoroethanol; DAR, differential absorption ratio; GalNAc, *N*-acetyl-D-galactosamine; CT, calcitonin; CT-R, calcitonin receptor; CTn-GalNAc, [Ser/Thr(GalNAc)^n]-calcitonin; Bn, benzyl.

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(0.1 mmol), we prepared each glycopeptide using an automated peptide synthesizer (Applied Biosystems, Foster City, CA) with the Boc strategy. Except for the condensation with Boc-Ser or Boc-Thr attached with tri-*O*-benzyl-*N*-acetylgalactosamine in the α anomeric configuration [Boc-Ser/Thr-(triBn-GalNAc)], the standard procedure of the Boc strategy programmed in the synthesizer was employed.

The introduction of Boc-Ser/Thr-(triBn-GalNAc) was done manually. Boc-Ser/Thr-(triBn-GalNAc) (0.15 mmol) was coupled to the solid-phase peptide with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (0.15 mmol) and 1-hydroxybenzotriazole (0.15 mmol). The resultant resin was returned to the automated synthesizer after this manual step.

The cleavage from the resin and the simultaneous deprotection were carried out with anhydrous HF (8:2 HF/*p*-cresol mixture, cysteine (3.0 mmol), −2 to −5 °C, 60 min). After the removal of HF, the residue was washed with ether and then extracted with a 0.1% trifluoroacetic acid (TFA) aqueous solution. The extract was lyophilized to give the crude glycopeptide powder. The disulfide bond was formed in a 50% acetic acid aqueous solution at 0 °C with a 0.1 M I_2 methanol solution (1 equiv), and the reaction was stopped with ascorbic acid after 2 min.

The product was purified by a HPLC LC8A instrument (Shimadzu, Kyoto, Japan) with an ODS column (YMC, 30 mm × 250 mm) with an acetonitrile/water (0.1% TFA) linear gradient. The purity of the product was examined by analytical HPLC with an ODS column (YMC, 4.6 mm × 150 mm). An acetonitrile/water (0.1% TFA) linear gradient (from 20 to 70% over the course of 25 min at 50 °C) was used. The structure was confirmed by amino acid analysis and MALDI-TOFMS. The chemical yields shown below in parentheses are based on the loading capacity of the starting resin and after the HPLC purification.

[Ser(GalNAc)^2]−CT (CT2-GalNAc) (21 mg, 5.8%) was obtained. The analytical HPLC retention time was 13.5 min. Amino acid analysis: Asp 2.00 (2), Thr 3.67 (4), Ser 2.63 (3), Glu 3.12 (3), Gly 2.97 (3), Ala 1.04 (1), Val 2.03 (2), Leu 5.16 (5), Tyr 1.05 (1), His 1.02 (1), Lys 2.07 (2), NH_3 5.16 (4), Arg 1.02 (1), Pro 1.99 (2), Cys 1.85 (2). MALDI-TOFMS (*m*/*z*): found, 3619.2 ($[M + H]^+$); calcd, 3619.1 ($C_{154}H_{255}O_{52}N_{44}S_2$).

[Ser(GalNAc)^5]−CT (CT5-GalNAc) (17 mg, 4.7%) was obtained. The retention time was 12.3 min. Amino acid analysis: Asp 2.00 (2), Thr 3.71 (4), Ser 2.61 (3), Glu 3.09 (3), Gly 2.96 (3), Ala 0.99 (1), Val 1.97 (2), Leu 5.12 (5), Tyr 1.04 (1), His 1.00 (1), Lys 1.99 (2), NH_3 4.99 (4), Arg 0.98 (1), Pro 1.93 (2), Cys 1.83 (2). MALDI-TOFMS (*m*/*z*): found, 3619.5 ($[M + H]^+$); calcd, 3619.1 ($C_{154}H_{255}O_{52}N_{44}S_2$).

[Thr(GalNAc)^6]−CT (CT6-GalNAc) (13 mg, 3.6%) was obtained. The retention time was 12.1 min. Amino acid analysis: Asp 2.00 (2), Thr 3.74 (4), Ser 2.63 (3), Glu 3.12 (3), Gly 2.98 (3), Ala 1.01 (1), Val 1.98 (2), Leu 5.17 (5), Tyr 1.08 (1), His 0.99 (1), Lys 1.99 (2), NH_3 5.03 (4), Arg 1.00 (1), Pro 2.01 (2), Cys 1.85 (2). MALDI-TOFMS (*m*/*z*): found, 3619.9 ($[M + H]^+$); calcd, 3619.1 ($C_{154}H_{255}O_{52}N_{44}S_2$).

[Ser(GalNAc)^{13}]−CT (CT13-GalNAc) (15 mg, 4.1%) was obtained. The retention time was 13.7 min. Amino acid analysis: Asp 2.00 (2), Thr 3.76 (4), Ser 2.65 (3), Glu 3.13 (3), Gly 3.01 (3), Ala 1.02 (1), Val 1.97 (2), Leu 5.10 (5), Tyr 1.16 (1), His 1.00 (1), Lys 2.01 (2), NH_3 4.93 (4), Arg 1.00 (1), Pro 1.99 (2), Cys 1.91 (2). MALDI-TOFMS (*m*/*z*): found, 3619.0 ($[M + H]^+$); calcd, 3619.1 ($C_{154}H_{255}O_{52}N_{44}S_2$).

[Thr(GalNAc)^{21}]−CT (CT21-GalNAc) (18 mg, 5.0%) was obtained. The retention time was 13.6 min. Amino acid analysis: Asp 2.00 (2), Thr 3.69 (4), Ser 2.63 (3), Glu 3.12 (3), Gly 2.92 (3), Ala 1.02 (1), Val 1.96 (2), Leu 5.13 (5), Tyr 1.00 (1), His 1.01 (1), Lys 2.07 (2), NH_3 5.33 (4), Arg 0.99 (1), Pro 2.01 (2), Cys 1.80 (2). MALDI-TOFMS (*m*/*z*): found, 3620.1 ($[M + H]^+$); calcd, 3619.1 ($C_{154}H_{255}O_{52}N_{44}S_2$).

[Thr(GalNAc)^{25}]−CT (CT25-GalNAc) (22 mg, 6.1%) was obtained. The retention time was 13.8 min. Amino acid analysis: Asp 1.98 (2), Thr 3.75 (4), Ser 2.62 (3), Glu 3.12 (3), Gly 2.92 (3), Ala 1.00 (1), Val 1.92 (2), Leu 5.07 (5), Tyr 1.04 (1), His 1.00 (1), Lys 2.00 (2), NH_3 5.12 (4), Arg 0.96 (1), Pro 1.98 (2), Cys 1.71 (2). MALDI-TOFMS (*m*/*z*): found, 3618.5 ($[M + H]^+$); calcd, 3619.1 ($C_{154}H_{255}O_{52}N_{44}S_2$).

[Thr(GalNAc)^{31}]−CT (CT31-GalNAc) (15 mg, 4.1%) was obtained. The retention time was 14.0 min. Amino acid analysis: Asp 2.00 (2), Thr 3.72 (4), Ser 2.63 (3), Glu 3.11 (3), Gly 2.85 (3), Ala 1.01 (1), Val 1.97 (2), Leu 5.17 (5), Tyr 1.10 (1), His 1.01 (1), Lys 2.00 (2), NH_3 5.02 (4), Arg 1.00 (1), Pro 1.97 (2), Cys 1.87 (2). MALDI-TOFMS (*m*/*z*): found, 3619.2 ($[M + H]^+$); calcd, 3619.1 ($C_{154}H_{255}O_{52}N_{44}S_2$).

Sample Concentration. The sample concentration of each derivative was determined by the HPLC peak area relative to that of CT.

CD Measurement. CD spectra were obtained using a JASCO J-720 spectropolarimeter in an aqueous trifluoroethanol (TFE) solution. The helical contents were estimated according to the method of Chen et al. (*10*).

Hypocalcemic Activity. A 0.2 mL aliquot of a 1.8 (low dose) or 3.6 (high dose) pmol/mL sample solution was injected into the tail vein of male Sprague-Dawley rats, and a blood sample was taken 60 min after the injection. The serum calcium concentration was determined by atomic absorption. The blank value is that of the untreated animal, and the standard value is that of the animal injected with elcatonin, a synthetic analogue of eel CT (*4*). Each value represents the average from 10 animals except for the blank value (the average from five animals).

Receptor Binding Assay. The competition toward the mouse osteoclast-like cell with 0.5 nM [^{125}I]elcatonin was assessed. Elcatonin has a binding affinity comparable to that of CT, and we have been using the labeled compound as an in-house standard.

Biodistribution. ^{125}I-labeled samples were injected into male ddY mice. The animals were killed with ether anesthesia, 5, 15, 30, or 60 min after the injection. Organs of interest were weighed, and the amount of radioactivity was measured. Tissue accumulation was calculated as the differential absorption ratio (DAR). Each value represents the average from five animals.

RESULTS

The amino acid sequence of eel CT and the seven O-glycosylation sites, with arrows, are shown in Figure 1. The conventional Boc strategy was employed in the syntheses of the glycopeptides. Though three Ser-GalNAc and four Thr-GalNAc derivatives were synthesized, a significant difference in their product yield was not observed.

FIGURE 1: Amino acid sequence of CT. The O-glycosylation sites are denoted with arrows.

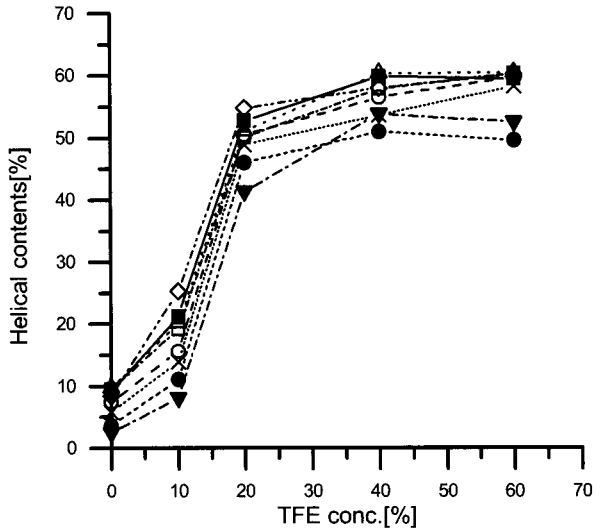

FIGURE 2: TFE concentration-dependent helical content change in CT (■), CT2-GalNAc (○), CT5-GalNAc (△), CT6-GalNAc (▼), CT13-GalNAc (◇), CT21-GalNAc (●), CT25-GalNAc (×), and CT31-GalNAc (□).

Table 1: Hypocalcemic Activity Shown in Serum Calcium Concentration (mg/dL)

sample	low dose	high dose
blank	9.79 ± 0.15	
elcatonin	8.48 ± 0.28	7.37 ± 0.24
CT	9.15 ± 0.24	8.19 ± 0.49
CT2-GalNAc	8.88 ± 0.30	7.41 ± 0.46
blank	9.69 ± 0.17	
elcatonin	8.63 ± 0.34	7.56 ± 0.25
CT5-GalNAc	9.28 ± 0.15	9.28 ± 0.17
CT6-GalNAc	9.37 ± 0.26	9.20 ± 0.21
blank	9.80 ± 0.35	
elcatonin	8.79 ± 0.40	7.53 ± 0.26
CT13-GalNAc	9.49 ± 0.23	9.17 ± 0.36
CT21-GalNAc	8.99 ± 0.23	7.86 ± 0.33
CT25-GalNAc	9.48 ± 0.34	9.11 ± 0.38
CT31-GalNAc	9.59 ± 0.24	9.26 ± 0.54

The TFE concentration-dependent change in the CD spectra is shown in Figure 2. The helical content of CT increases as the concentration of TFE increases (*5*). The helical content reaches an ~60% plateau at a TFE concentration of 40%. All of the GalNAc derivatives exhibited similar behavior in their helical content accumulation as can be seen from the figure. CT6-GalNAc and CT21-GalNAc, however, always exhibited lower helical contents than the other derivatives, and the final helical content was ~50%, 10% lower than those of the others. We also studied the temperature dependence of CD in a 40% TFE aqueous solution, but there was no significant difference among the derivatives.

The hypocalcemic activity data are summarized in Table 1. Because the assay was performed on three different days, the table is separated into three subsets. Each subset has its own blank and standard value. From the table, it is obvious that only CT2-GalNAc and CT21-GalNAc retained hypocalcemic activity and that the others lost their activities drastically. The activity of CT2-GalNAc and CT21-GalNAc even exceeded that of the native CT.

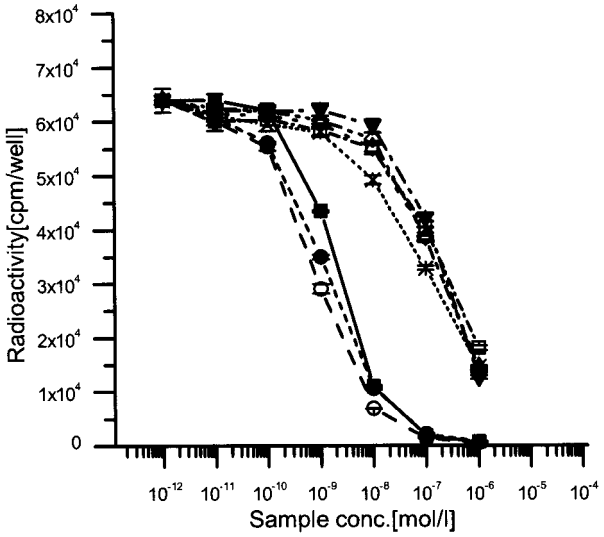

FIGURE 3: Calcitonin receptor binding activity of CT (■), CT2-GalNAc (○), CT5-GalNAc (△), CT6-GalNAc (▼), CT13-GalNAc (◇), CT21-GalNAc (●), CT25-GalNAc (×), and CT31-GalNAc (□).

Figure 3 shows the result of the CT−receptor (CT−R) binding assay. The activities of CT2-GalNAc and CT21-GalNAc slightly exceeded that of CT, while the other derivatives lost their binding activities by ~2 orders of magnitude. This result corresponds quite well to that of the hypocalcemic activity. Even the slight gain in both activities coincided.

No drastic difference was observed in the biodistribution among the GalNAc derivatives as shown in Figure 4. Nevertheless, a slight site dependence can be seen. Levels of CT2-GalNAc and CT21-GalNAc were lower in the blood and higher in the bone and levels of CT25-GalNAc and CT31-GalNAc higher in the liver and the level of CT2-GalNAc was lower and that of CT21-GalNAc higher in the kidney than the other derivatives.

DISCUSSION

CT has several advantages as a model peptide as previously discussed (*5*). One of the advantages, which we did not mention explicitly, is that we can obtain pure glycosylation effects, because CT is not glycosylated in its natural form. We have shown that the N-glycosylation at Asn3 affected the biological activity of CT depending on the carbohydrate structure. In this study, we synthesized seven GalNAc derivatives to examine the positional effect. It must be pointed out that the glycosylation sites are different from the natural O-glycosylation sequences, and the observed effects may not be seen in a natural system, though we think the results may help in understanding some aspects of the O-glycosylation function.

We chose the attachment of only a single GalNAc residue, not only because the synthesis is practically feasible but also because it is known that the first carbohydrate residue attached to the peptide backbone has the most fundamental influence on the conformation of glycopeptides (*11*). O-Glycosylation has an advantage over N-glycosylation in the study of the positional effects, because the number of

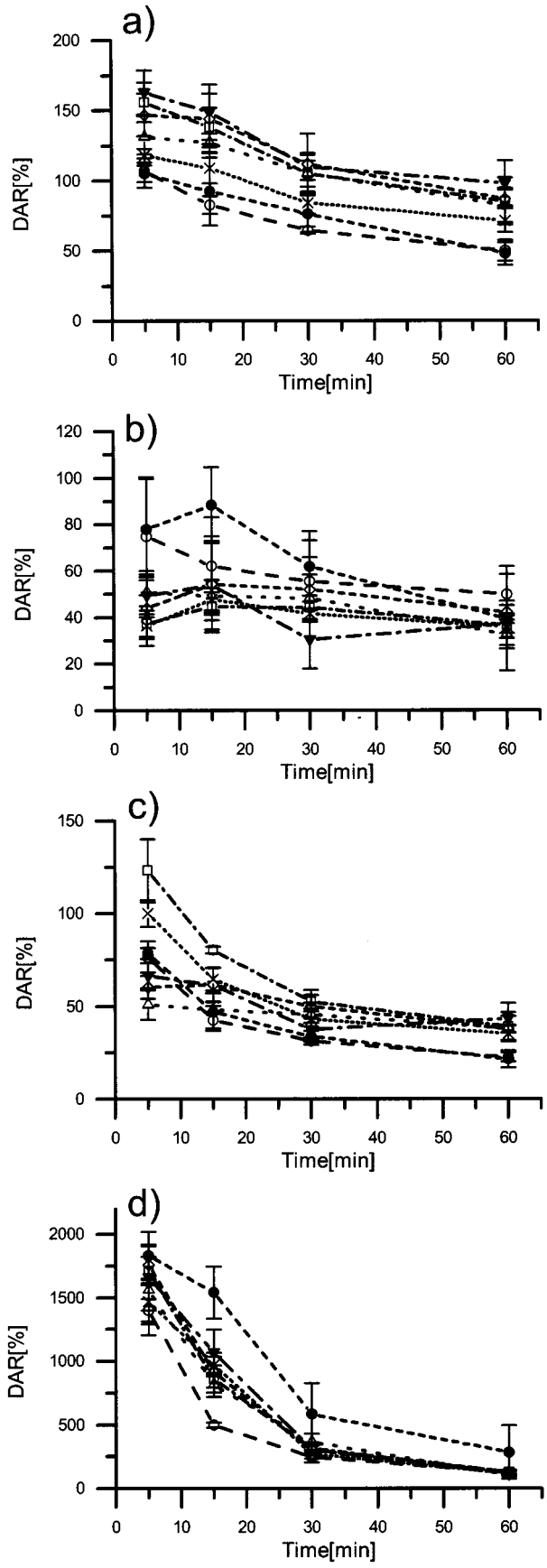

FIGURE 4: Biodistribution of CT2-GalNAc (○), CT5-GalNAc (△), CT6-GalNAc (▼), CT13-GalNAc (◇), CT21-GalNAc (●), CT25-GalNAc (×), and CT31-GalNAc (□) in blood (a), bone (b), liver (c), and kidney (d).

glycosylation sites is seven and the sites are scattered all along the CT sequence as can be seen in Figure 1. From the NMR studies (*12*, *13*), CT is known to assume a helical structure in hydrophobic environments. Though the TFE-induced structure is an artificial one, the helical structure is considered to be biologically important, because CT preforms a similar structure by the interaction with membrane before its binding with the CT−R species. The O-glycosylation sites are both in and out of the helical region, and this distribution was indeed a great advantage.

The CD spectra of all samples exhibited a two-state-like TFE concentration dependence; CT2-GalNAc, CT5-GalNAc, CT13-GalNAc, CT25-GalNAc, and CT31-GalNAc behave in a manner quite similar to that of CT even quantitatively, but CT6-GalNAc and CT21-GalNAc are unique in that they lose 10% helical content even at the 60% TFE concentration. This result clearly shows that the glycosylation affects the conformation of the peptide backbone depending on the attachment position. Because Thr6 is close to the N-terminal end of the helix and Thr21 to the C-terminal end of the helix, the result in which the glycosylated amino acid residue functions as a stronger helix breaker than the original amino acid residue can be understood.

As is well established in protein chemistry, each amino acid has its own propensity to make or perturb secondary structures, but the resultant secondary structure depends on the sequence in which the amino acid resides. In our case, Thr6 and Thr21 are on the edge and affected the helix, Ser13 is at the center and did not affect the helix, and the other sites are out of the helical region. Thus, the glyocosylated amino acid residue actually functioned as the helix breaker depending on the sequence context.

Several groups have reported the NMR structure of CT (*13*), and their assignment of the helical region differs slightly from one to another partly due to the differences in the species and the experimental conditions. We described the helical structure between Ser5 and Leu19 in our NMR paper (*12*), but we could detect weak signals that indicated a partial helical structure as far as Tyr22 in an aqueous TFE condition (*5*). At present, we estimate that the glycosylation at Thr21 perturbs this fragile portion of the helical structure.

A preliminary NMR study showed that the GalNAc residue attached to Thr6 and Thr21 had local intramolecular contacts and that the exchange rate of the helix amide protons became faster only around the glycosylation site. These NMR data indicate that the glycosylated amino acid residue locally perturbs the helical structure.

Though debates are still ongoing about the relationship between the native and TFE-induced structures, it is generally accepted that TFE stabilizes the local structures and enhances the secondary structural features (*14*). Our result has to be understood within this limitation. The helical structure of CT is induced by TFE and is not the native one. CT and the glycosylated derivatives assume a random conformation in aqueous solution as indicated by CD. Nevertheless, the helix-breaking tendency of the glycosylated amino acid may be generalized, because the helical propensity of amino acids holds even in the TFE solution at least as the local structural feature.

There has been superficial conflict regarding the structural effect of glycosylation. Some groups reported structural changes caused by glycosylation (*15*, *16*). Others did not

observe any change in the structure (*17*, *18*). This conflict can now be settled, if we assume that the effect of glycosylation is sequence-dependent. There has been only one report, as far as we know, that describes a glycosylated residue as a helix breaker in a TFE aqueous solution, though the residue is an N-glycosylated Asn (*19*). We think that the helix-breaking nature of glycosylated amino acid residues has seldom been reported simply because natural glycosylation sites are almost always out of the helical regions. Thanks to our artificial system, we can clearly demonstrate that a glycosylated residue locally perturbs a helical structure.

The N-glycosylation at Asn3 of CT caused a carbohydrate structure-dependent change in the hypocalcemic activity, but the change was rather mild (*5*). The effect of O-glycosylation was far more drastic. Only CT2-GalNAc and CT21-GalNAc retained, or slightly gained, activity. The activity of the other derivatives was reduced considerably. This result corresponds fairly well to the CT−R binding activity. Therefore, CT5-GalNAc, CT6-GalNAc, CT13-GalNAc, CT25-GalNAc, and CT31-GalNAc must have decreased their hypocalcemic activity due to the steric hindrance of the GalNAc residue in the interaction with the CT−R species, and the GalNAc residue of CT2-GalNAc and CT21-GalNAc must be outside of the recognition site. CT6-GalNAc may have lost its activity due to the structural change in the helical region. The slight increase in the hypocalcemic activity of CT2-GalNAc and CT21-GalNAc can also be ascribed to the increase in the binding activity. O-Glycosylation at Ser2 or Thr21 may have added favorable interactions with the receptor.

The results of the biological activity study are in general agreement with the former structure−activity studies of CT in that the important portions are scattered all along the sequence from the N-terminus to the C-terminus (*4*). Precise comparison, however, reveals that O-glycosylation affects the biological activity more than other modifications. For example, the deletion of Ser13 from salmon CT retains the hypocalcemic activity (*20*), but our CT13-GalNAc decreased in activity. From the truncation experiment of the C-terminal part of eel CT (*21*) and the comparison between human and salmon CT sequences (*22*), the importance of Thr31 is demonstrated (*22*). Thus, the activity loss of CT31-GalNAc is reasonable, but the magnitude is higher than the reported examples. In this sense, glycosylation can be used as a sensitive tool in the structure−activity relationship study of peptides. The activity retention with the glycosylation at Ser2 and Thr21 also has corresponding reports. The deletion of Ser2 from salmon CT does not change the activity (*23*, *24*). The apparent conflict between the structural change and the activity retention by the O-glycosylation at Thr21 can also be explained on the basis of the reported result that the C-terminal portion of the helix is not essential for biological activity (*25*). Hitherto, most of the structure−activity relationship studies on CT have been concerned with the N-terminal, C-terminal, and helical segments. The importance of Ser5, Thr6, and Thr25 is revealed for the first time in this report.

Because the GalNAc residue is common among the derivatives and the main determinant of biodistribution is usually the structure of the carbohydrate portion, we did not expect the biodistribution to change much among them. As can be seen from Figure 4, the expectation was right in that all of the derivatives exhibited similar biodistributions. Still, there were some notable differences. Levels of CT2-GalNAc and CT21-GalNAc were higher in the bone, the target tissue, but lower in the blood. The accumulation of CT2-GalNAc and CT21-GalNAc in the bone and the opposite trend in the blood can be explained as a consequence of their high receptor binding activity. CT25-GalNAc and CT31-GalNAc accumulated in the liver, probably because the glycosylation did not cover the hydrophobic part of CT, as was apparent in their retention times on reverse-phase HPLC. Hepatic clearance of peptides is known to depend on their hydrophobicity (*26*). In the kidney, the concentration of CT2-GalNAc was low and that of CT21-GalNAc was high. Though we cannot explain this behavior with simple causes, it is interesting that the two active derivatives exhibited quite different tendencies in the kidney. The complex balance of the biodistribution must have resulted in this apparent difference.

In conclusion, we can show that the effect of glycosylation is clearly site-dependent. For example, even within the small disulfide loop, glycosylation at Thr6 affected both the structure and activity, at Ser5 only the activity, and at Ser2 none, indicating that the site dependence is very strict. The structural consequence of glycosylation is understood if we assume that glycosylation causes an amino acid residue to be a stronger helix breaker than the original amino acid. The drastic difference in the hypocalcemic activity can be explained by the steric inhibition of the interaction with the receptor. Therefore, we must be very careful in choosing the attachment site when we use glycosylation in the design of biologically active peptides. Retrospectively, we were lucky in selecting Asn3 as the N-glycosylation site in the first study of the glycosylated CT (*5*). If the position is right, the function of carbohydrates can be utilized. Currently, we are investigating the detailed structure of CT6-GalNAc and CT21-GalNAc by NMR.

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