Inhibitory Effect of a Self-derived Peptide on Glucosyltransferase of Streptococcus mutans

POSSIBLE NOVEL ANTICARIES MEASURES*

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Glucosyltransferase (GTF) plays an important role in the development of dental caries. We examined the possible presence of self-inhibitory segments within the enzyme molecule for the purpose of developing anticaries measures through GTF inhibition. Twenty-two synthetic peptides derived from various regions presumably responsible for insoluble-glucan synthesis were studied with respect to their effects on catalytic activity. One of them, which is identical in amino acid sequence to residues 1176–1194, significantly and specifically inhibited both sucrose hydrolysis and glucosyl transfer to glucan by GTF-I. Double-reciprocal analysis revealed that the inhibition is noncompetitive. Scramble peptides, composed of the identical amino acids in randomized sequence, had no effect on GTF-I activity. Furthermore, the peptide is tightly bound to the enzyme once complexed, even in the presence of sodium dodecyl sulfate (SDS). Kinetic analysis using an optical evanescent resonant mirror cuvette system demonstrated that the enzyme-peptide interaction was biphasic. These results indicate that the peptide directly interacts with the enzyme with high affinity and inhibits its activity in a sequence-specific manner. This peptide itself could possibly be an effective agent for prevention of dental caries, although its effectiveness may be improved by further modification.

Glucan synthesis catalyzed by glucosyltransferases (GTFs; sucrose 6-glucosyltransferase, EC 2.4.1.5) of the oral streptococci is critical in the development of dental caries. This is the first step in the formation of dental plaque, which mediates the aggregation of cariogenic microorganisms, which in turn leads to acid demineralization of the tooth enamel. Therefore, GTF activity is a potential up-stream target in the pathological cascade (1, 2).

GTFs catalyze two sequential reactions: the cleavage of sucrose into fructose and an enzyme-bound glucosyl moiety (sucrase activity), and the subsequent transfer of the latter to the C-3/C-6 position of the glucose residue of glucan (transerfase activity) or to water (3). In accordance with these enzymatic properties, GTFs have two relatively independent structural domains, a catalytic domain comprised of the N-terminal two-thirds of the protein and a glucan-binding domain comprised of the remaining one-third (4). Although these domains are common among GTFs found in streptococci and the sequences of the active sites are highly conserved (3, 5), each GTF exhibits distinct enzyme activity that differs in position of glucosyl linkage of product and/or primer requirement for catalytic activity (1, 3).

Streptococcus mutans (S. mutans), a major causative organism of human dental caries, produces three GTFs, i.e. GTF-I (gtfB) and GTF-SI (gtfC), which catalyze primarily the synthesis of α-1,3-linked water-insoluble glucan and low molecular water-soluble glucan in a primer-independent manner, and GTF-S (gtfD), which catalyzes the synthesis of α-1,6-linked water-soluble glucan in a primer-dependent manner (6–8). Disruption analysis of genes encoding these GTFs revealed that the synthesis of water-insoluble glucan is essential to the cariogenesis of S. mutans (9).

GTF-I is composed of 1475 amino acids with a signal peptide of 34 residues at the N-terminal (10). The sequence responsible for sucrose binding was proposed to be DSIRVDAVD (residues 446–454), and Asp651 was identified as one of the active centers for catalytic activity (11, 12). The C-terminal glucan-binding domain consists of six highly homologous repeating units of approximately 65-amino acid residues each, and deletion analysis has revealed that the presence of more than three of these units is necessary for GTF to be able to catalyze glucan synthesis (13). However, details of the mechanism of catalytic action and structure-function relationships of GTF remain yet unknown.

In the present study, we paid special attention to the fact that several enzymes, for example protein kinase C, have been reported to contain autoinhibitory sequences (14, 15). In addition, peptides corresponding to the partial sequences of proteins can mimic the original function of the region and can be used to characterize the functional domains (16, 17). Based on these facts, we synthesized 19-mer peptides corresponding to various regions of GTF-I and examined their ability to modulate the catalytic activity of the enzyme. Here, we demonstrate that a peptide, corresponding to residues 1176–1194, inhibits GTF-I activity in a sequence-specific manner and three other peptides, covering residues 902–943, increase GTF-I activity. We further report the examination of the interaction between GTF-I and the inhibitory peptide.

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The abbreviations used are: GTF, glucosyltransferase; GTF-I, insoluble-glucan synthesis glucosyltransferase; PAGE, polyacrylamide gel electrophoresis; QAE, diethyl[2-hydroxypropyl]aminoethyl.
**Effect of Peptide on GTF Activity**

### Table I

| Peptide No. | Amino acid residue | Sequence | Net charge | Sucrase activity<sup>a</sup> | GTF-I activity<sup>a</sup> | Transferase activity ratio<sup>b</sup> |
|-------------|-------------------|----------|------------|-----------------------------|---------------------------|-----------------------------------|
| 1           |                  |          |            |                             |                           |                                   |
| 10          |                  |          |            |                             |                           |                                   |
| 20          |                  |          |            |                             |                           |                                   |
| 30          |                  |          |            |                             |                           |                                   |
| 40          |                  |          |            |                             |                           |                                   |

<sup>a</sup> GTF activity in the presence of 30 μg/ml of peptide. Values are means of triplicate determinations, and S.D. are shown in parentheses.

<sup>b</sup> GTF activity in the absence of a peptide.

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**Experimental Procedures**

**Peptide Synthesis and Purification**—Peptides were synthesized by the solid phase Merrifield method (18) in a model 350 peptide synthesizer (Advanced Chemtech, Louisville, KY) and purified by reverse phase high performance liquid chromatography on a TSKgel column (ODS-Prep, Tosoh Co., Tokyo, Japan). The molecular masses of peptides were confirmed by mass spectrometry (JMS-HX-110A/110A, JEOL Co., Tokyo, Japan).

**Enzyme Preparation**—GTF-I was prepared from *Streptococcus milleri* transormant KSB8 cells expressing the gtfB gene (19). *S. milleri* is a noncaroiogenic oral streptococcus variant that cannot synthesize extracellular glucans natively and therefore is more suitable than *S. mutans* for characterization of GTFs employing recombinant protein expression systems (19, 20). In brief, the cells cultured anaerobically overnight at 37 °C were collected by centrifugation, washed, and sonicated in 20 mM phosphate buffer (pH 6.0) at 25 °C for 1 ho r4° C overnight unless otherwise stated in the figure legends. The reaction was started by addition of sucrose and, after incubation for 1 h at 37 °C, was stopped by heating at 80 °C for 5 min. The amounts of glucose and fructose in the reaction mixture were measured by use of an F-kit (Roche Molecular Biochemicals, Mannheim, Germany). The amount of fructose in the reaction mixture represents the level of sucrase activity, and the difference between the amount of free glucose and that of free fructose in the reaction mixture represents the amount of glucan, the level of total GTF-I activity (21), and the ratio of the amount of glucan to that of fructose represents the level of glucosyl transferase activity of GTF-I (transferase activity ratio).

For activity staining, 50 milliunits of GTF-I was preincubated in the presence or absence of 30 μg/ml synthetic peptide in 50 mM phosphate buffer (pH 6.0) at 25 °C for 1 h or 4 °C overnight unless otherwise stated in the figure legends. The reaction was started by addition of sucrose and, after incubation for 1 h at 37 °C, was stopped by heating at 80 °C for 5 min. The amounts of glucose and fructose in the reaction mixture were measured by use of an F-kit (Roche Molecular Biochemicals, Mannheim, Germany). The amount of fructose in the reaction mixture represents the level of sucrase activity, and the difference between the amount of free glucose and that of free fructose in the reaction mixture represents the amount of glucan, the level of total GTF-I activity (21), and the ratio of the amount of glucan to that of fructose represents the level of glucosyl transferase activity of GTF-I (transferase activity ratio).

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**Fig. 1.** Schematic diagram of the structure of GTF-I showing the location of the synthetic peptide sequences. The diagram of the structure of GTF-I and the sequences of the active site are shown at the top (11). The numbers indicate the amino acid positions in GTF-I. The aspartic acid (Asp<sup>451</sup>) indicated in *boldface type* is one of the active centers of sucrose hydrolysis (12). Glucan-binding domain consists of six highly homologous repeating units: residues 1096–1159, 1160–1223, 1224–1288, 1289–1353, 1354–1418, and 1419–1475 (13). The domains from which the sequences of the synthetic peptides are derived are shown at the bottom.
EDTA, and 0.01% Thimerosal, at 25 °C overnight. The synthesized glucan was observed as a white band on the polyacrylamide gel (6).

Preparation of Antibody and Western Blot Analysis—An anti-GTF-I antibody was raised against a synthetic 25-mer peptide, CGEFVTDRY-GRISYYDANGERVRIN, conjugated to keyhole limpet hemocyanin (Calbiochem) as described previously (23). This peptide corresponds to the C-terminal portion of GTF-I (10). 10 milliunits of GTF-I was subjected to SDS-PAGE, and Western blot analysis using anti-GTF-I antibody was performed as described previously (23).

Co-precipitation of GTF-I with Peptide 14—Synthetic peptide was coupled to support matrix, Affi-Gel 10 gel (Bio-Rad) in 100 mM HEPES (pH 7.0). The gel was preincubated in phosphate-buffered saline containing 1% bovine serum albumin and 0.5% Triton X-100 at 4 °C for 1 h, followed by incubation with crude GTF-I solution at 4 °C overnight in the presence or absence of 500 µg/ml peptide 14. The gel was washed by subjecting it to 10 min of agitation in phosphate-buffered saline containing 1% Triton X-100, followed by centrifugation and discard of the supernatant, repeated 5 times. The gel was suspended in SDS-PAGE solubilization buffer and incubated at 100 °C for 15 min. After centrifugation, the supernatant was analyzed by Western blot analysis using anti-GTF-I antibody as described above.

Interaction between GTF-I and Peptide 14 Analyzed by an Optical Evanescent Resonant Biosensor—An optical evanescent resonant mirror cuvette system (IAsys, Affinity Sensors, UK) was used to measure the interaction of peptide with GTF-I as described (24–26). 5 mg/ml peptide 14 in acetate buffer (pH 4.5) was immobilized to an aminosilane cuvette at 25 °C for 10 min following cuvette activation by bis(sulfosuccinimidyl)suberate (Pierce). The cuvette surface was inactivated by 1 M ethanolamine/HCl (pH 8.5) and blocked with bovine serum albumin. The association reaction of GTF-I to the immobilized peptide was started by addition of the enzyme into the cuvette, and the dissociation was by replacement of GTF-I with blank buffer. The traces of the association and dissociation process were analyzed using Fastfit analysis software supplied with the instrument. The pseudo first-rate constant, $k_{on}$, was obtained for each concentration of the analyte protein.

The slope of the plot of $k_{on}$ against the analyte protein concentration

![FIG. 2. Kinetic analysis of the inhibitory effect of peptide 14 on GTF-I activity. Panel A, sucrase activity. Panel B, GTF-I activity. The activity in the presence (open circles) and absence (closed circles) of peptide 14 at the indicated concentration of sucrose was examined. GTF-I was preincubated with buffer alone or 30 µg/ml peptide 14, and the reaction was initiated by addition of sucrose of various concentrations to the reaction mixture. The values are means of triplicate determinations, and the error bars indicate S.D. (n = 3). Panel C, transferase activity ratio. The ratio of glucose moiety to glucan by GTF-I in the presence (open circles) and absence (closed circles) of peptide 14 was shown. Panel D, Lineweaver-Burk double reciprocal plot of sucrase activity. The values in presence (open triangle) and absence (open rectangle) of peptide 14, which were deduced from the results in panel A, are shown. Panel E, Lineweaver-Burk double reciprocal plot of GTF-I activity. The values in presence (open triangle) and absence (open rectangle) of peptide 14, which were deduced from the results in panel B, are shown.](image-url)
Effect of Peptide on GTF Activity

RESULTS

Effect of GTF Peptides on GTF-I Activity—Table I shows the code numbers of the synthetic peptides, their amino acid sequences, the corresponding amino acid positions in GTF-I, their net charge, and their effects on GTF-I activity. We synthesized 19-mer peptides derived from various GTF-I regions highly conserved among GTFs of oral streptococci (3, 5) because we speculated that these regions were of structural and/or functional importance. The sequences of peptides 32, 33, 1, and 2 are located upstream of the active center of sucrose hydrolysis, those of peptides 13 and 14 are located in the glucan-binding domain at the C terminus, and those of the others are located between the active center for sucrose hydrolysis and the glucan-binding domain (Fig. 1). The role of the region between the active center and the glucan-binding domain has not yet been reported. Some peptides, including the one corresponding to the active site sequence, were not examined for their effects on GTF-I activity because of their low solubility in buffer solution. Of 22 peptides tested, peptide 14 (residues 1176–1194) had significant effects on GTF-I activity (Table I). Peptide 14 inhibited both sucrase activity and transferase activity of the enzyme, resulting in a marked reduction in the amount of glucan. No inhibitory effects of the peptides on hexokinase and glucose-6-phosphate dehydrogenase activities used for the biochemical assay were detected even at a higher concentration of the peptides than that used in Table I. On the other hand, peptides 23, 24, and 31, corresponding to residues 902–943, increased GTF-I activity by about 20–35%. These peptides primarily affected sucrase activity, whereas in the presence of these peptides the transferase activity ratio remained essentially unchanged.

Lineweaver-Burk Double Reciprocal Analysis of Inhibition by Peptide 14—Next, we examined the mechanisms of inhibition of GTF-I activity by peptide 14. GTF-I activity in the presence or absence of peptide 14 at various concentrations of sucrose was examined, and the results and deduced Lineweaver-Burk double reciprocal plots are shown in Fig. 2. Peptide 14 inhibited both sucrase and GTF-I activities in a noncompetitive manner, indicating that peptide 14 interacted with enzyme and enzyme-substrate complex (Fig. 2, D and E). In the absence of peptide 14, about 70–80% of glucose moiety was transferred to glucan, and in the presence of peptide 14, only 5–30% of glucose moiety was transferred at any concentration of sucrose (Fig. 2C). The secondary plots deduced from the double-reciprocal plots yielded an apparent Ki of 10.5 μM for GTF-I activity (mean of three independent experiments, S.D. = 4.5). The inhibition of GTF-I activity by peptide 14 was dose-dependent, and the concentration of the inhibitor giving 50% inhibition (IC50) was about 3 μM (Fig. 3).

Effect of Peptide 14 on Purified GTF-I—To rule out the possible involvement of other putative components which might interact with peptide 14 to affect GTF-I activity, we examined the effect of peptide 14 on purified GTF-I. The crude enzyme solution was applied to an anion exchange HPLC, and the fraction eluted at the NaCl concentration of 0.65 M yielded a...
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The enzyme preincubated in the presence or absence of synthetic peptide was subjected to SDS-PAGE without heating of the samples, and after electrophoresis, the polyacrylamide gel was incubated in sucrose buffer. As shown in Fig. 6A, peptide 14 inhibited glucan synthesis by GTF-I on the polyacrylamide gel. pep14/sc1 had no effect on enzyme activity, consistent with the results in Fig. 5. In addition, we examined the interaction of GTF-I and peptide 14 using the support gel (Fig. 6B). GTF-I was co-precipitated with peptide 14 coupled to the gel as revealed by Western blot analysis, whereas it was not co-precipitated with pep14/sc2. Furthermore, addition of excess free peptide 14 in the reaction solution resulted in the disappearance of co-precipitated GTF-I. Moreover, the interaction between GTF-I and peptide 14 was confirmed using an optical evanescent resonant mirror cuvette system (IAsys). GTF-I of various concentrations from 100 nM to 1 μM was examined for association/dissociation to peptide 14 immobilized on the aminosilane cuvette. We did not add the substrate into the enzyme solution in this experiment because the glucan product caused aggregation of the enzyme and would interfere with the analysis. As shown in Fig. 7, the interaction was dependent on the enzyme concentration. The traces of the association and dissociation were analyzed using Fastfit analysis software. Analysis revealed that the interaction was biphasic and yielded first and second rate constants, $k_{on(1)}$, $k_{off(1)}$, $k_{on(2)}$, $k_{off(2)}$, $k_1$, and $K_D$, obtained from the results of $k_{on(1)}$, were $8.2 \times 10^4 M^{-1} s^{-1}$, $9.8 \times 10^{-3} s^{-1}$, and $120$ nM, respectively, and $8.3 \times 10^3 M^{-1} s^{-1}$, $2.3 \times 10^{-3} s^{-1}$, and $277$ nM, respectively, from $k_{off(2)}$. These results strongly indicate that GTF-I and peptide 14 directly interacted in a biphasic manner, although the critical region of interaction of both was not identified, and that this complex could not be separated by sodium dodecyl sulfate during electrophoresis.

**DISCUSSION**

In this report, we have demonstrated that peptides derived from GTF-I affected the enzyme activity; those corresponding to residues 902–943 (peptides 23, 24, and 31) elevated GTF-I activity, and the one corresponding to residue 1176–1194 (peptide 14) inhibited GTF-I activity. Peptide 14 directly interacted with GTF-I in a biphasic manner to inhibit the enzyme activity (Figs. 4, 6, and 7), and this inhibition was noncompetitive with the substrate sucrose (Fig. 2). Furthermore, inhibition was in a sequence-specific manner, and the net charge of the peptide

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**Fig. 6. The direct interaction of GTF-I with peptide 14.** Panel A, the effects of peptide 14 as determined by activity staining. GTF-I was preincubated with buffer (lane 1), or peptide 14 (lane 2), or pep14/sc1 (lane 3), and subjected to SDS-PAGE as described under “Experimental Procedures.” Bands of synthesized glucan could be visualized after incubation of polyacrylamide gel in sucrose buffer. The arrow indicates the relative molecular weight of GTF-I, 150,000, as confirmed by Western blot analysis using anti-GTF-I antibody. The bars indicate that peptide 14 inhibited glucan synthesis by GTF-I on the polyacrylamide gel. pep14/sc1 had no effect on enzyme activity, consistent with the results in Fig. 5. Panel B, co-precipitation of GTF-I with peptide 14. GTF-I was incubated with peptide 14 (lanes 1 and 3) or pep14/sc2 (lane 2) coupled to the support gel in the absence (lanes 1 and 2) or presence (lane 3) of free peptide 14. The supernatant eluted from the washed gel was subjected to Western blot analysis. The results are representative of three independent experiments.
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does not explain the inhibition because neither peptides with similar net charge (Table I) nor scramble peptides (Fig. 5) were effective.

Several proteinaceous inhibitors of α-amylase, a GTF-related enzyme, have been identified, for example tandemstat (30). It is interesting that the complex of tandemstat and amylase cannot be separated by sodium dodecyl sulfate (30), similar to the case for peptide 14 and GTF-I (Fig. 6A). However, no marked amino acid sequence homology was found between tandemstat and peptide 14, and peptide 14 does not contain the consensus sequence WRY, which is typical of the amylase inhibitors that bind to the catalytic site (31).

The residues 1176–1194, represented by peptide 14, are located in the second repeating unit in the glucan binding domain. Because the mode of inhibition by peptide 14 is non-competitive (Fig. 2) and because the peptide is tightly bound to the enzyme once complexed (Fig. 6), this segment may play a crucial role in regulation of the enzyme activity.

The binding experiments using IAsys demonstrated that the enzyme-peptide binding processes were composed of complex molecular-to-molecular interactions (Fig. 7). The double rate constants seem to represent intrinsic biomolecular interactions, such as some conformational changes occurring after the first binding or double binding site on the enzyme, rather than the steric hindrance (27, 28). We suppose that the second rate binding may be important for inhibition compared with the $K_i$ and IC50 values (Figs. 2, 3, and 7) although we do not have a rationale for the difference of these values at present. However, we have cogitated that the most possible reason for the difference is the lack of the substrate sucrose in the binding experiment using IAsys. The presence of sucrose should have some influences on the enzyme-peptide interactions in the non-competitive inhibitory reactions (Fig. 2). Also, the difference could be because of immobilization of the peptide.

How the ability of the peptide to inhibit GTF-I activity is related to the function of the region corresponding to it in the molecule remains to be elucidated. However, residues 902–943 are located in the most homologous region among streptococcal GTFs (3) and also have sequence similarity to barley α-amylase (approximately 70% similarity in this region) (10, 32, 33), suggesting its functional importance. Therefore, it is possible that interactions of the synthetic peptides with the enzyme region in a homophilic manner may have resulted in modulated activity. Alternatively, these peptides may have interfered with conformational changes and/or intramolecular interaction induced in GTF catalytic process by mimicking the function of the enzyme region.

Our results demonstrate the possibility that the use of a peptide derived from the enzyme would be effectual to modulate enzyme activity, as well as to identify the functional domains necessary for protein-protein interactions as previous studies have shown (16, 17). We expect that these peptides, besides clinical applications, could be strong tools to elucidate the structure-function relationships of GTF-I, which would provide us with clues to clarify why GTF is responsible for α-1,3/1,6-glucosidic linkage, whereas most α-glucosidase, including amylase, are responsible for α-1,4/1,6-glucosidic linkage. For both these purposes, we would also like to emphasize that the effectiveness of the peptide may be further improved by additional modifications such as acetylation, amidation, and amino acid substitution/addition.

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