Subsite Specificity of the Active Site of Glucosyltransferases from Streptococcus sobrinus*

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Oral bacterial glucosyltransferases use sucrose as a substrate in synthesis of either 1,3-α-glucans (GTF-I) or 1,6-α-glucans (GTF-S). The binding specificity of the glucosyl and fructosyl subsites of the sucrose-binding site was examined to identify ligands that bind exclusively to each subsite. Such compounds can be used as reporter ligands to localize the subsite binding of any reversible or irreversible active site inhibitor. In examining potential subsite-specific ligands, binding affinity to GTF-I was consistently stronger than binding to GTF-S. Fructose was found to be a moderate GTF inhibitor, but free glucose, α-methylglucoside, and glucose epimers were very weak inhibitors. In contrast, glucose transition-state analogues, α-glucano-1,5-lactone, 1-deoxynojirimycin (dNJ), and most N-alkyl derivatives of dNJ were moderate to strong inhibitors; in particular N-methyl-dNJ was found to be the strongest GTF inhibitor identified to date. Multiple inhibitor kinetic analysis established nonexclusive binding of fructose and dNJ at the respective subsites. Binding of fructose and N-alkyl-dNJ derivatives was, to a small degree, partially exclusive. Fructose and dNJ were used as reporter ligands to localize the subsite specificity of two test inhibitors: a reversible inhibitor, Zn²⁺, and an irreversible inhibitor, diethyl pyrocarbonate (DEP). Zn²⁺ paired with dNJ in multiple inhibitor kinetic analysis showed no competition between the inhibitors, while Zn²⁺ paired with fructose decreased ligand affinity 7-fold, establishing Zn²⁺ binding exclusively at the fructose subsite. Analogous experiments adapted to the irreversible inhibitor DEP indicated that it reacts at both subsites or induces a protein conformational change at one subsite that alters ligand binding at the adjacent subsite.

Members of the prevalent family of oral bacteria referred to as mutants streptococci are remarkable for their central role in the initiation of dental caries on smooth enamel surfaces. They derive this potential in large measure from the production of extracellular glucosyltransferases. The enzymes tap the high free energy of the sucrose glycosidic bond to synthesize long-chain glucans. The glucans accumulate on tooth enamel surfaces and form a scaffolding for bacterial colonization. In the protected environment of the glucans, the mutants streptococci and other oral microorganisms form a stable community (dental plaque) and may release sufficient quantities of metabolic acids to demineralize tooth enamel and initiate dental caries (1–3).

The two most common glucosyltransferases secreted by mutants streptococci synthesize different glucan products: sucrose: 1,3-α-glucan 1,3-a-α-glucosyltransferase (GTF-I), which synthesizes a water-insoluble 1,3 glucan, and sucrose: 1,6-α-glucan 1,6-a-α-glucosyltransferase (GTF-S), which synthesizes a water-soluble 1,6 glucan. The enzymes are highly selective for sucrose as the glucosyl donor. This substrate is the only abundant, readily available saccharide with a free energy (~6600 cal/mol) sufficient for synthesis of an 1,3 or 1,6 glycosidic bond. The enzyme catalyzes glucan synthesis, sucrose hydrolysis, and isotope exchange between fructose and sucrose. In glucan synthesis, the steady state reaction flux follows a hybrid rapid equilibrium random/ping-pong mechanism (4).

Little is known of the enzyme structure. The amino acid sequences of no less than six glucosyltransferases from mutants streptococci are known from the gene sequence (5–10). They are large and highly homologous proteins of approximately 1,500 residues organized into two relatively independent structural and functional domains, a catalytic domain comprised of the N-terminal two-thirds of the protein and a glucan-binding domain formed from the remaining one-third (5, 11).

The sucrose-binding catalytic site has been explored only minimally, with the exception of a recently identified catalytic aspartate that functions to stabilize the oxocarbonium transition state in glucosyl transfer (12, 13). In this study we have taken a kinetic approach to explore the specificity of the catalytic site. In the course of the study tight binding ligands were identified as selective for the glucosyl or fructosyl subsites. By applying multiple inhibition kinetics, it was possible to use the ligands to localize the subsite specificity of any reversible or irreversible inhibitor that bound or reacted at the enzyme active site.

**EXPERIMENTAL PROCEDURES**

*The abbreviations used are: GTF-I, sucrose: 1,3-α-glucan 1,3-a-α-glucosyltransferase; dNJ, 1-deoxynojirimycin; GTF, glucosyltransferase; GTF-S, sucrose: 1,6-α-glucan 1,6-a-α-glucosyltransferase; MBS, 2-α-d-morpholinoethanesulfonic acid; DEP, diethyl pyrocarbonate.*

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Methods

Bacterial Culture and Purification of Glucosyltransferases—S. sobrinus was cultured in defined media for 48 h at 37 °C as previously described (4). GTF-I and GTF-S were purified by a modification of a previously reported procedure (4). Cell-free culture supernatant was directly loaded onto a 5-cm diameter chromatography column containing dextran-agarose affinity resin equilibrated with 0.1 M sodium phosphate buffer, pH 6.8. All protein except GTF-I and GTF-S was eluted with 0.5 M guanidine hydrochloride in 0.1 M sodium phosphate buffer, pH 6.8. GTF-I and GTF-S were eluted with 4.0 M guanidine hydrochloride in the same buffer. Diazaoyzed enzyme fractions were purified to homogeneity with a Synchropak, Inc. HAP-5 high performance liquid chromatography column equilibrated with 0.05 M sodium phosphate, and the reaction and 0.3 mM CaCl₂, pH 6.8. GTF-S eluted from the column without retention, and GTF-I was eluted with a step gradient at 0.5 M sodium phosphate and 0.1 mM CaCl₂, pH 6.8.

Kinetic Assays—All kinetic experiments were based on 14C-isotope transfer from uniformly labeled sucrose to glaucan, similar to a previously described procedure (4). Fifty-mL reaction mixtures were buffered in 0.1 M sodium phosphate, pH 6.5, or 0.1 M MES at pH 6.0 when metal ions were used as inhibitors. Four inhibitor concentrations (including zero) and four sucrose concentrations were used to determine Kᵢ. Multiple inhibition kinetics were performed at 10 mM sucrose and four or five concentrations (including zero) of each of the two inhibitors.

Kinetic Data Analysis—Kinetic data were resolved by nonlinear regression based on the algorithm described by Cleland (14). The specific program used for multiple inhibition kinetics was kindly supplied by Dr. W. W. Cleland and was translated from FORTRAN into BASIC. Kinetic data were graphed in reciprocal form to establish a linear relationship before resolving the data by nonlinear regression. The following equations were used.

Linear competitive inhibition data were resolved according to Equation 1.

\[ v = \frac{V_{\text{max}} K_i}{K_i + [I]} + A \]  

(Eq. 1)

where v is the initial velocity, V is the maximum velocity, Kᵢ is the inhibition constant for inhibitor I, and A is the substrate concentration.

The degree of interaction between two competitive inhibitors was determined by multiple inhibition kinetic analysis according to Equation 2.

\[ v = \frac{V_{\text{max}}(1 + K_i n + J K_i n + J(\alpha K_i K_j))}{[S]} \]  

(Eq. 2)

where α is the interaction factor, and I and J are the inhibitor concentrations.

Protection of the Enzyme from Inactivation by Diethyl Pyrocarbonate—Enzyme protection was carried out by preincubating the enzyme (4 μg) with inhibitors (0.8 μM dNJ or 1.2 μM fructose) in sodium phosphate buffer, pH 6.5, for 10 min at room temperature. After incubation, 40 μM diethyl pyrocarbonate was added, and the reaction mixture was further incubated for 10 min at room temperature. The reaction was stopped by the addition of 50 mM histidine. A 5 μL aliquot was removed and assayed for glucan synthesis activity after diluting the reaction mixture 285-fold. The dilution reduced all inhibitor concentrations to low levels.

RESULTS

Table I lists 13 potential inhibitors of GTF-I and GTF-S, each assayed at sufficiently high concentration (100 mM) to detect weak binding. All of the compounds are monosaccharides or monosaccharide derivatives, which might bind to either the glucose or fructose subsite of the active site. Among the compounds with potential to bind to the glucose subsite are free β-glucose, α-methyl-β-d-glucopyranoside, and glucose epimers. All were very weak inhibitors, indicating that the glucosyl moiety of sucrose contributes relatively little to the initial sucrose Michaelis complex. Free α-fructoset is a substantially stronger inhibitor even without accounting for the fact that the β-fructose form of fructose represents only 9% of the four solution forms of fructose (αβ, pyranose/furanose) (15). While β-glucose (Fig. 1, D) is a weak binding ligand, the glucosyl transition-state analogues, β-glucosyl-1,5-lactone (II), 1-deoxyxojirimycin (5-imino-1,5-dideoxy-β-glucose (dNJ)) (III, R=H), and N-alkyl-dNJ derivatives are strong GTF-I inhibi-

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>GTF-I</th>
<th>GTF-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Aldohexoses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Glucose</td>
<td>85 ± 7</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>α-Methylglucopyranoside</td>
<td>44 ± 6</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>α-Mannose</td>
<td>68 ± 3</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>α-Galactose</td>
<td>74 ± 17</td>
<td>79 ± 9</td>
</tr>
<tr>
<td>Ketohexoses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Fructose</td>
<td>9 ± 0.1</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>α-Allose</td>
<td>65 ± 3</td>
<td>88 ± 13</td>
</tr>
<tr>
<td>α-Psicose</td>
<td>49 ± 5</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>Glucose transition-state analogues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Glucosyl-1,5-lactone</td>
<td>17 ± 1</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>dNJ</td>
<td>22 ± 7</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>N-methyl-dNJ</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>N-ethyl-dNJ</td>
<td>5 ± 0.3</td>
<td>59 ± 12</td>
</tr>
<tr>
<td>N-buty1-dNJ</td>
<td>8 ± 0.1</td>
<td>91 ± 20</td>
</tr>
<tr>
<td>α-Glucal</td>
<td>72 ± 7</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>α-GlacalA</td>
<td>74 ± 2</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>α-GlacalB</td>
<td>72 ± 4</td>
<td>99 ± 2</td>
</tr>
</tbody>
</table>

* Preincubated with enzyme for 5 min.
* Preincubated with enzyme for 60 min.

Among the three ketohexose epimers in Table I, fructose inhibition was the strongest, although as noted above, when comparing the inhibition values, the differences in relative abundance of the four solution forms must be considered. It is not known which of the ketose structure form(s) is inhibitory with the possible exception of β-fructofuranose. What is clear is that formation of the Michaelis complex is essentially the domain of the fructosyl moiety of sucrose and independent of the very weak binding glucosyl moiety.

Finally, Table I reveals a consistent pattern of stronger ligand affinity with GTF-I compared with GTF-S, a characteristic that is particularly pronounced with high affinity ligands. The Ki values of some of the ligands are quantified in the next section as well as the magnitude of interaction between glucose and fructose subsite ligands when both subsites are occupied.

Single Inhibitor Kinetics—Representative ligands that are likely to bind at the glucose subsite of the fructose subsite were

Inhibition of GTF-I and GTF-S with monosaccharides and monosaccharide analogues at high inhibitor concentration

Assays were performed at 100 mM inhibitor and 10 mM sucrose concentration, pH 6.5. Percent activity values are the mean of four measurements ± S.E.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percent activity</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Aldohexoses</td>
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<tr>
<td>α-Glucose</td>
<td>85 ± 7</td>
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<tr>
<td>Ketohexoses</td>
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<td>α-Fructose</td>
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</tr>
<tr>
<td>α-Allose</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>α-Psicose</td>
<td>49 ± 5</td>
</tr>
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<td>Glucose transition-state analogues</td>
<td></td>
</tr>
<tr>
<td>α-Glucosyl-1,5-lactone</td>
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</tr>
<tr>
<td>dNJ</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>N-methyl-dNJ</td>
<td>&lt;2</td>
</tr>
<tr>
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<tr>
<td>α-GlacalB</td>
<td>72 ± 4</td>
</tr>
</tbody>
</table>

* Preincubated with enzyme for 5 min.
* Preincubated with enzyme for 60 min.
chosen to measure inhibition constants and interactions between subsites. Both GTF-I and GTF-S were assayed using fructose as a potential fructose subsite ligand and dNJ, N-methyl-dNJ, N-ethyl-dNJ, and N-butyl-dNJ as potential glucose subsite ligands. The ligand dissociation constants, calculated according to Equation 1, are listed in Table II. The data confirm the disparity between GTF-I and GTF-S ligand affinities. The most pronounced examples are the glucose subsite ligands, dNJ and its derivatives. These ligands are nonexclusive as reported by the interaction factor, Interaction between I and J can be nonexclusive, exclusive, or partially exclusive as reported by the interaction factor.

Notable among the inhibition constants listed in Table II is the strongest GTF reversible inhibitor reported to date. The kinetic pattern for two competitive inhibitors is shown in Scheme 1. I and J are competitive inhibitors of substrate, S. Interaction between I and J can be nonexclusive, exclusive, or partially exclusive as reported by the interaction factor, &alpha;: &alpha; is 1 when I and J are nonexclusive, greater than 1 when I and J are mutually or partially exclusive, and less than 1 when I and J induce a conformational change that enhances binding at the adjacent subsite (19).

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The kinetic pattern for two competitive inhibitors is shown in Scheme 1. I and J are competitive inhibitors of substrate, S. Interaction between I and J can be nonexclusive, exclusive, or partially exclusive as reported by the interaction factor, &alpha;: &alpha; is 1 when I and J are nonexclusive, greater than 1 when I and J are mutually or partially exclusive, and less than 1 when I and J induce a conformational change that enhances binding at the adjacent subsite (19).

The steady state kinetic equation for Scheme 1 is given in Equation 4, where v is the initial velocity, &kappa; is the enzyme-substrate dissociation constant, and V_{max} is the maximum velocity.

\[
v = \frac{V_{\text{max}}[S]}{K_{\text{e}}\left(1 + \frac{[I]}{K_a} + \frac{[J]}{K_J} + \frac{[IJ]}{K_{IJ}}\right) + [S]}
\]

A typical GTF-S analysis with two variable inhibitors, fructose and dNJ, is shown in Fig. 2. The complementary plot of the same data set differing only in reversing the assignment of the variable and changing fixed inhibitors is not shown. The y-axis coordinate of the intersection point in Fig. 2 is \(-aK_{N\text{-methyl-dNJ}}\). The interaction factor, &alpha; cannot be calculated directly from this data but can be calculated from either a data set that includes multiple sucrose concentrations or by dividing aK_{N\text{-methyl-dNJ}} and aK_{dNJ} by the independently calculated inhibition constants.
The glucose and fructose subsites have unique and independent properties that forecast their function in catalysis. The fructose subsite has moderate affinity for \( \alpha \)-fructose and apparently functions exclusively to tether and align the glucosyl moiety in position for catalysis. There is very limited or no interaction between the glucose and fructose subsites. This is clear from multiple inhibitor kinetics using a glucose and fructose subsite inhibitor (e.g., dNJ paired with fructose), where the \( \alpha \) value was near 1.0. The absence of a fructose effect on glucose affinity implies that the catalytic process is not enhanced by a subsite-induced conformational change at the active site.

Catalysis is dependent on the native protein architecture at the glucose subsite. \( \alpha \)-Glucose, \( \alpha \)-methylglucoside, and glucose epimers have very weak affinity for the glucose subsite, but the glucose transition-state analogues, dNJ, \( N \)-alkyl-dNJ derivatives, and \( \alpha \)-glucono-1,5-lactone have strong GTF-I affinity. This is wholly consistent with concepts of enzyme rate acceleration, where the architecture of the glucose subsite is designed to distort and trap the transition state of the glucosyl moiety. The fructosyl moiety serves solely to extend the period of glucosyl occupancy in the catalytic site and increase the probability of capturing the transition state (21, 22).

1-Deoxynojirimycins are potent inhibitors of a wide range of glycosidases, some of which bind with dissociation constants 10-fold lower than the native monosaccharide analogues. Enzymes susceptible to the relevant dNJ epimer include \( \alpha \)- and \( \beta \)-glucosidases (23, 24), \( \alpha \)- and \( \beta \)-galactosidases (25, 26), \( \alpha \)- and \( \beta \)-mannosidases (27), and \( \alpha \)-l-fucosidase (28, 29). In some instances, particularly with enzymes with very high affinity, the enzyme-inhibitor complex forms very slowly, which implies a protein conformational change in formation of the tight enzyme-inhibitor complex (23, 30). dNJ is a relatively strong GTF inhibitor but not as potent as reported for several glycosidases, and there is no indication of a slow multistep process in attaining the final form of the complex.

The magnitude of GTF inhibition by \( N \)-alkyl-dNJ derivatives is highly dependent on the length of the \( N \)-alkyl chain. The \( N \)-methyl derivative has the lowest \( K_i \), followed by the \( N \)-ethyl and \( N \)-butyl derivatives. Steric constraints are obvious explanations. 

**DISCUSSION**

Several conclusions emerge from the survey of carbohydrates and carbohydrate derivatives binding to the GTF catalytic site. The glucose and fructose subsites have unique and independent properties that forecast their function in catalysis. The fructose subsite has moderate affinity for \( \alpha \)-fructose and apparently functions exclusively to tether and align the glucosyl moiety in position for catalysis. There is very limited or no interaction between the glucose and fructose subsites. This is clear from multiple inhibitor kinetics using a glucose and fructose subsite inhibitor (e.g., dNJ paired with fructose), where the \( \alpha \) value was near 1.0. The absence of a fructose effect on glucose affinity implies that the catalytic process is not enhanced by a subsite-induced conformational change at the active site.

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for this effect. For example, all sequenced mutans streptococci

glucosyltransferases have a conserved catalytic aspartate, which

ion pairs with the oxocarbonium cyclic oxygen and as such is

in very close proximity to the dNJ N-alkyl chain (2).

Also characteristic of the ligand binding to GTF-S and GTF-I is

the consistently stronger ligand affinity of GTF-I compared

with GTF-S. The explanation in the context of the thermody-

namics is not clear. However, tighter ligand binding including

substrate binding may have functional consequences, because

the glucan product of GTF-I, unlike GTF-S, is insoluble and far

more effective in aiding bacterial colonization of the host.

The extracellular oral streptococcal glucosyltransferases dis-

cussed here are arguably the most significant virulence factor

in initiation of dental caries on smooth enamel surfaces and

also root surfaces as well (2). For this reason, details of the

enzyme structure and function are important in evaluating the

nature of the virulence. Substantial kinetic and catalytic de-

tails have been resolved (3–5), but acquisition of detailed struc-

ture information is limited by the large size of the enzymes

(~1,500 residues), which presents a major challenge to x-ray

crystallography and is only theoretically possible with multi-

dimensional nuclear magnetic resonance. Thus, structure in-

formation is scant consisting of knowledge of the location in

the primary structure of a catalytic residue (13) and the gross

organization of the enzyme into an N-terminal catalytic domain

and a C-terminal glucan-binding domain (5, 11). An alternative

to gain further insight into significant features of the protein

structure is three-dimensional structure modeling, which we

are pursuing. In addition, the nonexclusive glucose and fruc-

tose subsite ligands presented here have the potential to aug-

ment details of the enzyme structure at the active site. Any

reversible or irreversible ligand binding at the sucrose site can

be easily localized to the glucose or fructose subsite. The simple

and practical analysis was successful in localizing two ligands,

the reversible inhibitor, Zn\(^{2+}\), which was found to bind at the

fructose subsite, and the irreversible inhibitor, DEP, which was

found to react at the interface between the two subsites or at

both subsites. In the context of adding structural details to our

knowledge of the enzymes, application of the glucose and fruc-

tose subsite ligands can lead to identification of peptides that

course through each subsite.

Acknowledgments—We are indebted to W. W. Cleland for supplying

the program to resolve multiple competitive inhibition kinetic data and
to Farooq Azam for translating the program into BASIC.

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