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Essential amino acids involved in glucan-dependent aggregation of *Streptococcus sobrinus*

J.S. Singh, K.G. Taylor and R.J. Doyle *

Academic Health Center, University of Louisville, Louisville, KY 40292 (USA)

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ABSTRACT

The active site of the glucan-binding lectin (or agglutinin) (GBL) of *Streptococcus sobrinus* was probed by specific amino acid modifying reagents. Reagents specific for carboxylates, imidazolium, phenolic, and lysyl residues inactivated the cell bound GBL, whereas agents specific for sulfhydryl, disulfide, and guanidinium groups had no effect on the lectin.

A low molecular weight α-(1 → 6)-glucan provided partial protection against the reagents which inactivated the protein, whereas an α-(1 → 4)-glucan, incapable of complexing with the lectin, afforded no protection. A reagent specific for tryptophan, 2-hydroxy-5-nitrobenzyl bromide (HNB) did not cause a loss of GBL activity, although *N*-bromosuccinimide, a reagent capable of oxidizing tryptophan and less selective than HNB, was a very effective inhibitor of the glucan-dependent cellular aggregation. In the latter case, α-(1 → 6)-glucan did not protect. Hydroxylamine partially restored the loss of lectin activity due to treatment of the cells with *N*-acetylimidazole (highly specific for tyrosine), glycine methyl ester plus water-soluble carbodiimide (specific for carboxylates), and diethylpyrocarbonate (specific for histidine). Because the soluble form of GBL rapidly loses activity when purified, it was necessary to perform the chemical modification of the amino acid side chains employing the cell-bound form of the lectin. Because specific ligand [α-(1 → 6)-glucan] protected against the inactivation of the agglutinin by selected reagents and because lectin activity could be restored in some cases, it was possible to identify likely essential amino acid residues needed for glucan binding. The results, taken together, suggest that aspartic (and/or glutamic) acid, histidine, lysine, and tyrosine are critical amino acids responsible for agglutinin activity. Present efforts are directed to the design and synthesis of glucan analogues which may serve as affinity inactivating agents of the lectin. Such glucan derivatives may be of value in studies on the role of the lectin in cariogenesis.

INTRODUCTION

Most bacteria have a tendency to adhere to surfaces. Adhesion may offer increased resistance to antibiotics, enzymes, and immune factors. In addition, adhesion may enhance the growth rate of a bacterium[1]. Some oral streptococci produce a glucan-binding lectin (GBL) which presumably functions to aid in the adhesion of the bacteria to α-(1 → 6)-glucans[2–5]. Most oral streptococci produce

* Corresponding author.

the glucans extracellularly by secreted glucosyltransferases (GTFs) (reviewed by Loesche[6]). Adherent microorganisms, glucan, salivary factors, and foodstuffs combine to create dental plaque. Plaque is required to initiate carious lesions in teeth. The exact role of GBLs in plaque genesis or maintenance is unclear, although it is thought that the lectins promote plaque formation by combining with glucans produced from dietary sucrose.

Drake et al.[7] and Landale and McCabe[8] have determined that the GBLs of *Streptococcus sobrinus* and *S. cricetus*, two bacteria thought to be cariogenic, possess unusually large combining sites. The lectins can bind 6–10 hexose units in linear α-(1 → 6)-glucans. Glucans rich in α-(1 → 3)-, α(1 → 2)-, or α(1 → 4)-linkages do not interact with the lectins. There have been numerous studies on the chemical modification of lectins, including lectin adhesins[9–16]. These studies have been directed at determining the nature of the contact amino acids of the lectins. For the lectins of the oral streptococci, it is difficult to study their properties in solution. When the lectins are purified[17,18] using Sephadex affinity chromatography, they rapidly lose activity[19]. Therefore, it is necessary to study the cell-associated form of the GBLs in order to obtain information about essential side chains. In this report, it is observed that several functional groups are required for activity of the lectin, including lysyl, carboxylate, phenolic, and imidazolinium. Indole, guanidinium disulfide, or sulfhydryl are not required for lectin activity.

MATERIALS AND METHODS

Bacteria and growth conditions.—*Streptococcus sobrinus* ATCC 6715 was the primary organism used in this study, although some experiments were carried out with *S. cricetus* AHT. Stocks were maintained on brain–heart infusion agar slants (Difco Laboratories, Detroit, MI) supplemented with calcium carbonate at room temperature. Cultures were inoculated into liquid broth (Trypticase soy broth, BBL Microbiology Systems, Cockeysville, MD) and incubated at 37°C in 5% CO_2 (ref 7). To eliminate trace sucrose and dextran contamination, the broth was incubated with yeast invertase (Sigma Chemical Company, St. Louis, MO) for 2 h at 55°C. A ratio of 5 mg enzyme per g of dry medium was used. The broth was also incubated with yeast dextranase [α-(1 → 6)-glucosidase, Sigma Chemical Company] at 37°C for 4 h employing a ratio of 1 mg of enzyme per 500 mg of dry medium. After cultures had reached early stationary phase, 0.5-mL aliquots were removed and added to 100 mL of fresh medium. The freshly inoculated medium was then incubated for 18 h at 37°C in 5% CO_2. Following incubation, the cells were harvested by centrifugation and washed twice in phosphate–saline buffer, pH 7.2 (20 mM potassium phosphate, 150 mM sodium chloride, PBS). Cells were finally suspended to an absorbance of 0.8–0.9 (1-cm pathlength, 540 nm).

Aggregation assays.—Three-mL aliquots of cells in PBS were added to rounded cuvettes, 50-μL aliquots of glucan T-2000 (a high mol wt α-(1 → 6)-glucan, Pharmacia, Piscataway, NJ) were added and the mixture was rapidly vortexed. Usually,

the final glucan concentration was 5 μg/mL. Control tubes received 50 μL of PBS. Decrease in absorbance caused by aggregation of the cells was monitored every 15–30 s for 5 min or once every 60 s for 20 min. Rate constants for the aggregation were obtained by first order plots of ln A/A_0 vs. time, where A_0 = absorbance at time zero and A = observed absorbance[7]. The best fitting curves were obtained through simple linear regression analyses.

Chemical modification experiments.—The general procedure was to wash cells in the buffer needed for a particular side chain modifying agent and suspend the cells to the original 0.8–0.9 absorbance. After the reactions had taken place, the cells were centrifuged, washed twice in PBS, and suspended in PBS for assay. In some cases, glucan-T10 [an α-(1 $\rightarrow$ 6)-glucan of 10000 average mol wt, Pharmacia], incapable of causing aggregation, was added to give a final concentration of 10 mg/mL prior to addition of the amino acid reagents. Another control was the substitution of amylose [α-(1 $\rightarrow$ 4)-glucan] for the glucan T-10. When cells were treated with hydroxylamine, its final concentration was 100 mM in PBS. Treatment with hydroxylamine was for 60 min at 3°C. Cells were washed twice with and suspended in PBS before use. In all cases, controls were run in which the cells had been subjected to the same conditions as the experimental samples, except the modifying agents were deleted.

Modification of ammonium groups.—Cells were suspended in 100 mM $NaHCO_3$ at 3°C, and acetic anhydride was added dropwise to achieve its final desired concentration[20]. After stirring for 30 min, the cells were centrifuged, washed twice, and suspended in PBS. Reaction with 2-methylmaleic anhydride (citraconic anhydride, Aldrich Chemical Company, Milwaukee, WI) was carried out in 100 mM sodium pyrophosphate at 3°C (ref 20). Appropriate pH was maintained by the addition of 100 mM NaOH.

Modification of arginine (or guanidinium side chains).—The procedure of Patthy and Smith[21] was followed using 1,2-cyclohexanedione in 200 mM sodium borate (pH 9.0). The mixtures were stirred 1 h at 37°C prior to centrifugation and washings. Some experiments were carried out with *p*-hydroxyphenylglyoxal at 85 mM in 100 mM sodium bicarbonate. In these cases, the incubation was for 1 h at room temperature[22].

Modification of carboxylate side chains.—Cells suspended in a solution of 400 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodimiide hydrochloride (EDC, Aldrich Chemical Company), adjusted to pH 4.75 with dil HCl, were added to 1.0 M glycine methyl ester for 60 min at room temperature[23], with the pH being maintained at pH 4.75. Some experiments employed ethylenediamine (ED) in place of glycine methyl ester, but the results were the same.

Modification of histidine side chains.—Reaction of cell suspensions with diethyl pyrocarbonate (DEP) (Aldrich Chemical Company) was carried out according to Fu and Robyt[24]. The reagent ethoxyformylates histidine residues specifically at pH < 6. Cells were suspended in 100 mM acetate (pH 4.5) and incubated at room temperature with the required concentrations of DEP for 20 min.

Modification of tryptophan residues.—Two methods were routinely used: one method involved treating cell suspensions in 50 mM acetate buffer (pH 4.0) with Koshland's reagent (2-hydroxy-5-nitrobenzyl bromide, HNB, Aldrich Chemical Company)[25] for 15 min at room temperature. In the other method, oxidation with *N*-bromosuccinimide was carried out in acetate buffer (100 mM, pH 4.5) at room temperature for 30 min[20].

Modification of phenolic groups.—*N*-Acetylimidazole (NAI, Aldrich Chemical Company) was added to cell suspensions in PBS according to Riordan et al.[26]. The suspensions were incubated at room temperature for 60 min prior to centrifugation. The NAI reagent *O*-acetylates tyrosine residues, but may *N*-acetylate ammonium groups at high concentrations. Acetylation of phenolic groups can be reversed by hydroxylamine. Tetranitromethane (TNM, Aldrich Chemical Company), which forms 3-nitro derivatives with tyrosine[27], was added to cell suspensions in 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer for various periods of time and at various concentrations. Nitrations were at room temperature.

Other modifying agents.—Reaction of cells with *p*-chloromercuribenzoate (PCMB) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), reagents specific for sulfhydryl groups, was done according to Means and Feeny[20]. Mercaptoethanol, a reagent specific for disulfides, was added to cell suspensions in PBS for 4 h at room temperature. Oxidation with H_2O_2 was done at room temperature in PBS employing H_2O_2 concentrations up to 200 mM for 3 h.

RESULTS

Interaction with acetic anhydride and citraconic anhydride.—*S. sobrinus* 6715 was suspended in bicarbonate buffer, and cold acetic anhydride added to a final concentration of 100 mM. Following acetylation, the cells were treated with hydroxylamine in order to remove any *O*-acetyl groups. The amino-acetylated cells were incapable of complexing with glucan T-2000 (Fig. 1). The reagent did not inactivate the GBL in the presence of glucan T-10, a ligand for the lectin. It must be considered that the glucan T-10 protected the GBL, because it may have quenched the acetic anhydride. This seems remote because amylose at the same concentration failed to protect the lectin activity (Fig. 1). Citraconic anhydride also inactivated the lectin, but glucan T-10 offered partial protection.

Involvement of carboxylate in GBL activity.—The carbodiimide nucleophilic interaction with proteins is highly specific for carboxylate residues[23]. Furthermore, when glycine methyl ester is used as the nucleophile, the ester can be removed by hydroxylamine[23]. When cells were modified with EDC–ED, there was an irreversible loss of GBL activity (Fig. 2). However, glucan T-10 afforded protection of the lectin at all EDC concentrations used. Amylose did not protect the lectin. Glycine methyl ester–EDC mixtures also inactivated the lectin, the activity of which could be restored (> 80%) by treatment of the cells with hydroxylamine. These results provide strong support for a role of carboxylate in the combining site

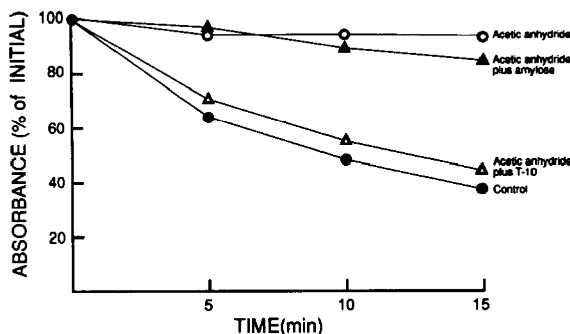

Fig. 1. Inactivation of the glucan-binding lectin of *S. sobrinus* 6715 by acetic anhydride. Results show that glucan T-10 partially protected the lectin from loss of activity. Closed circles, controls with no acetic anhydride; open circles, cells treated with 1.0 mM acetic anhydride; open triangle, cells treated with acetic anhydride in the presence of 10 mg/mL of glucan T-10; closed triangles, cells treated with acetic anhydride in the presence of amylose.

of the GBL. In the data shown in Fig. 2, the first-order rate constant for control cells was 0.73 min^{-1}, whereas the rate constant for cells treated with 5 mM EDC–ED was 0.08 min^{-1}. (First-order rate constants, or "*k*" values, are not shown, but were used in calculating the percent GBL activity shown in Fig. 2.)

Reaction of Streptococcus sobrinus *with diethylpyrocarbonate.*—The DEP reagent is highly specific for imidazolinium groups at pH < 6 (ref 24). Furthermore, the ethoxyformylation can be reversed by hydroxylamine[24]. Only very low concentrations of DEP were required to inactivate the GBL of *S. sobrinus* 6715 (Fig. 3). When the cells were treated with hydroxylamine, GBL activity was fully to

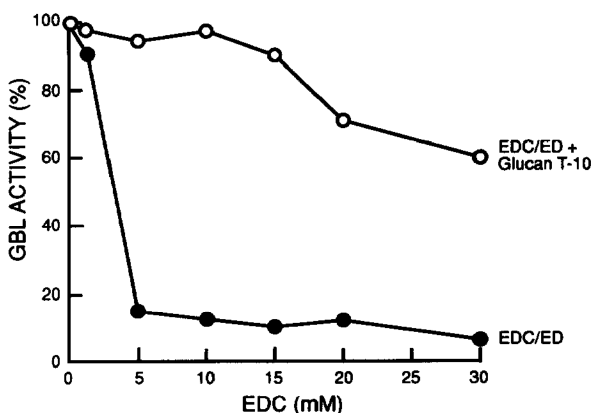

Fig. 2. The glucan-binding lectin of *S. sobrinus* 6715 is inactivated by a carboxylate-modifying agent. The reagents employed were ethylenediamine (ED) and 1-(3-dimethylaminopropyl)-3-ethylcarbodimiide hydrochloride (EDC). Glucan T-10 was used at a 10 mg/mL concentration. All reagents were removed by centrifugations and washings prior to assay for GBL activity. Closed circles, controls; open circles, modified cells.

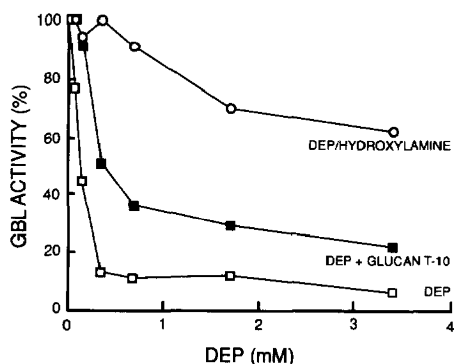

Fig. 3. Effect of diethylpyrocarbonate (DEP) on GBL activity of *S. sobrinus* 6715. The cells were treated with the reagent for 1 h at room temperature and pH 4.5. Reactivation of GBL with hydroxylamine (100 mM) was performed for 1 h at 4°C and pH 7.4.

partially restored at all DEP concentrations. In addition, glucan-T10 afforded partial protection against the effects of DEP.

Phenolic residues and GBL activity.—Two reagents, known to be highly specific for phenolic residues, were used to study the role of tyrosine residues in the GBL activity of *S. sobrinus*. One reagent, *N*-acetylimidazole (NAI), *O*-acetylates the phenolic ring, whereas the other, tetranitromethane (TNM), forms 3-nitrotyrosine derivatives. Fig. 4 shows the results of incubating cells with 10 mg/mL of NAI for

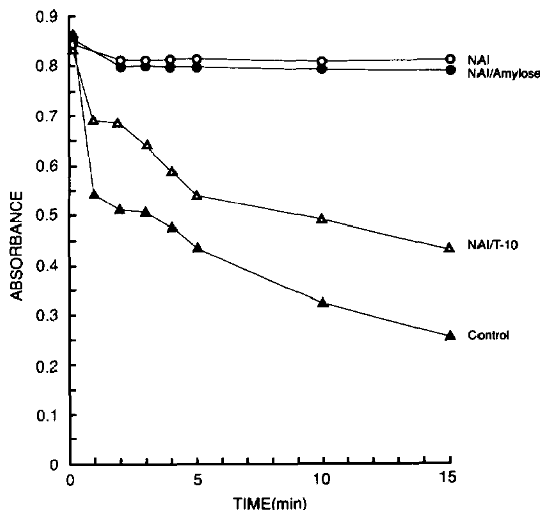

Fig. 4. Effect of *N*-acetylimidazole on GBL activity of *S. sobrinus* 6715. The cells (in PBS) were treated with the reagent (10 mM) for 1 h at room temperature and pH 7.3. Deblocking with hydroxylamine (100 mM) was carried out for 1 h at 4°C. Glucan T-10, but not amylose (10 mg/mL), protected GBL from inactivation.

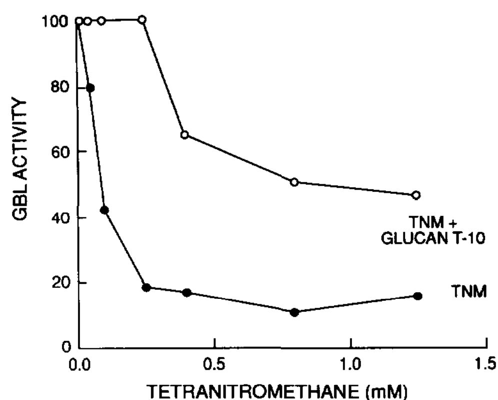

Fig. 5. Inactivation of GBL by tetranitromethane. Presence of glucan T-10 protected the lectin from inactivation.

TABLE I

Interaction of the glucan-binding lectin of *S. sobrinus* 6715 with amino acid side chain probes [a]

Modifying agent	Side chain modified	GBL inactivation	Inactivation reversible with hydroxylamine	Glucan T-10 protection
Acetic anhydride	Lysyl	Yes	No	Yes
N-Acetyl	Tyrosine	Yes	Yes	Yes
N-Bromosuccinimide	Tryptophan	Yes	NA	No
p-Chloromercuribenzoate	Sulfhydryl	No	NA	NA
Citraconic anhydride	Lysyl	Yes	Partial	Partial
1,2-Cyclohexanedione	Guanidinium	No	NA	NA
Diethylpyrocarbonate	Histidine	Yes	Yes	Yes
Ethylenediamine plus carbodiimide	Carboxylate	Yes	NA	Yes
Hydrogen peroxide	Tryptophan, histidine, methionine, cysteine	No	NA	NA
2-Hydroxy-5--nitrobenzyl bromide	Tryptophan	No	NA	NA
p-Hydroxyphenyl glyoxal	Guanidinium	No	NA	NA
Glycine methyl ester plus carbodiimide	Caboxylate	Yes	Yes	Yes
Mercaptoethanol	Disulfide	No	NA	NA
Tetranitromethane	Tyrosine	Yes	NA	Yes

[a] Similar results were obtained when *Streptococcus cricetus* AHT was used instead of *S. sobrinus* 6715. NA, not attempted or not applicable.

60 min. It is clear that NAI abolished the ability of the cells to aggregate with glucan T-2000. Glucan T-10 afforded complete protection of lectin activity. Control experiments established that amylose did not prevent the NAI from reducing the lectin activity. When the NAI-treated cells were incubated with 100 mM hydroxylamine, there was a partial restoration of activity.

Low concentrations of TNM were also able to significantly reduce the interaction between *S. sobrinus* and glucan T-2000 (Fig. 5). At concentrations of TNM up to 0.4 mM, glucan T-10 completely prevented inactivation of the lectin. There was also considerable protection at higher TNM concentrations. These results, coupled with those with NAI, provide evidence to suggest a role for tyrosine in the combining site of the GBL of *S. sobrinus*. Although TNM is highly specific at low concentrations for Tyr, other side reactions have been reported[28].

Role of thiol, disulfide, guanidium and indole.—Reagents, such as *p*-chloromercuribenzoate, specific for thiol groups, and mercaptoethanol, specific for disulfides, failed to inhibit the GBL of *S. sobrinus*. Concentrations of these agents ranged from 1–100 mM. Treatment of cells with 1,2-cyclohexanedione or *p*-hydroxyphenyl glyoxal did not result in loss of activity when the reagents were used at a final concentration of 25 mM. When cells were treated with 0.1–2.0 mM HNBB ("Koshland's reagent"), no loss of GBL activity occurred. However, *N*-bromosuccinimide (NBS) at pH 4.5 at 56 μM completely abolished lectin activity. Concentrations even as low as 5.6 μM caused a ten-fold decrease in activity, but glucan T-10 failed to protect against loss of binding. Finally, hydrogen peroxide, at a 200 mM concentration in PBS, had no effect on the cellular lectin activity. Taken together, it appears that thiol, disulfide, guanidinium, and indole are not associated with the combining site(s) of the glucan-binding lectin. Table I summarizes the results for all of the reagents used in this study.

DISCUSSION

The purpose of this study was to determine the essential amino acids needed for GBL activity in *S. sobrinus*. The general strategy was to chemically modify whole cells with reagents known to have specificity for particular amino acid side chains. When a reagent was shown to reduce lectin activity, experiments were run to determine if an inhibitor of the lectin, glucan T-10, would afford protection. Further experiments were then run to determine if the activity could be restored by deblocking agents. When possible, more than one agent was employed to confirm results obtained with another agent.

The general conclusions that can be reached are that lysyl, carboxylate, imidazolinium, and phenolic residues are essential for GBL activity. Two reagents, acetic anhydride and citraconic anhydride, abolished the GBL activity. In both cases, glucan T-10 partially protected against the loss of binding. Similarly, when carbodiimide–activated carboxylates were modified with ethylenediamine, there was a loss of lectin activity, but the loss was prevented by low mol wt glucan.

Substitution of ethylenediamine by glycine methyl ester also resulted in loss of the lectin binding, but in this case, hydroxylamine regenerated lectin activity. Glucan T-10, but not amylose, protected against loss of activity by the carbodiimide–glycine methyl ester. The same approach was taken when diethylpyrocarbonate was employed as a GBL modifying agent. The agent caused a loss in GBL activity, but the loss was partially reversible with hydroxylamine and glucan T-10 was protective. Two reagents specific for phenolic side chains gave virtually the same results. Both *N*-acetylimidazole and tetranitromethane caused significant reductions in lectin activity, but glucan T-10 was able to render partial protection. Hydroxylamine restored the lectin activity of NAI-treated cells, probably by removing *O*-acetyl groups. A composite interpretation of the results is that lysyl, carboxylate, imidazolinium, and phenolic side chains are essential for lectin activity. Because agents known to modify guanidinium, disulfide, thiol, and indole were without effect on the lectin or because glucan T-10 did not offer protection, it is concluded that these groups are not involved in glucan binding.

There is a disadvantage in using cell-bound lectin in chemical modification studies because it is impossible to quantitate the extent of amino acid substitution. It must be assumed that a particular reagent behaves against cell-bound proteins in a manner similar to that against soluble proteins. The main advantage is that the group-specific reagent, the glucan T-10, amylose or deblocking agent (hydroxylamine) can be rapidly removed from the cells by centrifugation and washing. The results are strengthened by protection and deblocking experiments.

The GBL of the oral streptococci is able to complex with 6–10 α-(1 $\rightarrow$ 6)-linked glucose units and has no affinity for nonreducing glucose termini[7]. As with many plant lectins, the GBLs depend on Mn^{2+} ion for activity. It is not known with certainty, however, that the GBL is a manganoprotein[31]. Carboxylate, histidine and phenolic residues are known to be essential for the activity of concanavalin A[14,15,29,30]. Some of these residues participate as contact amino acids as well as function to bind Ca^{2+} and Mn^{2+} (ref 30). Cernakova et al.[9] and Vancurova et al.[10] have performed extensive chemical modification studies on lectins and have emphasized the critical roles of Asp, Glu, His, and Tyr in lectin binding. Other studies on lectins by Ashford et al.[11] and Ziska et al.[12] further describe a role for either Asp, Glu, His, or Tyr as contact amino acids required for carbohydrate binding. Sikdar and Chatterjee[13] have found that Tyr, Asp, Glu, and Trp are essential for the activity of the blood group A-specific lectin, crotalarin. Mehlbauer et al.[32] found that periodate-oxidized α-(1 $\rightarrow$ 6)-glucans irreversibly inhibited the GBL of *S. sobrinus* (and *S. cricetus*) when the cell–gluan mixture was treated with sodium borohydride. They concluded that the active site of the GBL contained a lysyl group that could form a Schiff's base with the dialdehyde glucan, a conclusion consistent with the results in this report implicating lysyl side chains. Kawagishi and Mori[16] have studied the chemical modification of the lectin from *Ischnoderma resinosum* (mushroom) and concluded that the essential amino acids are Arg, His, Tyr, and Asp (Glu). Based on current knowledge, it is impossible to predict all the

amino acids in a lectin combining site, although carboxylate, histidine, and tyrosine seem to be the most common. The most extensive studies on carbohydrate–protein interactions have been performed by Quiocho and colleagues. Quiocho[33] has pointed out that most carbohydrate-binding proteins provide electronegative sites (phenolic, carboxylate) to form hydrogen bonded structures with the carbohydrate. The L-arabinose binding protein of *Escherichia coli* interacts with the pentose via at least four hydrogen bonds. In some cases, an anomeric hydrophobic group (methyl, phenyl, etc.) may contribute to the stability of the complex by interacting with hydrophobic amino acid side chains. The GBL of *S. sobrinus*, however, does not recognize α-(1 $\rightarrow$ 6)-glucans substituted with methyl groups (results not shown).

The α-(1 $\rightarrow$ 6)-glucan synthetase from *S. sobrinus* is known to have a glucan-binding domain in addition to the catalytic domain[34–36]. Wong et al.[34] were able to isolate a peptide–glucan complex from the enzyme that contained a carboxylate function associated with the binding of the glucan. The insertion mechanism for the synthesis of α-(1 $\rightarrow$ 6)-glucan from sucrose by α-(1 $\rightarrow$ 6)-glucan synthetases is compatible with the presence of a glucan-binding domain on the enzyme, but does not demand it[24]. The glucan-binding domain of the α-(1 $\rightarrow$ 6)-glucan synthase is unique from the glucan-binding lectin involved in aggregation of the streptococci by high mol wt α-(1 $\rightarrow$ 6)-glucans.

Very few studies have been performed on the chemical modification of bacterial lectin adhesins. Jacobs et al.[37,38] have described the chemical modification of fibrillar adhesins of enterotoxigenic strains of *E. coli*. They found that the lectin activity could be destroyed by agents specific for Lys, Arg, Tyr, Asp (or Glu), and Cys. The most critical amino acid seemed to be Lys as the modification of only one residue per subunit destroyed the ability of the fibrillar preparations to bind glycolipids.

One of the goals to follow this study is to design and synthesize affinity labels for the GBL. McAlister et al.[39] showed that aldehydo derivatives of sucrose were good affinity probes for the glucosyltransferases of *S. sobrinus*. Glycosyltransferases contribute to adhesion by catalyzing the conversion of sucrose into glucan right on the surfaces of teeth[40]. If appropriate affinity labels become available, it may be possible to find an effective inhibitor of dental caries. Present studies are directed to agents which irreversibly modify Lys, Asp, (or Glu), His, and Tyr.

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