INFECTION AND IMMUNITY, Dec. 1987, p. 3011–3016
0019-9567/87/123011-06$02.00/0

Vol. 55, No. 12

Characterization by Affinity Electrophoresis of an α-1,6-Glucan-Binding Protein from *Streptococcus sobrinus*

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Received 13 April 1987/Accepted 25 August 1987

Glucan-binding protein 1 (GBP1), the most abundant glucan-binding protein isolated from culture supernatants of *Streptococcus sobrinus* 6715-49, has been purified by affinity chromatography on Sephadex G-50 followed by gel permeation chromatography with Bio-Gel P-10. The specificity and affinity of GBP1 for glucans were assessed by affinity electrophoresis. GBP1 did not detectably bind to glucans lacking linear arrays of α-1,6 linkages. The association constant for the linear α-1,6-glucan Dextran T2000 was 3×10^7 M^{-1}. Providing small isomaltosaccharide ligands to compete with this dextran indicated that the binding site maximally accommodated isomaltosaccharides with a degree of polymerization of 8. When glucans produced by purified *S. sobrinus* glucosyltransferases were tested, GBP1 displayed the highest affinity for the glucan from the soluble-product, primer-independent glucosyltransferase.

The synthesis of extracellular glucans by *Streptococcus sobrinus* and *Streptococcus mutans* potentiates the cariogenicity of these bacteria (13). The glucans contribute to the accretion of dental plaque and to bacterial adherence on the tooth surface (28). While it is possible that microorganisms are, to some extent, passively trapped in the plaque matrix, most attention has focussed on proteins that could specifically bind the bacteria to the glucans (14).

Other experimental systems corroborate both the contribution of extracellular polysaccharides to bacterial adherence and persistence (7, 33) and specific contributions of carbohydrate-binding proteins to pathogenesis (18, 20). Indeed, the importance of such lectinlike proteins has also been shown in attachment-related processes as diverse as the reaggregation of sponges (26), the development of slime molds (2) and vertebrate nerve cells (11), and the scavenging of desialyated glycoproteins from the blood (27).

The interaction between the mutans group streptococci and the plaque matrix has been attributed to the glucosyltransferases which synthesize the exocellular glucans and thus clearly have the potential to bind to them (12, 13, 22). However, the agglutination by glucan of glucosyltransferase-depleted cells (25) suggests that other proteins may also be involved in these interactions. We have purified a candidate for such a protein (23), hereafter referred to as glucan-binding protein 1 (GBP1), by affinity chromatography on Sephadex G-50. GBP1 can comprise as much as 50% of the total protein specifically bound to the affinity column. Our results show that this protein specifically binds to α-1,6-linked glucans, including those synthesized by the *S. sobrinus* soluble-product glucosyltransferases (GTF-S).

(Preliminary results of this work have been presented [E. C. Landale and M. M. McCabe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B91, p. 39].)

(This work constitutes a portion of the dissertation of E. C. Landale, submitted in partial fulfillment of the requirements for the Ph.D. degree from the University of Miami, Miami, Fla., 1987.)

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MATERIALS AND METHODS

Bacteria. *S. sobrinus* 6715-49 (8) was a gift of R. J. Fitzgerald (Dental Research Unit, Veterans Administration Hospital, Miami, Fla.). Growth medium was composed of the following (per liter): Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 20 g; glucose (Sigma Chemical Co., St. Louis, Mo.), 10 g; yeast extract (BBL Microbiology Systems), 4 g; sodium carbonate (Fisher Scientific Co., Pittsburgh, Pa.), 4 g, supplemented with freshly prepared cysteine hydrochloride (Sigma), 50 mg. Cultures were incubated anaerobically at 37°C.

Purification of GBP1. Protein was prepared by a modification of the method of McCabe et al. (23). Proteins were precipitated from the culture supernatant by adding ammonium sulfate (Schwarz/Mann, Orangeburg, N.Y.) to 50% saturation. The precipitate was dissolved in 0.02% sodium azide (Eastman Kodak Co., Rochester, N.Y.) at 10% of the original volume. This solution was applied to a bed (26 by 300 mm) of the ether-cross-linked α-1,6-glucan Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N.J.), and glucan-binding proteins were eluted from the extensively washed column with 6 M guanidine (1). Proteins eluting with the guanidine front were dialyzed within a 3,000-molecular-weight cutoff membrane, concentrated on a Diaflo YM2 membrane (Amicon Corp., Lexington, Mass.), and applied to a bed (26 by 1,000 mm) of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.). Development with distilled water eluted GBP1 in the second peak. Proteins in solution were quantified by the Coomassie brilliant blue G-250 (Eastman Kodak Co.) assay of Sedmak and Grossberg (29).

Affinity electrophoresis. GBP1 was electrophoresed through polyacrylamide gels containing a polysaccharide ligand of sufficient size to be immobilized. The relative mobility observed in these gels was compared with that observed in control gels lacking ligand to quantify the affinity of GBP1 for the ligand. As the protein is unable to migrate through the gel when bound to an immobilized ligand, the observed retardation by a specific ligand is proportional to the strength

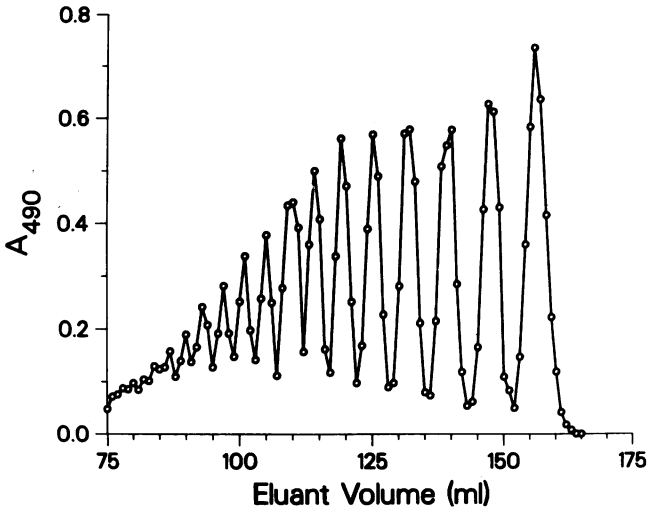

FIG. 1. Chromatography of Dextran T10 hydrolysate on Bio-Gel P-4. One-milliliter fractions (eluant volume) were collected and assayed for carbohydrate (A_{490}) to permit pooling of the fractions containing each isomaltosaccharide from DP3, the third peak from the right, to DP12, the twelfth peak from the right.

of the interaction between GBP1 and that ligand (17). When gels were poured with an immobilized ligand to retard GBP1 as well as competitor ligands that were too small to be immobilized, the latter would restore the protein's relative mobility (see Fig. 5). The quantitative analysis of such experiments has been described before (16).

Native gel electrophoresis in tubes (5 by 125 mm) used a Tris-glycine discontinuous buffer system (9). Separating gels of 10% acrylamide with 5% cross-linker were approximately 100 mm long, and stacking gels of 3% acrylamide with 20% cross-linker were approximately 25 mm long. Ligands to be tested for binding by GBP1 were immobilized, at concentrations of <10 g/liter, by being mixed with the separating gel prior to polymerization of the acrylamide (17). As required to suspend insoluble polysaccharides in the gel column, polymerization was accelerated by doubling the concentration of *N,N,N',N'*-tetramethylethylenediamine (Bio-Rad Laboratories) and ammonium persulfate (Bio-Rad Laboratories) to 0.1%. When small free ligands were to be tested, Dextran T2000 (Pharmacia Fine Chemicals) was immobilized at concentrations of 0.1 to 0.2 g/liter in conjunction with 0.1 to 0.2 g of these isomaltosaccharide ligands per liter (16) (see Fig. 5). Protein concentration was standardized by always loading 2 μg onto a gel tube. Protein samples were mixed with 20%, by volume, of a solution of 0.4% bromophenol blue (Bio-Rad Laboratories) in glycerol before application to the gels. Gels were electrophoresed at a constant 160 V within 1 h of applying the stacking gel. Temperature was maintained at 10°C. The operative pH of the separating gel was taken to be 9.0 based on Jovin's reanalysis of this buffer system (19). Gels were stained with Coomassie brilliant blue R-250 (Eastman Kodak Co.).

Soybean trypsin inhibitor, 21,500 molecular weight and pI 4.5, exhibited electrophoretic mobility similar to that of GBP1 and was electrophoresed in conjunction with GBP1 to provide a noninteracting control. The distances travelled by both protein and dye front were measured, and the former was divided by the latter to yield the relative mobility.

Polysaccharides. Polysaccharides tested for binding by GBP1 included Ficoll, corn starch, oyster glycogen, and pullulan (Sigma Chemical Co.); nigeran (E. E. Smith, University of Miami School of Medicine); cellulose (Whatman, Inc., Clifton, N.J.); amylose (ICN Pharmaceuticals Inc., Irvine, Calif.); and Dextran T2000. Also tested were the three types of glucan produced by the *S. sobrinus* GTFs. These glucans were synthesized in vitro by enzymes purified to homogeneity from the Bio-Gel P-10 void volume pool (22, 24). The GTFs were incubated separately with 1% sucrose (Mallinckrodt, Inc., St. Louis, Mo.) that had been purified by passage through a 12,000-molecular-weight-cutoff dialysis membrane. The reaction was buffered at pH 6 with 0.05 M sodium phosphate. After 48 h, unpolymerized sucrose was removed by dialysis against distilled water with 12,000-molecular-weight-cutoff membranes and the glucan products were lyophilized for storage. Carbohydrate was quantified by the phenol-sulfuric acid assay (10).

Isomaltosaccharide series. Dextran T10 (Pharmacia Fine Chemicals) was hydrolyzed to the extent of 20% by the following protocol: 20 g of Dextran T10 was dissolved in 400 ml of water at 100°C and mixed with an equal volume of 0.5 M sulfuric acid at 100°C. After 4 h at 100°C, the reaction mixture was cooled, neutralized with 45 g of barium hydroxide, and stirred overnight. The final pH was 6.9. After centrifugation and filtration to remove the barium sulfate formed during neutralization, the reaction mixture was lyophilized, redissolved in 40 ml of water, refiltered, and stored frozen. The sample was assayed for carbohydrate and reducing groups to determine the extent of hydrolysis. Samples of 0.5 ml of this partial hydrolysis mixture were applied to a bed (16 by 2,000 mm) of Bio-Gel P-2 (Bio-Rad Laboratories) or a bed (16 by 1,000 mm) of Bio-Gel P-4 (Bio-Rad Laboratories) and eluted with distilled water to resolve glucose and the isomaltosaccharide series from degree of polymerization 2 (DP2), isomaltose, to DP12 (Fig. 1). Peak fractions of each isomaltosaccharide were pooled and lyophilized for storage.

Data analysis. Regression lines, linear calibration, and associated confidence intervals were calculated as described by Cochran and Snedecor (6). The standard error for the slope of a line was calculated by A. Koroos (University of Georgia, Athens), using the SAS computer program for statistical analysis of scientific data (SAS Institute Inc., Cary, N.C.).

To assess the protein-polysaccharide interaction (see Fig. 3) the relative mobility of the noninteracting control, soybean trypsin inhibitor, was subtracted from the relative mobility of GBP1. The results were thereby normalized for any slight alteration in the density or resistivity of the gel matrix caused by the included polysaccharide. The association constant (K_A) of GBP1 for Dextran T2000 was determined from the reciprocal of the 50% retardation concentration. This was calculated by linear calibration of the logarithm of the dextran concentration and the corresponding retardation of GBP1. The free energy of the association was calculated from the relationship $\Delta G^\circ = -R\,T\ln K_A$, where R is the universal gas constant and T is the absolute temperature. The data from experiments involving isomaltosaccharide ligands were analyzed by two methods. The statistical analysis was based on the first method (see Fig. 6). The remobilization of GBP1 was expressed as the fraction of restoration $[(d - D_i)/(D - D_i)$, where d is relative mobility in the presence of both free and immobilized ligands, D is relative mobility in the presence of free ligand only, and D_i is relative mobility in the presence of immobilized ligand only] and was regressed on the concentration of restoring isomaltosaccharide (c) for pooled data compiled from all experiments in which that isomaltosaccharide was assayed.

In the second method, after (16) (see Table 1), the observed mobility Hořejší divided by the potential remaining restoration $[d/(D - d)]$ was regressed on the concentration of isomaltosaccharide for each experiment. Extrapolating this regression line to the x intercept permitted evaluation of the dissociation constant (K_D) from the equation $K_D = -X/[d_0/d + (c_i D_i/K_i D)]$, where X is the x axis intercept determined from regressing $[d/(D - d)]$ on c, d_0 is relative mobility in the absence of ligands, c_i is concentration of Dextran T2000, and K_i is K_D for Dextran T2000 (3.0×10^{-8} M; see below).

RESULTS

GBP1. Bio-Gel P-10 peak 2 pool produced a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2), nondenaturing polyacrylamide gel electrophoresis, and isoelectric focussing. Calibration of these gels indicated that GBP1 is a homodimer of 7,500-molecular-weight subunits and has a pI of 4.6 (E. C. Landale, Ph.D. dissertation, University of Miami, Miami, Fla., 1987). This preparation had no detectable enzymatic activity towards sucrose or glucans.

Specificity for glucans. Because GBP1 can be purified by affinity chromatography on a bed of the α-1,6-glucan Sephadex G-50, it is clear that the protein will bind to such glucans. To discover whether glucans with other linkage patterns are also bound by GBP1, 10 g/liter of various polysaccharides were immobilized in 10% acrylamide gels, and GBP1 and soybean trypsin inhibitor were electrophoresed through the resulting matrix. Among the tested polysaccharides, Ficoll, a branched polymer of sucrose (alternating fructose and glucose monomers), as well as the glucans nigeran (1,3-α-D-glucan), cellulose (1,4-β-D-glucan), starch (1,4-α-D-glucan), amylose (1,4-α-D-glucan), and glycogen (1,4-α-D-glucan with α-1,6-branch linkages), had essentially no effect on the mobility of GBP1 (Fig. 3). The presence of pullulan, a linear glucan chain consisting of repeating units of three to four α-1,4 linkages connected by a single 1,6-α-D-glucosyl linkage (4), resulted in a decreased mobility of GBP1. The presence of Dextran T2000, a linear α-1,6-linked glucan, resulted in an even greater reduction in the mobility of GBP1. These data indicate that, of the polysaccharides tested, only glucans containing α-1,6 linkages in linear arrays were bound by GBP1.

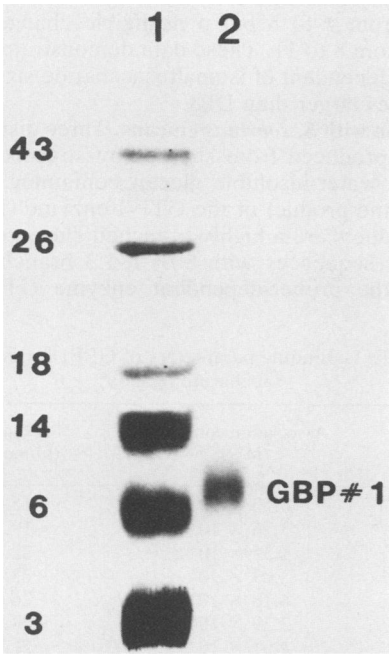

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of GBP1 and protein standards. Molecular weight (in thousands) is given (lane 1) for (top to bottom) ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme, bovine trypsin inhibitor, and insulin to predict the weight and to assess the homogeneity of (lane 2) GBP1.

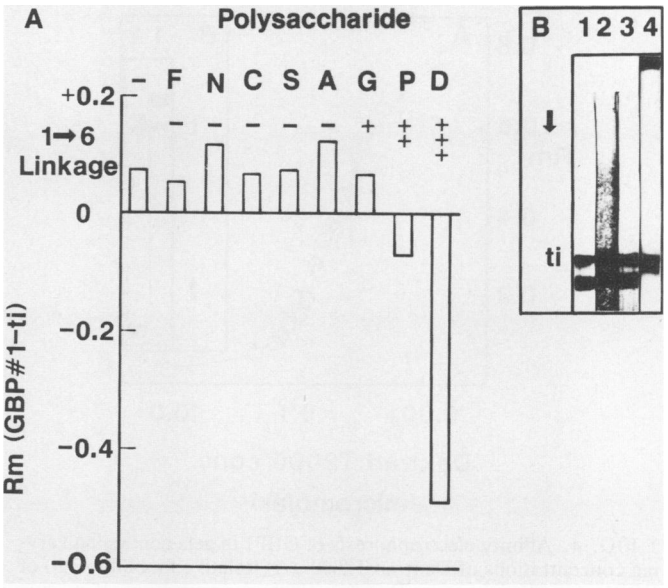

FIG. 3. Affinity electrophoresis of GBP1 and soybean trypsin inhibitor in gels containing different immobilized polysaccharides. (A) The relative mobility of soybean trypsin inhibitor was subtracted from that of GBP1 [Rm (GBP1−ti)]. The relative amount of the 1,6 structure of the polysaccharides is indicated by the minus and plus signs above the bars. At the top is indicated the immobilized polysaccharide: no polysaccharide, −; Ficoll, F; nigeran, N; cellulose, C; starch, S; amylose, A; glycogen, G; pullulan, P; Dextran T2000, D. (B) Soybean trypsin inhibitor (ti) and GBP1 electrophoresed in the direction of the arrow through gels containing no polysaccharide (lane 1), Ficoll (lane 2), glycogen (lane 3), and Dextran T2000 (lane 4).

Affinity for dextran. The Dextran T2000 concentration was varied from 100 pM to 10 μM to determine the relationship between concentration and relative mobility (Fig. 4). While the mobility of soybean trypsin inhibitor was unaffected, the relative mobility of GBP1 (Y) varied with the logarithm of the Dextran T2000 concentration (X) according to the following relationship: $Y = -0.356X - 0.067$. The predicted association constant was 3.3×10^7 M^{-1}, with a 95% confidence limit of 1.4×10^7 M^{-1} to 4.0×10^7 M^{-1}, and the free energy of binding ($\Delta G°_{pH\ 9}$) at 10°C was −42 kJ/mol.

Affinity for isomaltosaccharide ligands. To determine the degree of polymerization of the isomaltosaccharide ligand that, on a molar basis, would be bound most strongly by GBP1, both immobilized and free ligands were mixed with the separating gel acrylamide (Fig. 5B and C), resulting in the two α-1,6-linked glucans, Dextran T2000 and the isomaltosaccharide, being competitively bound by GBP1 during electrophoresis. The small free isomaltosaccharides were not large enough to be immobilized and caused only slight retardation of the protein to which they were bound even for the highest concentration of the largest isomaltosaccharide (Fig. 5, lane 3). Thus, these ligands remobilized the protein

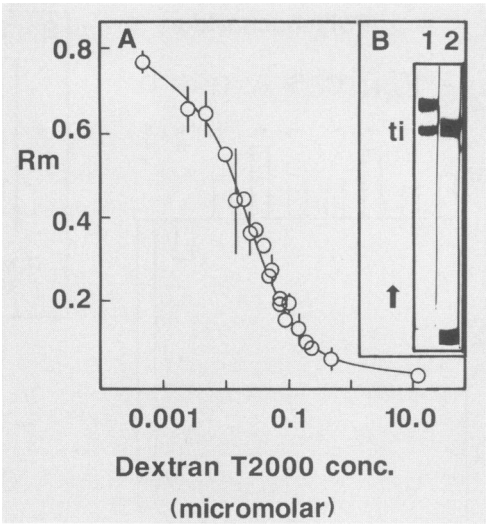

FIG. 4. Affinity electrophoresis of GBP1 in gels containing varying concentrations of Dextran T2000. (A) Relative mobility (Rm) of GBP1 plotted against the Dextran T2000 concentration. Bars give 95% confidence intervals larger than circles. (B) Soybean trypsin inhibitor (ti) and GBP1 electrophoresed in the direction of arrow through gels containing 1 nM (lane 1) and 10 μM (lane 2) Dextran T2000.

in a manner dependent on both the concentration (Fig. 5B) and the size (Fig. 5C) of the isomaltosaccharide. The slopes of the fraction of restoration versus concentration regression lines, for each isomaltosaccharide, were plotted against the degree of polymerization of the restoring isomaltosaccharide

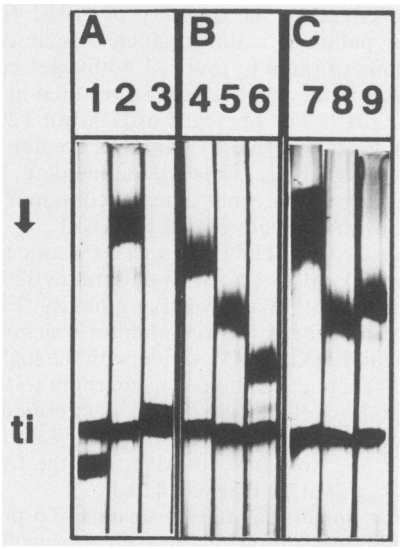

FIG. 5. Inhibition by small isomaltosaccharides of GBP1 binding of Dextran T2000 measured by affinity electrophoresis. Soybean trypsin inhibitor (ti) and GBP1 were electrophoresed in the direction of the arrow. (A) Effect of ligands individually: no ligand (lane 1), 0.15 g of immobilized dextran per liter only (lane 2), and 0.20 g of isomaltosaccharide of DP12 per liter only (lane 3). (B) Restoration of mobility by increasing concentrations of isomaltosaccharide of DP8: dextran at 0.15 g/liter and DP8 at 0.05 (lane 4), 0.20 (lane 5), and 0.50 (lane 6) g/liter. (C) Restoration of mobility by different sizes of isomaltosaccharides: dextran at 0.15 g/liter and isomaltosaccharide at 0.20 g/liter—DP5 (lane 7), DP8 (lane 8), and DP12 (lane 9).

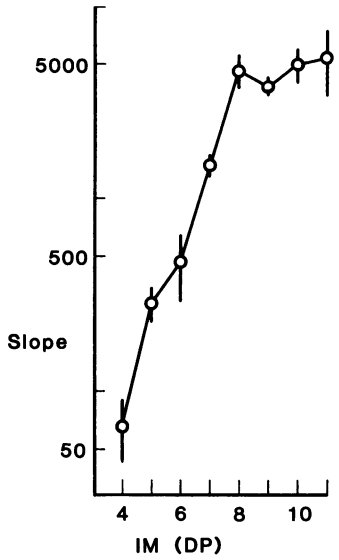

FIG. 6. Slopes of restoration lines determined from affinity electrophoresis with small isomaltosaccharides (IM) inhibiting GBP1 binding of Dextran T2000. Slopes were determined for the restoration lines obtained by regressing the fraction of restoration on the molarity of restoring isomaltosaccharide and were plotted against the degree of polymerization of the restoring isomaltosaccharide. Bars give the 90% confidence intervals. The number of observations is the same as for Table 1.

(Fig. 6). Also, the association constants and free energies of association for each isomaltosaccharide were determined (16) (Table 1). There was a marked increase in the slopes of the restoration curves, the association constants, and the free energies of association as the isomaltosaccharide DP increased from 4 to 8 but a negligible change as the DP increased from 8 to 11. These data demonstrate that affinity becomes independent of isomaltosaccharide size for isomaltosaccharides larger than DP8.

Interaction with *S. sobrinus* glucans. Three distinct types of glucan are produced from sucrose by streptococcal GTFs (24, 32). A water-insoluble glucan containing linear α-1,3 linkages is the product of the GTF-I enzyme (15). The two soluble products are a highly branched glucan consisting of α-1,6 linear sequences with 30% α-1,3 branches (21) produced by the primer-dependent enzyme GTF-Sd and a

TABLE 1. Binding parameters of GBP1 for isomaltosaccharide ligands[a]

Isomaltosaccharide (DP)	Association constant (M^{-1})[b]	$\Delta G°_{pH\ 9}$ (kJ/mol)[c]	n[d]
4	8.50×10^2	−15.9	6
5	3.76×10^3	−19.4	6
6	1.35×10^4	−22.4	12
7	2.09×10^4	−23.4	6
8	4.18×10^4	−26.0	6
9	3.39×10^4	−24.5	3
10	4.01×10^4	−25.0	6
11	3.89×10^4	−24.9	3

[a] In 10% polyacrylamide gels with immobilized Dextran T2000, at 10°C and pH 9.
[b] Analyzed after Hořejší (16) for the general case, with $K_i = 3 \times 10^{-8}$ M.
[c] $\Delta G° = -RT \ln K_A$.
[d] Each datum is the average of two gels.

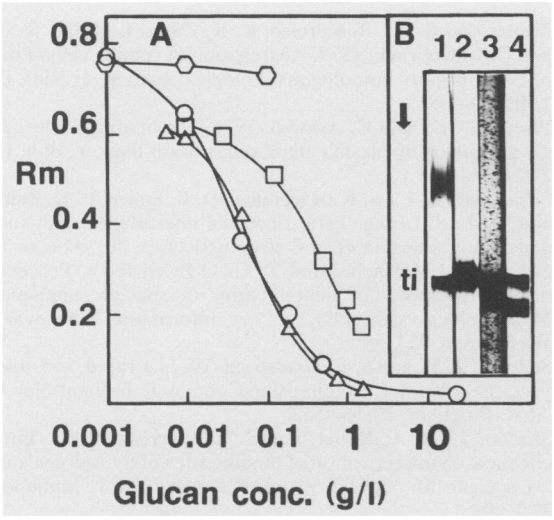

FIG. 7. Affinity electrophoresis of GBP1 in the presence of immobilized glucans synthesized by *S. sobrinus* GTFs. (A) Relative mobility (Rm) of GBP1 in gels containing GTF-I product (hexagons), GTF-Sd product (squares), and GTF-Si product (triangles) and, for comparison, dextran T2000 (circles) plotted against the glucan concentration. (B) Soybean trypsin inhibitor (ti) and GBP1 electrophoresed in the direction of the arrow through GTF-Si product (lane 1), GTF-Sd product (lane 2), and GTF-I product (lane 3), all at 0.1 g/liter, and in the absence of glucan (lane 4).

slightly branched α-1,6-glucan (22, 32) produced by the primer-independent enzyme GTF-Si. Glucans of each of the three structural patterns were synthesized in vitro by the purified GTFs and immobilized for affinity electrophoresis (Fig. 7). GBP1 was only slightly retarded by 0.1 g of the GTF-I product per liter, indicating a low-affinity interaction. The concentrations of the GTF-Sd and GTF-Si products required to retard GBP1 to 50% of its unfettered mobility were 0.2 and 0.08 g/liter, respectively. The latter was similar to the equivalent concentration for Dextran T2000, 0.06 g/liter.

DISCUSSION

These data pertaining to the specificity of the interaction between GBP1 and polysaccharides suggest that GBP1 recognizes linear arrays of sequential α-1,6-linked glucose residues. Ligands without α-1,6 linkages were not bound (Fig. 3), while pullulan, a linear partial α-1,6-glucan, was bound slightly (Fig. 3) and the GTF-Sd product, an α-1,6 backbone with branches, was bound with intermediate affinity (Fig. 3 and 7). The ligands bound with highest affinity, Dextran T2000 and the GTF-Si product (Fig. 4 and 7), are both essentially linear α-1,6-glucans. Of all of the potential linkage patterns, α-1,6 provides the greatest degree of rotational freedom and spaces the monomers further apart (3), either or both of which might facilitate the binding by GBP1.

As the size of the isomaltosaccharide exceeds DP8, the affinity for GBP1 ceases to increase (Fig. 4). This is consistent with the binding site of GBP1 being of sufficient size to accommodate an isomaltosaccharide of DP8 because, as the binding site is being progressively filled by larger and larger isomaltosaccharides, the affinity would increase, whereas once the size of the binding site is exceeded, any additional residues would not be accommodated and thus would not contribute to the strength of the interaction. Such a size is close to the DP5 to DP7 size range that has been determined for numerous anti-dextran antibodies (5, 30, 31). The free energies of association of these antibodies, about −20 kJ/mol for isomaltosaccharides of DP7, are similar to the value of −23 kJ/mol determined in this study (Table 1). The association constant of GBP1 for isomaltopentaose, K_A = 3,800 M^{-1}, is close to that of the plant lectin concanavalin A for maltopentaose, K_A = 1,100 M^{-1} (34), and that of the snail lectin from *Helix pomatia* for *N*-acetyl-D-galactosamine, K_A = 5,700 M^{-1} (35). Thus, with respect to both binding site size and affinity for ligands, GBP1 is similar to other carbohydrate-binding proteins.

This study of the specificity and affinity of GBP1 clearly demonstrates that it will bind to the glucan products of the streptococcal GTFs (Fig. 7), especially the GTF-Si product. An earlier investigation has shown that the protein can be released from the cell surface by sonication (23). These data suggest a possible role for GBP1 in anchoring the glucans produced by the GTF-S enzymes to the cell surface, and they provide an explanation for the observation that bacteria depleted of GTFs can still be agglutinated by dextran (25). GBP1 could thus complement the glucan-binding activities of the GTFs in facilitating the persistence of the bacteria in the plaque matrix. The participation of the extracellular glucans in the accretion of this matrix and in the pathogenesis of dental caries argues for the importance of proteins such as GBP1 that are capable of specifically binding the glucan polymers produced by the streptococcal GTFs.

ACKNOWLEDGMENT

This study was supported by Public Health Service grant DE 04321 from the National Institute for Dental Research.

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