Use of the photoaffinity cross-linking agent *N*-hydroxysuccinimidyl-4-azidosalicylic acid to characterize salivary-glycoprotein–bacterial interactions

E. James BERGEY, Michael J. LEVINE,* Molakala S. REDDY, Steven D. BRADWAY and Ibtisam AL-HASHIMI

Department of Oral Biology, 109 Foster Hall, State University of New York at Buffalo, Buffalo, NY 14214, U.S.A.

The present study has utilized the iodinated cross-linking agent *N*-hydroxysuccinimidyl-4-azidosalicylic acid (ASA) to examine the specific interaction between the proline-rich glycoprotein (PRG) of human parotid saliva and *Streptococcus sanguis* G9B. The binding of ¹²⁵I-ASA-PRG to *Streptococcus sanguis* G9B displayed saturation kinetics, reversibility and was inhibited by unlabelled PRG. Inhibition studies with other glycoproteins and saccharides indicated that binding was mediated by a bacterial adhesin with specificity towards *N*-acetylneuraminic acid, galactose, and *N*-acetylgalactosamine. After cross-linking, the ¹²⁵I-ASA-PRG–adhesin complex could be extracted with SDS and separated from uncoupled ¹²⁵I-ASA-PRG by gel filtration on Sepharose CL-6B. Approx. 1% of the ¹²⁵I-ASA-PRG was cross-linked to the bacterial surface. Examination of the ¹²⁵I-ASA-PRG–adhesin complex by SDS/polyacrylamide-gel electrophoresis/fluorography on 5%-(w/v)-polyacrylamide gels revealed that PRG was bound to two bacterial components. These findings support our previous suggestion that human salivary glycoproteins can specifically interact with oral streptococci and that these interactions occur between the glycoprotein's carbohydrate units and lectin(s) on the bacterial cell surface.

INTRODUCTION

Bacterial attachment to surfaces of animal tissues has gained increasing attention as an important initial event in the pathogenesis of bacterial infections (Gibbons & van Houte, 1980; Beachey, 1981). The selectivity of this process can be clearly demonstrated in the oral cavity, where salivary glycoproteins function to modulate bacterial colonization on various oral surfaces (Tabak *et al.*, 1982; Levine *et al.*, 1985). *Streptococcus sanguis* is among the earliest colonizers of the tooth surface (Carlsson *et al.*, 1975), and consequently this micro-organism appears to have a pivotal role in the cascade of events leading to the formation of plaque-mediated disease (Gibbons & van Houte, 1980). Available evidence indicates that the adherence of this bacterium to saliva-coated hydroxyapatite surfaces is complex and involves both specific (Murray *et al.*, 1982; Gibbons & Etherden, 1982; Gibbons *et al.*, 1983; Morris & McBride, 1984) and non-specific interactions (Hogg & Embury, 1981; Doyle *et al.*, 1982; Nesbitt *et al.*, 1982; Westergren, 1981; Westergren & Olsson, 1983). However, the exact molecular mechanisms of these interactions are not fully understood. Recent studies on the interactions of *Streptococcus sanguis* and salivary glycoproteins have begun to elucidate possible mechanisms of attachment. Although electrostatic and hydrophobic forces are clearly involved in streptococcal binding to a saliva coated tooth surface, these types of interactions do not fully account for the tissue specificity/selectivity observed in bacterial adherence. Therefore, it has been proposed (Gibbons *et al.*, 1985; Levine *et al.*, 1985; Rosan *et al.*,

1985) that the bacteria possess a recognition system that is superimposed upon such forces which may function to align salivary glycoproteins with bacterial surface adhesins.

Collective data suggests that the carbohydrate moieties of salivary glycoproteins play a role in bacterial interaction (Gibbons & Qureshi, 1978; Levine *et al.*, 1978; Shibata *et al.*, 1980; Nagata *et al.*, 1983), and early studies (McBride & Gisslow, 1977) implicated sialic acids as the determinants necessary for certain types of salivary-glycoprotein–bacterial-cell interactions. Investigations by Levine *et al.* (1978) and Stinson *et al.* (1982) have demonstrated that salivary mucin interaction with strains of *S. sanguis*, but not *S. mutans*, is dependent upon the presence of *N*-acetylneuraminic acid. More recently, this laboratory has presented evidence for a lectin on the surface of *Streptococcus sanguis* with specificity for a NeuAc α 2-3Gal β 1-3GalNAc sequence (Murray *et al.*, 1982).

Although the specificity of the interaction between bacterial cells and salivary glycoproteins has been examined, the assays used to define these interactions have failed either to quantify or to identify the bacterial cell-surface lectin(s). Recently, macromolecular photoaffinity labelling utilizing a two-step (heterobifunctional) process has been used successfully to identify receptors and binding sites for various macromolecular ligands, including concanavalin A (Vanin & Ji, 1981; Ji & Ji, 1982; Baenziger & Fiete, 1982), insulin (Yip *et al.*, 1978), glucagon (Johsson *et al.*, 1981), vasoactive intestinal polypeptide (Wood & O'Dorisio, 1984) and lutropin (Ji & Ji, 1980). Similar approaches have been used for

Abbreviations used: PRG, proline-rich glycoprotein; ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; Lf, lactoferrin.

* To whom correspondence and reprint requests should be addressed.

delineating the structure of β -adrenergic receptors by the incorporation of heterobifunctional photoaffinity probes derived from high-affinity radiolabelled antagonists (Shorr *et al.*, 1982). Photoaffinity probes constructed from simple monosaccharides for the labelling of cell-surface lectins have exhibited decreased affinities and thus have limited or undefined specificity (Beppu *et al.*, 1975; Fraser *et al.*, 1976). Recently, Baenziger & Fiete (1982) developed methods for coupling ^{125}I -labelled glycopeptides with photoactivatable aryl azide derivatives and used these derivatives to compare the selectivity and specificity of different lectins. The present study describes the derivation of the salivary proline-rich glycoprotein (PRG) with an iodinated heterobifunctional photoaffinity cross-linking reagent (the *N*-hydroxysuccinimide ester of 4-azidosalicylic acid) and the use of this probe to quantify and identify the bacterial binding components present on the surface of *Streptococcus sanguis* G9B.

EXPERIMENTAL PROCEDURES

Materials

The following reagents were obtained from the indicated sources: *N*-acetylneuramin-lactose, *N*-acetyl-lactosamine, *N*-acetylneuraminic acid (type VII), insoluble neuraminidase (type X-A), human salivary amylase (IX-A), bovine serum albumin (A7906), human transferrin (T2252), protein A (P9267) and chloramine-T, were obtained from Sigma. Lactoferrin was obtained from Calbiochem-Behring. Bio-Sil TSK guard columns, Bio-Sil TSK 250 columns, nitrocellulose-blotting membranes, Bio-Gel PDG-6, and Bio-Gel P60 were obtained from Bio-Rad. Sephadex G-10, Sephadex G-25 and Sepharose CL-6B were purchased from Pharmacia. *N*-Hydroxysuccinimide ester of 4-azidosalicylic acid was from Pierce. Na^{125}I (13–17 mCi/ μg) was purchased from Amersham International. Rabbit anti-(human colostral lactoferrin) serum (A186) was purchased from Accurate Chemical Co. Rabbit anti-(human salivary amylase) serum was prepared as previously described (Shomers *et al.*, 1982).

General chemical and analytical methods

Protein and neutral sugars were determined by the Lowry procedure (Lowry *et al.*, 1951) and the anthrone reaction (Roe, 1955) respectively. Amino acid and carbohydrate analyses were carried out by previously described procedures (Reddy *et al.*, 1982). Amino acids and hexosamines were quantified on a Beckman model-120 C amino acid analyser. Neutral sugars were quantified as their alditol acetates on a Varian model-3700 gas chromatograph through a Gilson model-620 data module interfaced to an Apple IIe computer. Polyacrylamide-gel electrophoresis was performed on SDS/7.5%-(w/v)-polyacrylamide slab (14 cm $\times$ 14 cm) gels as described by Laemmli (1970) and samples stained for protein with Coomassie Blue. M_r values were calculated from plots of the log M_r versus relative mobility of standard proteins.

Preparation of glycoproteins and derivatives

The proline-rich glycoprotein (PRG) was isolated from the citric acid-stimulated parotid saliva of a single male donor (age 35) by methods previously described (Levine *et al.*, 1969). Asialo-PRG was prepared by treatment with insoluble neuraminidase. Insoluble neuraminidase

(200 μl) was equilibrated by washing with 3×4 ml of 0.1 M-sodium acetate buffer, pH 5.0 (500 g, 1 min, 4 °C). PRG (4.0 mg in 400 μl of 0.1 M-sodium acetate, pH 5.0) was added to the neuraminidase-agarose, followed by an additional 200 μl of buffer. The mixture was incubated at 37 °C for 18 h with gentle shaking. The supernatant containing asialo-PRG was collected by centrifugation (500 g, 1 min, 4 °C) and the agarose was washed with 3×1.0 ml of buffer. The combined supernatants were dialysed against distilled water at 4 °C and freeze-dried. On the basis of hexosamine content, a quantitative recovery of asialo-PRG was obtained. After hydrolysis with 25 mM- H_2SO_4 for 1 h at 80 °C, the sialic acid content of intact and asialo-PRG was determined by the thiobarbituric acid assay (Warren, 1959). No sialic acid was detected with asialo-PRG, whereas intact PRG contained 0.51 $\mu\text{mol}/\text{mg}$. Periodate-treated PRG was prepared as described by Reddy *et al.* (1982). PRG (4.0 mg) was dissolved in 400 μl of water, after which 400 μl of 0.1 M-sodium acetate, pH 5.0, containing 0.16 M-sodium periodate, was added. The reaction was allowed to proceed in the dark for 72 h at 4 °C and was then terminated by the addition of 40 μl of ethylene glycol for 1 h at room temperature. The pH of the sample was then raised to 8.0 by the addition of 1 M-NaOH and reduction accomplished by the addition of 8 mg of NaBH_4 in 400 μl of water. After 18 h at 4 °C, the pH of the sample was lowered to 5.0 with 4 M-acetic acid, dialysed against water at 4 °C and then freeze-dried. Neutral sugar and hexosamine analyses of periodate treated-PRG indicated that all of the sialic acid, fucose and galactose were destroyed, with one residue of mannose and *N*-acetylglucosamine being lost.

Human colostral lactoferrin (Lf) was purified further by CM-52 cellulose chromatography (Querijnje *et al.*, 1971) and h.p.l.c. by using a Bio-Sil TSK guard column (7.5 mm $\times$ 75 mm) coupled to a Bio-Sil TSK 250 column (7.5 mm $\times$ 300 mm). H.p.l.c. columns were equilibrated with 0.01 M-potassium phosphate buffer, pH 6.5, containing 0.4 M-sodium sulphate. Lf samples (10 mg/ml) were dissolved in equilibrating buffer and applied to the h.p.l.c. columns by using a 100 μl sample loop. Samples were eluted at 0.5 ml/min. Eluates were monitored at 230 nm and collected (0.5 ml fractions) into a Pharmacia Frac-100 collector. Lf-containing fractions eluted between 19 and 21 min were pooled, dialysed extensively against distilled water at 4 °C, and then freeze-dried. Human salivary amylase was purified further by gel filtration on columns (1.5 cm $\times$ 110 cm) of Bio-Gel P60 equilibrated in 0.1 M-Tris/HCl, pH 7.4, with 1.0 M-NaCl. Approx. 13.9 mg of amylase (protein equivalents) were dissolved in 5 ml of equilibrating buffer, stirred overnight at 4 °C, centrifuged at 12000 g for 30 min at 4 °C, and the supernatant applied to a column of Bio-Gel P60. Fractions (1.4 ml) were collected at room temperature and protein elution monitored at 280 nm. Void-volume fractions were pooled, dialysed against distilled water at 4 °C, and then freeze-dried. Approx. 8.7 mg of protein was recovered in the pooled amylase fractions. Human transferrin was used without further purification.

To evaluate further the purity of glycoprotein preparations after SDS/polyacrylamide-gel electrophoresis, materials were electrophoretically transferred on to nitrocellulose membranes and then blotted with specific antiserum. The antigen-antibody complex was next incubated with ^{125}I -protein A and then detected by

autoradiography at -70°C for 24–48 h using Kodak X-Omat film employing a du Pont Cronex intensifying screen (Towbin *et al.*, 1979; Smith & Summers, 1981).

Preparation of ASA-PRG

PRG (40 nmol) was dissolved in 200 μl of 0.1 M sodium borate buffer, pH 8.5, and derivatized with a 100 molar excess (based on 5 residues/100 total residues of PRG) of *N*-hydroxysuccinimide ester of 4-azidosalicylic acid (10 mg/ml in dioxan). The reaction was performed by using $5 \times 5 \mu\text{l}$ aliquots over a period of 2 h at room temperature in the absence of light (unless otherwise stated, all reactions involving the ASA photoaffinity probe were carried out in the dark or under a Kodak safe lamp). After incubation for an additional 3 h, the unchanged ASA was removed by desalting on columns (1.5 cm $\times$ 110 cm) of Sephadex G-25 equilibrated in 0.1 M- NH_4HCO_3 , pH 8.5, at room temperature. The ASA-PRG was recovered by repeated freeze-drying from water and stored in an actinic container at -30°C (Ji & Ji, 1982; Baenziger & Fiete, 1982).

Preparation of ^{125}I -ASA-PRG and ^{125}I -protein A

For iodination, 50 μg of ASA-PRG or protein A were dissolved in 50 μl of 0.1 M-potassium phosphate, pH 7.4. To this was added 10 μl of Na^{125}I (1 mCi in 10 μl of 0.01 M-NaOH), followed by 5 μl of chloramine-T (0.8 mg/ml in water) (Greenwood *et al.*, 1963). The sample was incubated for 1.0 min at room temperature and then the reaction terminated by the addition of 5 μl of sodium metabisulphite (2.0 mg/ml) and 5 μl of KI (2.0 mg/ml) (both in water). The iodinated product was separated from free iodine and reagents by gel filtration on 10 ml disposable columns of either Bio-Gel PDG-6 (for protein A), using 0.1 M-potassium phosphate, pH 7.4, containing 0.1% bovine serum albumin or Sephadex G-25 (for ASA-PRG), using 0.1 M-potassium phosphate, pH 7.4. The fractions containing the iodinated ASA-PRG or protein A were pooled and stored at 4°C . Specific radioactivities of $(2-3) \times 10^7$ c.p.m./nmol for ^{125}I -ASA-PRG were obtained.

Bacteria and culture conditions

Streptococcus sanguis strain G9B (obtained through the courtesy of Dr. B. Rosan, University of Pennsylvania, Philadelphia, PA, U.S.A.) was employed in these studies. Bacteria were grown for 16–18 h (mid- to late-stationary phase) at 37°C in FMC chemically defined medium freshly buffered with 0.01 M each of Na_2CO_3 and

NaHCO_3 (Terlecky *et al.*, 1975). Cells were harvested at 4°C by centrifugation at 6500 g for 10 min and washed three times in cold phosphate-buffered saline (0.01 M-sodium phosphate, pH 7.4, containing 0.15 M-NaCl). Before use in binding assays, bacteria were gently sonicated for 30 s to obtain short chains (5–15 cells).

Binding assays using ^{125}I -ASA-PRG

For the binding assays, the bacterial cells were suspended in phosphate-buffered saline at a concentration of 2×10^{10} cells/ml. The photoaffinity cross-linking of iodinated ASA-PRG to *Streptococcus sanguis* G-9B involved the incubation of 2.5–160 pmol of iodinated reagent with 1×10^{10} bacterial cells suspended in 100 μl of phosphate-buffered saline containing 1% bovine serum albumin for 2 h at room temperature in the dark. The unchanged iodinated ASA-PRG was removed by centrifugation at 13 000 g (Beckman Microfuge, model B). The cell pellet was washed with 3×1.0 ml of phosphate-buffered saline, resuspended in 1.0 ml of deionized water, and the ^{125}I -ASA-PRG cross-linked to the bacterial surface by exposure to a xenon strobe light for 1 min. The bacteria were centrifuged, and bound radioactivity in the resulting bacterial pellet measured in a Beckman 5500 γ -radiation counter. The reversibility of ^{125}I -ASA-PRG binding to *S. sanguis* was demonstrated by initial incubation of 10 pmol of ^{125}I -ASA-PRG with 1×10^{10} bacterial cells as described above. After 2 h at room temperature, a 200-fold excess of unlabelled PRG was added. The cells were incubated for an additional 2 h and then the bound radioactivity determined as described above. For inhibition studies, the bacteria were first incubated with various concentrations of inhibitor for 2 h at room temperature, after which ^{125}I -ASA-PRG (5–10 pmol) were added and incubated for an additional 2 h. Bound radioactivity was determined as described above.

To quantify the amount ^{125}I -ASA-PRG cross-linked to bacteria, cells were extracted with 1% SDS in deionized water at 50°C for 1–2 h. The extract was centrifuged (13 000 g) and the supernatant collected and subjected to chromatography on calibrated columns (1.5 cm $\times$ 110 cm) of Sepharose CL-6B equilibrated in 0.1 M- NH_4HCO_3 , pH 7.8, containing 0.1% SDS. Fractions of 1.1 ml were collected at room temperature and monitored for radioactivity on a Beckman 5500 γ -radiation counter. Fractions containing radioactivity were pooled and freeze-dried. Pools were then examined by SDS/polyacrylamide-gel electrophoresis on 5%-polyacrylamide

Table 1. Properties of (glyco)proteins used in the binding assays

Property	PRG	Lf	Transferrin	Amylase*
M_r	36 [†]	76 [‡]	77 [§]	56
Carbohydrate content (%)	37.5	5.4	5.7	0
No. of carbohydrate units	6	2	2	
Biantennary	—	+ [¶] , **	+ [§]	
Triantennary	+ ^{††}	—	—	
GlcNAc (mol/mol) ^{††}	28 (30)	7 (8)	8 (8)	0

* Non-glycosylated. [†] Levine *et al.* (1969). [‡] Querinjean *et al.* (1971). [§] Debray *et al.* (1981). ^{||} Keller *et al.* (1971). [¶] Prieels *et al.* (1978). ** Matsumoto *et al.* (1982). ^{††} Reddy *et al.* (1982). ^{‡‡} Calculated amounts; values in parentheses represent theoretical amounts.

Fig. 1. Saturation and inhibition of the binding of ^{125}I -ASA-PRG to *Streptococcus sanguis* G9B

(a) Binding of ^{125}I -ASA-PRG to *S. sanguis*; (b) inhibition of ^{125}I -ASA-PRG binding to *S. sanguis* with increasing concentrations of unlabelled PRG. The final concentration of inhibitor is based on a reaction volume of 0.15 ml.

gels and the iodinated components detected by autoradiography. Autoradiograms were then scanned with a LKB model-2202 Ultrascan laser densitometer interfaced to an Apple IIe computer.

RESULTS AND DISCUSSION

The salient properties of the various (glyco)proteins used in the binding assays are shown in Table 1. The purity of each molecule was ascertained by h.p.l.c. and SDS/polyacrylamide-gel electrophoresis. Western blotting further verified the identity of the Lf and non-glycosylated amylase preparations. PRG contains six triantennary *N*-linked oligosaccharide units (Reddy *et al.*, 1982) per molecule. Three of these units contain one sialic acid residue linked $\alpha 2,3$ to galactose on the $\alpha 1,6$ mannose branch. Lf and transferrin were used as additional examples of *N*-linked glycoproteins. Each contains two biantennary units. For transferrin, each biantennary unit contains two sialic acid residues linked $\alpha 2,6$ to galactose (Debray *et al.*, 1981). Lactoferrin has only one sialic acid residue linked $\alpha 2,6$ to galactose per oligosaccharide unit (Prieels *et al.*, 1978; Matsumoto *et al.*, 1982).

A typical binding curve of ^{125}I -ASA-PRG to *S. sanguis* is shown in Fig. 1(a). Saturation of the bacterial binding sites was observed at ^{125}I -ASA-PRG concentrations of approx. 25 pmol/ 10^{10} bacterial cells. The binding of ^{125}I -ASA-PRG (10 pmol/ 10^{10} bacterial cells) was inhibitable by the addition of various amounts of unlabelled

Fig. 2. Scatchard analysis of the binding of ^{125}I -ASA-PRG to *S. sanguis*

PRG (Fig. 1b). Maximum inhibition was achieved at an inhibitory concentration of 2.5 μM -PRG. Scatchard analysis (Fig. 2) of the binding data shown in Fig. 1(a) indicated that 1.96 pmol of ^{125}I -ASA-PRG saturated the binding sites on 1×10^{10} bacterial cells (confidence coefficient 0.97). On the basis of these data it is estimated that approx. 120 molecules of PRG can be bound to each bacterial cell. To ascertain the reversibility of the ^{125}I -ASA-PRG-bacterial interaction, bacteria were first incubated with ^{125}I -ASA-PRG (10 pmol) and then 2 h later incubated with a 200-fold molar excess of unlabelled PRG. This competitive desorption resulted in the release of 63% of the bound ^{125}I -ASA-PRG when compared with the control (absence of unlabelled PRG).

The specificity of PRG binding with *Streptococcus sanguis* G9B was examined by using inhibition assays.

Table 2. Inhibition of ^{125}I -ASA-PRG binding to *Streptococcus sanguis* G-9B by PRG derivatives and other glycoproteins

Inhibitor	Final [inhibitor] (μM)*	Inhibition† (%)
PRG	19	77 (4.0)‡
	1.0	64 (1.0)
Asialo-PRG	1.1	68 (6.9)
Periodate-treated PRG	1.0	17 (4.6)
Transferrin	19	48 (1.7)
	1.9	7 (2.0)
Lf	17	43 (6.4)
	1.7	0
α -Amylase§	19	8 (2.6)

* Calculated from hexosamine content (see Table 1); a final reaction volume of 0.15 ml was used.

† Values were expressed as a percentage of control (no inhibitor) and represent the average of four assays.

‡ Values in parentheses represent the s.d.

§ Non-glycosylated family.

Table 3. Inhibition of the binding of ^{125}I -ASA-PRG to *Streptococcus sanguis* G-9B by saccharides

Inhibitor	Final [inhibitor] (mM)*	Inhibition† (%)
<i>N</i> -Acetyl-lactosamine	1	51 (4.3)‡
<i>N</i> -Acetylneuramin-lactose	1	43 (9.7)
Galactose	1	30 (5.0)
<i>N</i> -Acetylgalactosamine	1	30 (2.9)
<i>N</i> -Acetylneuraminic acid	1	24 (2.9)
<i>N</i> -Acetylglucosamine	1	3 (2.0)
Glucose	1	0

* Calculated based on a final reaction volume of 0.15 ml.
† Values are expressed as a percentage of control (no inhibitor) and represent the average for three assays.
‡ Values in parentheses represent the S.D.

Initial inhibition studies assessed the effect of modifying PRG's carbohydrate moieties (Table 2). Removal of terminal *N*-acetylneuraminic acid residues did not effect PRG's ability to inhibit ^{125}I -ASA-PRG binding, presumably owing to the low content of *N*-acetylneuraminic acid in PRG (Reddy *et al.*, 1982). However, periodate treatment of PRG resulted in a marked decrease in this molecule's inhibitory capacity, thus implicating the role of the non-reducing terminal galactose in the binding reaction. For comparison, two other *N*-linked glycoproteins, Lf and transferrin, were tested for their ability to inhibit the binding of ^{125}I -ASA-PRG to *S. sanguis*. On a molar basis, transferrin and Lf were weaker inhibitors of ^{125}I -ASA-PRG binding to *S. sanguis*. Non-glycosylated salivary amylase had no inhibitory activity.

The effects of saccharide inhibitors on the binding of ^{125}I -ASA-PRG to the bacterial cells are outlined in Table 3. The tri- and di-saccharides *N*-acetyl-lactosamine and *N*-acetylneuramin-lactose were the most potent inhibitors tested ($51 \pm 4.3\%$ and $43 \pm 9.7\%$ respectively). The monosaccharides sialic acid, galactose and *N*-acetylgalactosamine exhibited intermediate inhibitory activity ($24.9 \pm 2.9\%$, $30 \pm 5.0\%$, and $30 \pm 2.9\%$ respectively), whereas *N*-acetylglucosamine and glucose were poor inhibitors.

To determine the efficiency of ^{125}I -ASA-PRG cross-linking to the bacterial cell surface and begin characterizing the bacterial adhesin, the cross-linked complexes were solubilized with 1% SDS at 50 °C for 2 h and subjected to chromatography on Sepharose CL-6B equilibrated in 0.1% SDS. The cross-linked complexes, which emerged in the void volume of the column, were pooled, and the percentage of the total extractable iodinated material was calculated to be approx. 1%, a value similar to the results obtained by others (Ji & Ji, 1980; Vanin & Ji, 1981; Baenziger & Fiete, 1982; Wood & O'Dorisio, 1984). A similar experiment where ^{125}I -ASA-PRG was first photoactivated and then added to the bacterial cells did not result in the appearance of high- M_r cross-linked components. In addition, gel filtration of photoactivated ^{125}I -ASA-PRG alone or ^{125}I -ASA-PRG + bovine serum albumin gave no detectable radioactivity in the void volume of the Sepharose CL-6B columns. These control experiments indicate that the ASA probe does not

Fig. 3. SDS/polyacrylamide-gel electrophoresis of ^{125}I -ASA-PRG and ^{125}I -ASA-PRG-*S. sanguis*-adhesin complex

Fractions obtained from Sepharose CL-6B chromatography were subjected to electrophoresis on 5% polyacrylamide gels under reducing conditions and subjected to autoradiography as described in the text. Laser densitometer scans of autoradiograms of: (a) void volume fraction containing ^{125}I -ASA-PRG-*S. sanguis*-adhesin complex (~ 22000 c.p.m./50 μl) and (b) ^{125}I -ASA-PRG (~ 36000 c.p.m./50 μl at 2.7×10^7 c.p.m./nmol).

promote cross-linking between PRG itself or with bovine serum albumin and suggests that the void-volume materials obtained are the result of the covalent coupling of ^{125}I -ASA-PRG to adhesin(s) on the bacterial cell surface. Indeed, photoactivation of the azido group incorporated on to PRG results in the formation of a highly active and unstable aryl nitrene that has sufficient energy to insert (non-specifically) into the adhesin, resulting in a covalent coupling to these molecules. Owing to the extremely short half-life of this nitrene (on the order of 5 ms), the probability of insertion into a site other than the adhesin is extremely low. SDS/polyacrylamide-gel electrophoresis of this ^{125}I -ASA-PRG-adhesin complex on 5% polyacrylamide gels in the presence of 2-mercaptoethanol revealed two components (Fig. 3). The higher- M_r species failed to penetrate the gel, whereas the lower- M_r component migrated slightly into the gel matrix. These findings are consistent with preliminary data (P. A. Murray, M. J. Levine, M. S. Reddy & L. A. Tabak, unpublished work), which suggest that the *S. sanguis* adhesin contains two carbohydrate-binding subunits.

The present study has demonstrated the potential use of photoreactive crosslinking reagents to examine, in a quantitative fashion, the nature of specific interactions between salivary glycoproteins and oral streptococci. The data demonstrate specific binding that is saturable, reversible and inhibited by structural analogues. The results obtained indicate that glycoprotein binding is mediated by a bacterial adhesin that recognizes, either separately or together, complex oligosaccharide structures consisting of sialic acid, galactose and *N*-acetylgalactosamine. These results are in agreement with our previous findings (Murray *et al.*, 1982), which demonstrated the

presence of a *S. sanguis* lectin having specificity for the trisaccharide NeuAc α 2-3Gal β 1-3GalNAc, the major acidic oligosaccharide of the lower- M_r salivary mucin (Reddy *et al.*, 1985). Even though both glycoproteins can interact with oral streptococci, previous studies have indicated that only the low- M_r salivary mucin, but not PRG, agglutinates these bacteria (Levine *et al.*, 1978). This difference may be attributed to the density or clustering of carbohydrate units along the peptide backbone (Weigel *et al.*, 1979; Stowell *et al.*, 1980). Indeed, the low- M_r salivary mucin contains 140–150 oligosaccharide units uniformly distributed over a single peptide backbone (Levine *et al.*, 1985), whereas PRG contains only six triantennary oligosaccharide units distributed over the length of a single peptide chain (Shimomura *et al.*, 1983; Loomis *et al.*, 1986). It is apparent, therefore, that differences in composition, quantity and distribution of oligosaccharide chains between different salivary glycoproteins provide variation for their interaction with oral bacteria.

This work was supported in part by U.S. Public Health Service Grants DE04971, DE04518 and DE07034.

REFERENCES

- Baenziger, J. U. & Fiete, D. (1982) *J. Biol. Chem.* **257**, 4421–4425.
- Beachey, E. H. (1981) *J. Infect. Dis.* **143**, 325–345.
- Beppu, M., Tuuo, T. & Osawa, T. (1975) *J. Biochem. (Tokyo)* **79**, 1013–1019.
- Carlsson, J., Grahnen, H. & Jansson, G. (1975) *Caries Res.* **9**, 333–339.
- Debray, H., Decout, D., Strecker, G., Spik, G. & Montreuil, J. (1981) *Eur. J. Biochem.* **117**, 41–55.
- Doyle, D. J., Nesbitt, W. E. & Taylor, K. G. (1982) *FEMS Microbiol. Lett.* **15**, 1–5.
- Fraser, A. R., Hemperly, J. J., Wang, J. L. & Edelman, G. M. (1976) *Proc. Natl. Acad. Sci.* **73**, 790–794.
- Gibbons, R. J. & Etherden, I. (1982) *Infect. Immun.* **36**, 52–58.
- Gibbons, R. J. & Qureshi, J. V. (1978) *Infect. Immun.* **22**, 665–671.
- Gibbons, R. J. & van Houte, J. (1980) in *Bacterial Adherence: Receptors and Recognition* (Beachey, E. H., ed.), vol. 6, pp. 60–104, Chapman and Hall, London.
- Gibbons, R. J., Etherden, I. & Moreno, E. C. (1983) *Infect. Immun.* **42**, 1006–1012.
- Gibbons, R. J., Etherden, I. & Peros, W. (1985) in *Molecular Basis of Oral Microbial Adhesion* (Mergenhagen, S. & Rosan, B., eds.), pp. 77–84, American Society of Microbiology, Washington.
- Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J.* **89**, 114–119.
- Hogg, S. D. & Embury, G. (1981) *Arch. Oral Biol.* **27**, 945–949.
- Ji, I. & Ji, T. H. (1980) *Proc. Natl. Acad. Sci.* **77**, 7167–7170.
- Ji, T. H. & Ji, I. (1982) *Anal. Biochem.* **121**, 286–289.
- Johsson, G. L., MacAndrew, V. I. & Pilch, P. F. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 875–878.
- Keller, P. J., Kauffman, D. L., Allan, B. J. & Williams, B. J. (1971) *Biochemistry* **10**, 4867–4874.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Levine, M. J., Weill, J. C. & Ellison, S. A. (1969) *Biochim. Biophys. Acta* **188**, 165–167.
- Levine, M. J., Herzberg, M. C., Levine, M. S., Ellison, S. A., Stinson, M. W., Li, H. C. & Van Dyke, T. (1978) *Infect. Immun.* **19**, 107–115.
- Levine, M. J., Tabak, L. A., Reddy, M. S. & Mandel, I. D. (1985) in *Molecular Basis of Oral Microbial Adhesion* (Mergenhagen, S. & Rosan, B., eds.), pp. 125–130, American Society for Microbiology, Washington.
- Loomis, R. E., Bergey, E. J., Levine, M. J. & Tabak, L. A. (1986) *Int. J. Protein Peptide Res.*, in the press.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
- Matsumoto, A., Yoshimi, H., Takasaki, S. & Kobata, A. (1982) *J. Biochem. (Tokyo)* **91**, 143–155.
- McBride, B. C. & Gisslow, (1977) *Infect. Immun.* **18**, 35–40.
- Morris, E. J. & McBride, B. C. (1984) *Infect. Immun.* **43**, 656–666.
- Murray, P. A., Levine, M. J., Tabak, L. A. & Reddy, M. S. (1982) *Biochem. Biophys. Res. Commun.* **106**, 390–396.
- Nagata, K., Nakao, M., Shibata, S., Shizukuishi, S., Nakamura, R. & Tsunemitsu, A. (1983) *J. Periodontol.* **54**, 163–172.
- Nesbitt, W. E., Doyle, R. J. & Taylor, K. G. (1982) *Infect. Immun.* **38**, 637–644.
- Prieels, J. P., Pizzo, S. V., Glasgow, L. R., Paulson, J. C. & Hill, R. L. (1978) *Proc. Natl. Acad. Sci.* **75**, 2215–2219.
- Querinjean, P., Masson, P. L. & Heremans, J. F. (1971) *Eur. J. Biochem.* **20**, 420–425.
- Reddy, M. S., Levine, M. J. & Tabak, L. A. (1982) *Biochem. Biophys. Res. Commun.* **104**, 882–888.
- Reddy, M. S., Levine, M. J. & Prakobphol, A. (1985) *J. Dent. Res.* **64**, 33–36.
- Roe, J. H. (1955) *J. Biol. Chem.* **212**, 335–343.
- Rosan, B., Eifert, R. & Golub, E. (1985) in *Molecular Basis of Oral Microbial Adhesion* (Mergenhagen, S. & Rosan, B., eds.), pp. 69–76, American Society for Microbiology, Washington.
- Shibata, S., Nagata, K., Nakamura, R., Tsunemitsu, A. & Misaki, A. (1980) *J. Periodontol.* **51**, 499–504.
- Shimomura, H., Kanai, Y. & Sanada, K. (1983) *J. Biochem. (Tokyo)* **93**, 857–863.
- Shomers, J. P., Tabak, L. A., Levine, M. J., Mandel, I. D. & Ellison, S. A. (1982) *J. Dent. Res.* **61**, 397–399.
- Shorr, R. G. L., Heald, S. L., Jeffs, P. W., Lavin, T. N., Strohsacker, M. W., Lefkowitz, R. J. & Caron, M. G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2778–2782.
- Smith, G. E. & Summers, M. D. (1981) *J. Virol.* **39**, 25–137.
- Stinson, M. W., Levine, M. J., Cavese, J. M., Pradabphol, A., Murray, P. A., Tabak, L. A. & Reddy, M. S. (1982) *J. Dent. Res.* **61**, 1390–1393.
- Stowell, C. P., Lee, R. T. & Lee, Y. C. (1980) *Biochemistry* **19**, 4904–4908.
- Tabak, L. A., Levine, M. J., Mandel, I. D. & Ellison, S. A. (1982) *J. Oral Pathol.* **11**, 1–17.
- Terlecky, J. B., Willet, N. P. & Schockman, G. D. (1975) *Infect. Immun.* **11**, 649–655.
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354.
- Vanin, E. F. & Ji, T. H. (1981) *Biochemistry* **20**, 6754–6760.
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975.
- Weigel, P. H., Schnaar, R. L., Kuhlenschmidt, M. S., Schmell, E., Lee, R. T., Lee, Y. C. & Roseman, S. (1979) *J. Biol. Chem.* **254**, 10830–10838.
- Westergren, G. (1981) *Arch. Oral Biol.* **26**, 1035–1039.
- Westergren, G. & Olsson, J. (1983) *Infect. Immun.* **40**, 432–435.
- Wood, C. L. & O'Dorisio, M. S. (1984) *J. Biol. Chem.* **260**, 1243–1247.
- Yip, C. C., Yeung, C. W. T. & Moule, M. L. (1978) *J. Biol. Chem.* **253**, 1743–1748.