An Intramolecular Interaction Involving the N Terminus of a Streptococcal Adhesin Affects Its Conformation and Adhesive Function*

Received for publication, February 7, 2013, and in revised form, March 27, 2013. Published, JBC Papers in Press, March 28, 2013, DOI 10.1074/jbc.M113.459974

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Background: P1 is an adhesin on the surface of Streptococcus mutans.

Results: Destroying the high affinity interaction between the N and C termini of S. mutans P1 creates a non-adherent phenotype.

Conclusion: The N terminus facilitates proper folding, function, and stability within recombinant P1.

Significance: The relationship between folding, maturation, and cell surface assembly is critical to understanding the P1 mechanism of action.

The adhesin P1 is localized on the surface of the oral pathogen Streptococcus mutans and facilitates an interaction with the glycoprotein complex salivary agglutinin that is comprised primarily of the scavenger receptor gp340. Recent crystal structures of P1 display an unusual structure in which the protein folds back upon itself to form an elongated hybrid helical stalk with a globular head at the apex and a globular C-terminal region at the base. The N terminus of P1 has not yet been characterized. In this report we describe the contribution of an interaction between the N-terminal and C-terminal portions of the protein that is required for proper function of P1 on the surface of S. mutans. Utilizing recombinant N-terminal and C-terminal fragments, we employed isothermal titration calorimetry and native gel electrophoresis to demonstrate that these fragments form a high affinity and stable complex in solution. Furthermore, circular dichroism and surface plasmon resonance measurements indicated that the N-terminal fragment contributes to the folding and increases the functionality of the C-terminal fragment in trans. Finally, we utilized circular dichroism, surface plasmon resonance, and differential scanning calorimetry to show that an N-terminal 106-amino acid segment within P1 contributes to the proper folding and function of the full-length recombinant molecule and increases the stability of its elongated hybrid helical stalk.

The primary structure of P1 is represented in Fig. 1a. P1 contains a signal sequence (which directs secretion), an unchar-

Dental caries (tooth decay) is the leading childhood infection in the United States and the most prevalent infectious disease within humans (1, 2). A primary causative agent of dental caries is Streptococcus mutans (3). The ability of S. mutans to adhere to host tissues, colonize the oral cavity, and ferment dietary carbohydrates into lactic acid leads to demineralization of the tooth surface (4, 5). The complete molecular mechanism by which S. mutans is able to adhere to host components has not been fully characterized.

S. mutans utilizes two methods of adherence: sucrose-dependent and sucrose-independent (6, 7). The 1561-amino acid residue cell surface antigen P1, also known as antigen I/II, Pac, and antigen B, is a virulence factor that mediates sucrose-independent adherence (8–14). The AgI/II family of adhesins is highly conserved among most oral streptococci and also has been found on other streptococcal species including Streptococcus pyogenes, Streptococcus agalactiae, and Streptococcus suis. Mutants of S. mutans lacking P1 display decreased cariogenicity in a gnotobiotic rat model (15). P1 functions by interacting with a variety of host constituents, the best characterized being the glycoprotein complex salivary agglutinin (SAG)2 comprised predominantly of the scavenger receptor gp340/DMBT1 (8–10, 12–14, 16–23). In the presence of fluid-phase SAG, the interaction with P1 induces bacterial aggregation. This is believed to represent an innate host defense mechanism (24) that would result in clearance of the bacteria from the oral cavity. On the other hand, when SAG is immobilized onto a surface such as hydroxyapatite, the interaction with P1 mediates adherence and subsequent colonization of the bacteria. S. mutans binding to fluid-phase or immobilized SAG are both P1-mediated events, and a P1-deficient mutant of S. mutans does not aggregate or adhere (15). However, these represent independent properties mediated by distinguishable interac-

2 The abbreviations used are: SAG, salivary agglutinin; aa, amino acids; RU, resonance units; ITC, isothermal titration calorimetry; MRE, mean residue ellipticity; rp1, recombinant P1; SRCR, scavenger receptor cysteine-rich domains; MBP, maltose binding protein.
acterized N-terminal region, three tandem alanine-rich repeats (A1–3), a variable region (V) that contains a segment where sequence differences among strains are clustered (34), three tandem proline-rich repeats (P1–3), a C-terminal region containing three distinct domains (C1–3), an LPXTG motif, and wall- and membrane-spanning regions (35, 36). P1 is linked to the *S. mutans* cell wall by sortase A, a transpeptidase found in numerous Gram-positive organisms that cleaves substrate proteins at the LPXTG consensus motif and subsequently covalently couples their C termini to the cell wall peptidoglycan (37). Recently solved crystal structures of truncated P1 polypeptides have allowed the development of a three-dimensional model of P1 that comprises the majority of the protein (36, 38, 39) (Figs. 1, b and c). The A- and P-regions interact to form an elongated hybrid α/polyproline type II helix, contributing to the majority of P1 length (38). The V-region is rich in β-structure and displays a globular β super sandwich at the apex of the molecule containing two β-sheets, each composed of eight-antiparallel strands. The C terminus (residues 1000–1486) contains three domains, each adopting a DE-variant immunoglobulin like (DEv-IgG) fold, with isopeptide bonds at similar positions in each of the three domains (36, 39). Isopeptide bonds are present in a variety of Gram-positive surface proteins and have been shown to contribute to stability and protease resistance (40–44). Like the V-region, the three C-terminal domains of P1 also display a great deal of β-structure, and the structure of the N-terminal 163 amino acids (∼20 kDa) preceding the A-region is currently unknown. Previous work in our laboratory has suggested that the N-terminal region of P1 is required for the proper assembly and function of the full-length adhesin on the surface of *S. mutans* (45).

In the current study our goal was to further elucidate the contribution of the N-terminal region to the function of P1 and to characterize the discontinuous interaction between the N- and C-terminal regions of the molecule. We demonstrate herein that disrupting the N-terminal/C-terminal interaction within P1 disproportionately abolishes the adherence compared with the aggregation properties of *S. mutans* in the presence of immobilized and fluid-phase SAG, respectively. We also demonstrate the formation of a functional complex formed by an N-terminal fragment (NA1) and a C-terminal fragment (P3C) (Fig. 1b) that reconstitutes a structure mimicking that of native P1 on the surface of *S. mutans*. Formation of this complex has high energetic favorability and displays increased adherence properties to immobilized SAG compared with that of P3C alone despite a lack of independent adherence to SAG by NA1. Last, an in-frame deletion polypeptide, lacking amino acids 86–190 of P1, displays decreased adherence to immobilized SAG, decreased thermal stability, and notable differences in secondary structure as compared with the full-length protein.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**

*S. mutans* serotype c strain NG8 (46) was used in these studies. The isogenic *spaP*-negative mutant PC3370 (15) was used as the negative control. The *S. mutans* strain NR7 that expresses P1 containing an internal deletion (Δaa 84–190) was created for this study. *S. mutans*, *spaP* insert DNA from the previously described pNR7 plasmid (32), was isolated from *Escherichia coli*, restricted, ligated to similarly digested *E. coli* streptococcal shuttle vector pDL289 (47), and used to transform PC3370 via natural transformation (48). *S. mutans* strain PC967, in which the expressed P1 contains additional isoleucine and aspartic acid residues at positions 826 and 827 and positions 999 and 1000, respectively (Fig. 1c), was previously described (45). *S. mutans* cultures were grown for 16 h at 37 °C in Todd-Hewitt yeast extract (THYE), Todd-Hewitt broth (Becton, Dickinson and Company, Sparks, MD) supplemented with 0.3% yeast extract (EMD Chemicals Inc.).

**Expression and Purification of Recombinant P1 and P1 Polypeptides**

DNA encoding the N terminus and first alanine-rich repeat of P1 (NA1, aa 39–308) (Fig. 1, a and b) was PCR-amplified from *spaP* of *S. mutans* NG8 using cggagaaggatttcACTTATGAAGCT-GCACCTAGA (the Xmn1 restriction site is in bold) and CGGaagatttTCAGTCATGTCATGTAGTGATGATGCCTTGTCGGCGGTGTGGGC (the HindIII restriction site is in bold, and the His$_{6}$ tag is underlined) as forward and reverse primers, respectively, cloned into pMAL-p2X (New England Biolabs, Ipswich, MA), and transformed into *E. coli* Top10 (Invitrogen) as described previously (49). The third proline-rich repeat through the C terminus of P1 (P3C, aa 921–1486) (Fig. 1, a and b) was similarly generated using cggagaaggattctAATATAACCACACCCGC-CGACC (the StuI restriction site is in bold) and CGGgagaccTTCAGTCATGTCATGTAGTGATGATGCCTTGTCGGCGGTGTGGGC (the BamHI restriction site is in bold, and the His$_{6}$ tag is underlined) as forward and reverse primers, respectively (49). NA1 and P3C were expressed and purified as follows: 20 ml terrific broth cultures were grown overnight at 37 °C and used to inoculate 1 liter of terrific broth the following day. Cultures were grown for 16 h at 37 °C with shaking. The overnight cultures were harvested the following morning, and the cell pellets were stored at −20 °C overnight. The following day the cell pellets were suspended in TALON equilibration buffer (50 mM Tris-HCl, pH 7.2, 300 mM NaCl, 40 mM imidazole) and sonicated on ice with a sonic dismembrator (Fisher Scientific, Rockford, IL), 10 mg of lysozyme, and 10 mM MgCl$_{2}$ and sonicated on ice with a sonic dismembrator (Fisher, Model 100) at maximum setting for 2 min using 5-s bursts. Cells were incubated at 4 °C on a rotary actuator for 1 h, and the sonication was repeated. The cell sonicates were centrifuged at 45,000 × g for 30 min, and the supernatant (soluble) fractions were filtered through a 0.22-μm syringe-driven filter (Millipore, Billerica, MA). Filtered samples were then applied to TALON Metal Affinity Resin (Clontech, Mountain View, CA), and bound protein was eluted with 150 mM imidazole. Elution fractions were subsequently passed over amyllose affinity matrix resin (New England Biolabs), and bound protein was eluted with 200 mM maltose. The purified protein was dialyzed overnight into 10 mM Tris, 150 mM NaCl, and 2 mM CaCl$_{2}$, concentrated using an Amicon filter (Millipore), 10-kDa molecular weight cutoff, and digested with Factor Xa (New England Biolabs) to remove the MBP tag. Digested proteins...
were separated from Factor Xa and free MBP first using TALON resin and elution with 150 mM imidazole followed by passing over amylose resin to remove residual free MBP or MBP fusion protein. NA1 and P3C were polished over an SEC-250 size-exclusion column (Bio-Rad). The NA1-P3C complex was purified as above, except before polishing NA1 and P3C were mixed together in equimolar ratios, incubated overnight at 4 °C, and the following day, purified using the SEC-250 column.

During the initial lysis process and purification of P3C over TALON resin, a stable breakdown product is produced. This product is no longer MBP-tagged but retains the C-terminal His6 tag. The breakdown product was separated from the P3C-MBP fusion protein using amylose affinity matrix resin and determined by N-terminal sequencing to represent the C terminus of P1 (aa 1000–1486).

Additional P1 constructs, full-length recombinant P1 lacking the secretion signal sequence (rP1, aa 39–1566), and recombinant NR7 (Δaa 84–190) were subcloned into pQE-30 and used to transform E. coli strain M15-pREP4 as described previously (32, 50). The P1 construct A3VP1 (aa 386–875) was subcloned into pET-30a (+) (EMD Millipore) and used to transform E. coli BL21 (DE3) cells. Sample preparation of rP1, NR7, and A3VP1 was performed as described above for NA1 and P3C, and the soluble fractions were applied to 10 ml of TALON Metal Affinity Resin (Clontech). Bound proteins were eluted with 150 mM imidazole followed by passing over amylose resin to remove residual free MBP or MBP fusion protein. NA1 and P3C were polished over an SEC-250 size-exclusion column.

Surface Plasmon Resonance; Adherence of S. mutans and Recombinant P1 and P1 Polypeptides

SAG was prepared from pooled unstimulated saliva from healthy human volunteers as previously described (13). Adherence of S. mutans whole cells was measured using Biacore surface plasmon resonance (Biacore Life Sciences, Uppsala, Sweden) on a Biacore 3000 instrument as described previously (33). S. mutans strains NG8 and PC3370 functioned as positive and negative controls, respectively.

Adherence of purified recombinant P1 and P1 polypeptides was performed as described above for whole cells. Briefly, recombinant P1 fragments were dialyzed overnight into adherence buffer (33). The following day each sample was diluted in adherence buffer to 4, 2, and 1 μM concentrations, and 40 μl of each sample was injected over the chip surface at a flow rate of 10 μl/min. Ten microliters of regeneration buffer (33) was used to regenerate the chip surface between runs. The uncoated surface FC1 was used as a control surface without SAG, and the background RU was subtracted from the observed signal for each injection. S. mutans NG8 and PC3370 were used to confirm a P1-specific interaction with the SAG-coated surface before measuring the adherence of each recombinant P1 protein.

SAG-mediated Aggregation of S. mutans

SAG-mediated aggregation was measured using a spectro-photometric assay as described previously (13). PBS without SAG served as a background control. NG8 and PC3370 were included as positive and negative controls, respectively.

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ELISA

Epitope Reconstitution—96-Well plates (Corning Inc., Corning, NY) were coated with 400 ng/well purified NA1 or P3C in 0.2 M sodium carbonate/bicarbonate, pH 9.4, as described previously (32). Plates were washed and blocked with PBS, 0.3% Tween 20 (PBST). Next, 400 ng of either P3C or NA1 in PBST was overlaid onto the NA1- or P3C-coated wells. Interaction between the coated and overlaid polypeptides was evaluated using anti-P1 mAbs 3–10E, 6–11A, and 4–10A followed by horseradish-peroxidase-labeled goat anti-mouse secondary antibody (MP Biomedicals, LLC, Santa Ana, CA) and development with O-phenylenediamine substrate solution. Absorbance was read at 450 nm.

Whole Cell Competition ELISA—S. mutans strain NG8 (~10^5 cfu per well) was immobilized overnight at 4 °C on a 96-well plate. Serial dilutions of polypeptides NA1, P3C, or the NA1-P3C complex, beginning at 1 μM, were mixed directly with anti-P1 mAb 3–10E IgG (1.8 mg/ml, diluted 1:1000 in PBST) and then added to wells for 1 h at 37 °C. After incubation, plates were developed as described above, and absorbance was read at 450 nm. Percent inhibition was calculated as [(Abs_450 without inhibitor – Abs_450 with inhibitor) ÷ Abs_450 without inhibitor] × 100.

Native Polyacrylamide Gel Electrophoresis

Five microliters of purified NA1, P3C, or NA1-P3C complex at a concentration of 0.2 mg/ml was mixed with 5 μl of loading buffer (125 mM Tris-HCl, 20% glycerol, 0.02% bromphenol blue, pH 8.0), electrophoresed at 4 °C through a 10% polyacrylamide gel, pH 8.0, in running buffer (190 mM glycine, 25 mM Tris-HCl, pH 8.3) for 4 h at 150 V, and blotted onto a Protran nitrocellulose membrane (Whatman, Dassel, Germany). Duplicate membranes were either stained with colloidal gold protein stain (Bio-Rad) or reacted with mouse anti-P1 mAbs 3–8D, 6–8C, or 4–10A ascites fluid diluted 1:500 in PBST and developed as described previously (31).

Isothermal Titration Calorimetry

Purified NA1 and P3C polypeptides were dialyzed overnight into PBS. The following day the interaction between NA1 (30 μM) and P3C (3 μM) was analyzed using a MicroCal VP-ITC calorimeter (MicroCal, Piscataway, NJ) as follows. P3C was loaded into the stir-cell and stirred at 300 rpm. NA1 was loaded into the syringe. After an initial injection of 2 μl (excluded in data fitting), 29 injections of 8 μl each were delivered at 250-s intervals. Data analysis and curve fitting was performed using Origin Software (Microcal). The experiments were performed in duplicate at 15, 20, 25, and 30 °C to determine the ΔC_p. A control run was also performed in which buffer-only was loaded into the stir-cell, and NA1 was loaded into the syringe and injected. This blank run was performed for all four temperature conditions, and background values were subtracted from each data set.

Circular Dichroism

Initial CD Measurements—All CD measurements were performed on an AVIV (Lakewood, NJ), model 202, CD spectrom-
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FIGURE 1. Schematic representation of the primary and modeled tertiary structure of P1. a, shown is the primary structure of P1 and location of peptides used in this study. b, shown are locations NA1 and P3C, which are predicted to be in juxtaposition based on the current tertiary model of P1 developed by Larson, M. R. et al. (36). These peptides interact to reconstitute the epitope recognized by anti-P1 mAb 3–10E (circled in red) that requires an interaction between the N-terminal and C-terminal regions. c, shown is a diagram displaying the location of the engineered Clal sites that resulted in the introduction of two extra amino acids, isoleucine and aspartic acid, flanking either side of the proline-rich region (circled in red, aa 826 and 827 and aa 999 and 1000) when DNA encoding the P-region was reintroduced into a P-region deletion construct to generate the recombinant plasmid pPC967.

RESULTS

Adherence and Aggregation Properties of S. mutans Expressing NR7 and PC967—Biacore surface plasmon resonance was used to evaluate adherence of S. mutans to SAG (33). The mutant strain, NR7, was engineered to express an internally deleted P1 variant (Δaa 84–190) (Fig. 1a) (32). Analysis of NR7 compared with the NG8 parent strain demonstrated that elimination of these 106 residues from P1 N terminus destroyed its ability to mediate adherence to immobilized SAG (Fig. 2a). This dramatic decrease in SAG binding was comparable with that of complete elimination of P1 from mutant PC3370. In contrast, a spectrophotometric assay that measures the P1-mediated interaction of S. mutans with fluid-phase SAG (13) showed NR7 is still capable of substantial residual aggregation activity, ~70% that of the wild type (Fig. 2b). In light of these results we also tested another S. mutans mutant, PC967, made previously in our laboratory (45) that was generated by reintroduction of DNA encoding the proline-rich region into the same site of plasmid-based spaP from which P-region residues 826–996 had been deleted. Because removal of the P-region was accomplished via in-frame ligation of upstream and downstream DNA by way of Clal sites engineered into the PCR primers, re-introduction of P-region DNA at this junction resulted in two extra amino acids, isoleucine and aspartic acid, that flanked this region. These occurred at positions 826/827 and 999/1000 (45, 50) and are localized on either end of the extended helical stalk in the tertiary structure model (Fig. 1c). An unusual feature of the PC967 polypeptide is its SDS-PAGE migration.
Unlike the M, 185 kDa of the unadulterated P1 protein, which runs ~20 kDa higher than predicted, PC967 migrates at its predicted size of 165 kDa. Thus, introduction of the additional amino acids had an inadvertent affect on an SDS-resistant feature of the P1 molecule that normally retards its electrophoretic migration. Upon eliminating 106 residues from the N-terminal sequence of NR7, the polypeptide also migrated as predicted and was not retarded in electrophoretic mobility. Like strain NR7, PC967 was completely deficient in the ability to bind immobilized SAG (Fig. 2a) and again retained >70% wild-type level aggregation in the presence of fluid-phase SAG (Fig. 2b). Therefore, the N-terminal region and the context of the proline-rich region, both, appear to play critical roles in P1-mediated bacterial adherence but are not essential for P1-mediated aggregation.

Reconstitution of Anti-P1 mAb Epitopes and mAb Competition—NR7 and PC967 appear to share a common structural change. Both are recognized by all 11 anti-P1 mAbs, except for 3–10E. This antibody is one of several that depends on an interaction between the alanine- and proline-rich repeat sequences for reactivity but is unique in that its epitope also requires pre-A and post-P regions of the protein (31, 32, 57). Two recombinant polypeptides, NA1 (aa 39–308) and P3C (aa 921–1486), were generated to further evaluate this interaction (Fig. 1a). The epitope recognized by mAb 3–10E was clearly reconstituted by incubating NA1 and P3C together on an ELISA plate (Fig. 3a). The epitope was restored irrespective of which binding partner was coated onto the plate and which was overlaid. Modest reactivity was detected against NA1 alone; however, the level of 3–10E reactivity increased notably to a level comparable to that of full-length rP1 when NA1 and P3C were incubated together. The interaction of NA1 and P3C restored the epitopes of other anti-P1 mAbs as well. mAb 4–10A and 6–11A also recognize discontinuous epitopes dependent on an interaction between the A- and P-regions; however, unlike 3–10E, mAb 4–10A depends solely on the A-P interaction, and 6–11A is contributed to by pre-A but not post-P sequence (32, 57–59). Taken together, these results indicate that NA1 and P3C interact to...
form a structure that involves both pre-A as well as post-P region sequences and not simply the previously characterized A- and P-region interaction (38). To confirm that the interaction of NA1 and P3C recreates a structure reflective of native P1 as it exists on the surface of S. mutans, a competition ELISA was employed. The polypeptides were tested singly and in combination for their ability to inhibit binding of mAb 3–10E to S. mutans whole cells by ELISA (Fig. 3b). When NA1 and P3C were mixed together the degree of inhibition was comparable with that of full-length rP1 over the 1–0.25 μM concentration range tested. However, neither NA1 nor P3C alone displayed any inhibition of 3–10E binding to surface-localized P1, even at the highest concentration. Taken together our results indicate that NA1 and P3C interact to form a complex whose structure is recognized by the highly conformation-dependent mAb, 3–10E, as well as other anti-P1 mAbs and that this complex mimics that of P1 as it exists on the cell surface.

Characterization of NA1–P3C Complex Formation—Formation of an NA1-P3C complex was also evaluated by native gel electrophoresis (Fig. 4a). A clear shift in migration was observed for the NA1-P3C complex compared with either NA1 or P3C in isolation. The corresponding Western blots of replicate gels confirm identification of the NA1-P3C complex band. mAb 4–10A recognizes a discontinuous epitope within the hybrid A/P helix. It was not reactive with either NA1 or P3C but was reactive with the complex. mAb 6–8C recognizes an epitope within the C terminus and was reactive with P3C as well as the NA1-P3C complex. In contrast, mAb 5–8D recognizes the A-region out of the context of the intact A/P hybrid helix. It was reactive with NA1 but not the NA1-P3C complex. These data confirm that NA1 and P3C form a stable complex that does not dissociate upon gel electrophoresis. Next, ITC was used to characterize heat release during the formation of the NA1-P3C complex over a range of temperatures from 15 to 30 °C (Table 1). The ΔH of NA1 titrated into buffer alone was negligible and was subtracted as background. A plot of the raw data at 20 °C (top) and the corresponding binding isotherm (bottom) is shown in Fig. 4b. The data were fit to a single binding site model using Origin software, and the $K_a$, $K_d$, $ΔH$, and stoichiometry ($n$) of binding were obtained (60–63). The $ΔS$ and $ΔG$ of binding were calculated from these measured values. All of the thermodynamic parameters are summarized in Table 1. The ITC data indicate that NA1 and P3C bind at a 1:1 molar ratio with a calculated dissociation constant of 1.63 nM at 20 °C. Upon binding, these polypeptides also exhibit a large change in enthalpy ($ΔH = -156.0 \text{ kcal/mol at 20 °C}$) accompanied by a large decrease in entropy ($ΔS = -493 \text{ kcal/mol/K}$). Furthermore, formation of the NA1-P3C complex is spontaneous and energetically favorable with an estimated $ΔG_{binding} = -11.5 \text{ kcal/mol}$. Plotting the $ΔH$ over the range of temperatures tested results in a calculated heat capacity of binding ($ΔC_p = -3.0 \text{ kcal/mol}$). In summary, both native gel electrophoresis and ITC experiments confirm that the NA1 and P3C fragments of S. mutans P1 form a stable and energetically favorable complex.

Circular Dichroism Measurements—Contributions of the N terminus of P1 to the secondary structure of the protein were estimated by CD. Far UV CD spectra were obtained for NA1, P3C, and the NA1-P3C complex from 250 to 200 nm (Fig. 5a). Deconvolution of the CD spectra (Fig. 5a) indicates that

![FIGURE 4. Characterization of the NA1-P3C complex by native gel-electrophoresis and isothermal titration calorimetry. a, native gel-electrophoresis displayed a shift in migration upon NA1-P3C complex formation. The anti-P1 mAbs used in the corresponding Western blots are shown below each panel. b, ITC was used to measure the energetics of the NA1-P3C interaction. Calorimetric measurements were performed at 20 °C, with multiple additions of 8 μl of 30 μM NA1 injected into a stir-cell containing 3 μM P3 (upper panel). The energy (kcal/mol) released during each injection is illustrated (lower panel).](image-url)

### TABLE 1

| Temperature °C | Number of sites | $K_a$ | $ΔH$ kcal/mol | $ΔS$ cal K⁻¹mol⁻¹ | $ΔG$ kcal/mol |
|---------------|----------------|-------|---------------|-------------------|---------------|
| 15            | 1.02 ± 0.004   | 2.86 ± 0.18 | -142.7 ± 1.00 | -456 | -11.3 |
| 20            | 0.933 ± 0.003  | 1.63 ± 0.19 | -156.0 ± 0.83 | -493 | -11.5 |
| 25            | 0.925 ± 0.008  | 1.11 ± 0.36 | -168.9 ± 3.37 | -525 | -12.4 |
| 30            | 1.01 ± 0.003   | 0.87 ± 0.25 | -186.4 ± 1.29 | -527 | -13.0 |

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NA1 is highly α-helical (61%), whereas P3C contains more β-structure (61%). These calculated values are in good agreement with the crystal structure and tertiary model (36, 39). The predicted secondary structure content of the NA1-P3C complex largely represents the calculated weighted average of NA1 and P3C, 33% α-helical and 37% β-structure, suggesting that both NA1 and P3C contribute to the overall structure of the complex. Far UV CD spectra from 260 to 185 nm were also obtained on purified NR7 (36–190) and rP1 polypeptides to estimate the secondary structure contribution of the N terminus within the full-length protein (Fig. 5b). Deconvolution of the CD spectra (Fig. 5b, inset) shows a modest difference between NR7 and rP1, with more obvious differences between several local minima and maxima within both spectra. Taken together these CD spectra suggest that the N terminus contributes to the overall structure of the P1 molecule but that CD lacks the resolution to determine exactly where the difference lies.

**Adherence of P1 to Immobilized SAG**—The adherence of P1 fragments to immobilized SAG was measured using Biacore surface plasmon resonance. P1 is known to have two regions located at opposite ends of the helical stalk that are both capable of independent binding to immobilized salivary agglutinin. Each fragment was tested at a concentration of 4 μM. Time in seconds is indicated on the x axis. The change in resonance (ΔRU) (arbitrary units) is indicated on the y axis. Proteins were injected over a period of 240 s at a flow rate of 10 μl/min. b, maximum binding values (ΔRU) measured for each polypeptide at 4, 2, and 1 μM concentrations are illustrated. S. mutans whole cells were used as positive and negative controls to ensure appropriate coating of SAG to the surface. NG8 is the wild-type positive control strain. PC3370 is the P1-deficient negative control strain. Each assay was performed in triplicate, and the S.E. is illustrated.
molecule. NA1, which does not contain either of the two known binding sites, did not interact with immobilized SAG. A3VP1, which contains one of the binding sites, and P3C and the isolated C terminus, which each contain the other, all displayed measurable adherence. The levels of binding of P3C and the C terminus were comparable to one another, but the isolated C terminus dissociated much more rapidly in the absence of the P-region sequence. The level of binding of NR7 was diminished by ~30% in the absence of the deleted N-terminal sequence despite the lack of evidence for a direct binding site within this segment. This suggests that a conformation conferred by the presence of the N-terminal sequence contributes to the overall adherence of P1 to SAG. Consistent with this, the NA1-P3C complex displayed ~2 times the level of adherence compared with the C terminus or P3C alone. These results were apparent over the range of protein concentrations tested (Fig. 6b). The increase in the ability of P3C to bind immobilized SAG in the presence of NA1 reiterates the importance of the N-terminal/C-terminal interaction to the proper function of P1.

**Thermal Stability and Refolding of P1**—Last, the thermal denaturation profiles of the purified rP1 and NR7 polypeptides were compared using differential scanning calorimetry to measure their ΔH of unfolding between 30 and 80 °C (Fig. 7a). The melting curves of rP1 and NR7 both displayed three large transitions that occurred during heating. Compared with rP1, NR7 displayed a small shift of ~0.62 °C in the first transition and a larger shift of ~2.14 °C in the second transition. The third transition was unaffected. To gain insight into which regions of NR7 were less stable compared with rP1, a CD spectrum of each protein was obtained from 25 to 70 °C to estimate changes in secondary structure upon thermal denaturation (Fig. 8a). NR7 and rP1 displayed slightly different CD spectra at 25 °C; NR7 denatured more quickly, but both proteins eventually showed very similar profiles and were both largely denatured at 70 °C. Comparing the changes in MRE of both NR7 and rP1 at specific fixed wavelengths over the range of temperatures demonstrates the unique melting curves for each protein (Figs. 8, b–d), further highlighting their differences in thermal sensitivity and secondary structure changes during denaturation. Deconvolution of the CD spectra (Table 2) confirmed that NR7 denatures ~2 °C lower than rP1. The decrease in helical content with increasing temperature suggests that this instability may largely be due to the unwinding of the P1 hybrid helical stalk. Next, the refolding of NR7 and rP1 was compared after thermal denaturation. When NR7 was renatured after thermal denaturation, it displayed a substantially different CD spectrum compared with renatured rP1 (Fig. 9). Again, deconvolution of these CD spectra (Table 3) indicates that much of this difference in secondary structure is the result of decreased helical content.

**DISCUSSION**

P1-mediated adherence and aggregation of *S. mutans* are distinct processes, both involving an interaction with the high molecular weight glycoprotein complex, salivary agglutinin (13, 15). Recent crystal structures of P1 have led to a nearly complete model in which two adherence domains lie at opposite ends of an extended hybrid helical stalk. One domain is contained within the A3VP1 fragment and the other within the C terminus (36, 38). The tertiary model highlights the unique nature of the P1 structure. Perturbation of that structure has been found to have dramatic affects upon the proper configuration of the protein on the surface of the cell (45, 50, 64). The sensitivity of the P1 function to structural alteration is exemplified by *S. mutans* mutants NR7 (∆84–190) as well as PC967, in which the expressed P1 contains only four additional amino acid but whose structure and function is substantially altered. Binding to immobilized SAG is completely abolished in both NR7 and PC967. Although the altered sequences are not located within SAG binding regions, they clearly have a devastating negative effect on the adherence capability of *S. mutans*. Despite the inability of the NR7 and PC967 strains to interact with immobilized SAG, they are still capable of substantial P1-mediated aggregation in the presence of fluid-phase SAG. The disproportionate effect of the mutations on adherence versus aggregation substantiates that these represent independent processes involving different regions of P1. Not only do *S. mutans* NR7 and PC967 share similar adherence and aggregation phenotypes, they also display similar reactivities with a panel of anti-P1 mAbs (13, 31, 32, 45, 50, 58).

Based upon the current tertiary model as well as epitope mapping data, we employed two discontinuous P1 fragments (NA1 and P3C), which are predicted to be in juxtaposition in the folded structure, as a tool to further characterize the contribution of the P1 N terminus to the adhesin structure. These fragments were shown to interact to reconstitute the epitope recognized by mAb 3–10E and, when present together, to compete as well as full-length rP1 for mAb 3–10E binding to P1 on *S. mutans* whole cells. Thus NA1 and P3C were shown to form a complex representative of the native adhesin structure as it exists on the bacterial surface. When NA1 and P3C are in isolation these two polypeptides appear to be inherently unstable. NA1 is degraded into several fragments, and P3C also breaks down, losing the proline-rich region to yield the isolated C terminus. However, upon formation of a complex, the two interacting polypeptides become highly stable and show no signs of degradation even when stored at 37 °C for several months (data...
Furthermore, these two fragments display quite a high affinity for one another. The $K_d$ of NA1 and P3C measured at 20 °C is 1.63 nM, more than 30 times greater than that of the previously characterized A/P hybrid helical stalk interaction (38). Thus, the very strong interaction between NA1 and P3C appears not only to occur via the alanine- and proline-rich regions but also is contributed to by an interaction involving the pre-A and post-P regions. ITC measurements of NA1 added to the C terminus alone showed no evidence of an interaction between these fragments (data not shown). However, the $\Delta C_p$ calculated for formation of the NA1-P3C complex in the current study was $-3.0 \text{ kcal/(K\cdot mol)}$ compared with $\Delta C_p = -1.9 \text{ kcal/(K\cdot mol)}$ reported for the A/P interaction (38). Such large changes in heat capacity have been shown to be proportional to the surface area of solvent excluded from non-polar amino acids (65–67). This exclusion of solvent is presumed to be evidence of an extensive folding event during which the non-polar amino acids become buried and water is subsequently released from the protein surface (67–71). Because the $\Delta C_p$ for NA1-P3C complex formation is substantially larger than that of the

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### FIGURE 8. Changes in circular dichroism spectra during thermal denaturation of NR7 compared with rP1.

- **a.** Far UV CD spectra were measured from 260 to 185 nm over a temperature range of 25–70 °C. Representative spectra are shown at specific temperatures during thermal denaturation. The dynode voltage remained below 800 V at all wavelengths over the entire temperature range.
- **b–d.** Changes in mean residue ellipticity at fixed wavelengths (190, 210, and 220 nm, respectively) were measured over the 25–70 °C temperature range.

### TABLE 2

**Predicted changes in secondary structure of recombinant NR7 and rP1 during thermal denaturation**

Changes were calculated by measuring the far UV CD spectra from 260–185 nm during heating and using the deconvolution algorithm CDSSTR (51–56). The protein reference set was SDP42.

| Secondary structure content | 25 °C | 40 °C | 45 °C | 50 °C | 52 °C | 54 °C | 56 °C | 58 °C | 60 °C | 65 °C | 70 °C |
|-----------------------------|------|------|------|------|------|------|------|------|------|------|------|
| rP1                         |      |      |      |      |      |      |      |      |      |      |      |
| α-Helical content           | 53%  | 53%  | 51%  | 49%  | 42%  | 37%  | 24%  | 20%  | 17%  | 8%   | 6%   |
| β-Sheet content             | 20%  | 20%  | 16%  | 14%  | 16%  | 14%  | 19%  | 17%  | 18%  | 17%  | 23%  |
| β-Turn content              | 12%  | 11%  | 14%  | 17%  | 19%  | 18%  | 21%  | 18%  | 17%  | 16%  | 19%  |
| Unordered content           | 15%  | 16%  | 19%  | 20%  | 23%  | 31%  | 36%  | 45%  | 48%  | 53%  | 46%  |
| NR7                         |      |      |      |      |      |      |      |      |      |      |      |
| α-Helical content           | 50%  | 50%  | 50%  | 43%  | 36%  | 24%  | 20%  | 15%  | 8%   | 7%   | 5%   |
| β-Sheet content             | 23%  | 24%  | 17%  | 15%  | 17%  | 17%  | 18%  | 22%  | 24%  | 25%  | 31%  |
| β-Turn content              | 14%  | 12%  | 15%  | 17%  | 18%  | 18%  | 19%  | 19%  | 17%  | 17%  | 18%  |
| Unordered content           | 13%  | 14%  | 18%  | 25%  | 29%  | 41%  | 43%  | 44%  | 51%  | 51%  | 46%  |
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A/P helical stalk, this suggests that the complete folding process cannot be attributed solely to the A/P stalk but includes an additional event involving adjacent regions of the protein. This appears to result in a substantial conformational change in both binding partners, changes that dictate the overall structure and function of the complete full-length P1 molecule.

Circular dichroism measurements demonstrated that NA1 and P3C each have substantial structure in isolation and are in good agreement with the available tertiary model of P1 (36, 38, 39). Upon formation of the NA1-P3C complex, the resulting CD spectrum largely represents the calculated weighted average of NA1 and P3C based upon the CD spectra of each individual fragment (data not shown). This implies that NA1 remains primarily helical in structure and P3C remains relatively β-structured upon complex formation. Despite the lack of obvious change in the CD spectra of the NA1 and P3C fragments after complex formation, comparison of the CD spectra of the longer NR7 and full-length rP1 polypeptides revealed several notable differences in local minima and maxima. These indicate the presence of distinct aberrations in the NR7 secondary structure compared with that of full-length rP1. Unfortunately, CD does not provide sufficient resolution to identify which regions within the protein were altered. However, taken together, the data do suggest that the N terminus of P1 contributes to the overall structure and folding of the molecule.

Although the observable differences in secondary structure resulting from the removal of a major portion of the P1 N terminus may be subtle, the functional effect is dramatic. NA1 does not itself adhere to immobilized SAG but increases the adherence of P3C by 2-fold when it is contained within a complex. Our Biacore data are in good agreement with previous reports that the C terminus of P1 represents an adherent domain and additionally show that for the C terminus to reach its full adherent capability, the N terminus must be present as well. The level of interaction of the NA1-P3C complex with immobilized SAG was comparable in magnitude to that of the A3VP1 fragment that contains the other adherent domain of the protein (38). In the current study the isolated C terminus did not display optimal binding to immobilized SAG and dissociated much more quickly than P3C or the NA1-P3C complex. This result is consistent with previously reported data in which a C-terminal fragment (C12), which contains the first two but not the third DEv-IgG domain, displayed greater binding and slower dissociation than the complete C terminus (36). This suggests that a relevant binding site may be partially masked in the isolated C123 polypeptide and that the intramolecular interaction between NA1 and P3C facilitates optimal display of the binding site contained within C12. In addition to enhanced adherence of the NA1-P3C complex to immobilized SAG, the purified recombinant NR7 polypeptide displayed a 30% reduction in adherence compared with the unadulterated molecule. This indicates that the N terminus also contributes to P1 interaction with immobilized SAG in the context of the full-length protein. Based upon the large ΔCp calculated from ITC experiments for NA1-P3C complex formation and the CD spectra, which suggest a folding event and indicate changes in secondary structure when P1 lacks a large portion of its N terminus, our results imply that the diminished binding affinity of NR7 with SAG stems from the loss of a critical conformation that is normally stabilized by sequence contained within amino acids 84–190. Our results suggest that the N terminus of P1 may function as an intramolecular chaperone. These serve as critical building blocks to help the proteins that contain them achieve a thermodynamically stable state during folding (72, 73). Thus, the coupling of a large folding event upon the interaction of NA1 with P3C suggests that the intramolecular recognition between these binding partners promotes a stable native conformation within P1 (73).

Previous studies have identified a single 16-amino acid segment (SRCRP2) within the consensus sequence of the 13 repeating scavenger receptor cysteine-rich domains (SRCR) of gp340/DMBT1 that aggregates and adheres to S. mutans (22). This SRCRP2 segment was designated as the major bacterial binding site within gp340 (74), which has been further narrowed down to an 11-amino acid region with residues VEV-LXXXXW identified to be of particular importance for the peptide interaction with S. mutans (23). Therefore, the SAG interaction stabilized by the N-terminal 106 amino acids of P1 may help expose a region within its C terminus that binds to the 11-amino acid segment present within the gp340/DMBT1 mol-
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eucle in such a way as to mediate adherence but that is less critical for bacterial aggregation.

The complete lack of adherence of *S. mutans* cells that express the NR7 polypeptide further implies that this conformational variant of P1 cannot be displayed on the cell surface in a way capable of mediating bacterial adherence. This also appears to be true of P1 expressed by the mutant strain PC967 in which formation of the pre-A/post-P interaction was inadvertently precluded as a result of introduction of two additional amino acids on either side of the P-region as evidenced by destruction of anti-P1 mAb 3–10E epitope within the recombinant polypeptide (45). The residual aggregation capacity as well as reactivity of these two strains with anti-P1 mAbs 4–10A and 3–8D but no others (data not shown) is further evidence that P1 is present on the cell surface of NR7 and PC967 but that it is substantially altered in its architecture and function. The stark contrast between antigenicity of the recombinant proteins expressed in *E. coli* compared with the same polypeptides localized on the surface of *S. mutans* suggests an additional cellular event that occurs in the context of the homologous organism. It appears that the intramolecular interaction responsible for formation of the mAb 3–10E epitope is absolutely critical for proper expression and function of P1 on the streptococcal surface. Given the increased stability of NA1 and P3C when they exist together as a complex, it is likely that surface-localized P1 lacking such an interaction is inherently unstable and unable to achieve or maintain numerous epitopes as well as an adherence-competent form.

Deconvolution of the CD spectra measured for NR7 and rP1 during their thermal denaturation revealed that the observed unfolding events were largely associated with a loss of helical content during heating and that they occur at ~2 °C lower in NR7 compared with rP1, in close agreement with the differential scanning calorimetry profile of each protein. The unique melting curves of NR7 and rP1 further demonstrate how differences in secondary structure manifest in varying thermal sensitivity and structural changes during denaturation. Assessment of refolding of NR7 after thermal denaturation also demonstrated a substantial loss of this protein helical structure. In contrast, rP1 refolded in a manner similar to that of the original undenatured protein. The pronounced loss of helical secondary structure in NR7 may well involve changes in the P1 unusual hybrid helix, further suggesting that the presence of the N terminus also helps stabilize the alanine- and/or proline-rich regions that interact to form the characteristic extended fibrillar stalk. Clearly, the N terminus of P1 plays an integral role in the folding, stability, and function of the adhesin and its assembly on the surface of the cell, thus advancing our understanding of this complex virulence factor and its association with human salivary agglutinin.

Acknowledgments—We thank Dr. Shyamasri Biswas in the University of Florida Department of Biochemistry and Molecular Biology for help with ITC initial data collection and analysis. We also thank Dr. Steve Hagen, Professor in the University of Florida Department of Physics, for use of the CD spectrometer.

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