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Energetics of the Initial Phase of Adhesion of *Streptococcus sanguis* to Hydroxylapatite

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The initial adhesion of *Streptococcus sanguis* 10556 to artificial salivary pellicle and to bare hydroxylapatite was studied at several temperatures between 18 and 37°C. When the natural logarithms of rate constants for adsorption and desorption were plotted against reciprocal temperatures in Arrhenius plots, curved lines were obtained, indicating that the thermodynamic quantities of enthalpy and entropy of activation were temperature dependent. For the bare hydroxylapatite system, the heat capacity (ΔCp = dH/dT) was large and negative. ΔCp was also negative for adhesion to saliva-coated hydroxylapatite, although its value was lower. Negative heat capacities, when coupled with favorable entropy, are often indicative of either electrostatic or hydrophobic interactions. When electrolyte (100 mM ammonium sulfate) was added to the cell-hydroxylapatite bead mixture, the rate and extent of adhesion were decreased. Addition of nonpolar *p*-dioxane (10% [vol/vol], final concentration) to the mixture enhanced binding. This suggests that electrostatic linkages participate in the primary adhesion of streptococci to both substrata. The strongly positive entropy values and the lesser temperature dependence of the saliva-coated hydroxylapatite system suggest that another entropy-driven process is imposed on the electrostatic linkages. This supports a role for hydrophobicity, suggesting that a combination of electrostatic and hydrophobic forces mediate the initial adhesion of *S. sanguis* to the salivary pellicle.

Significant advances have recently been made in the description of cellular adhesion mechanisms. Mammalian (23) and bacterial (6) cells have been shown to adhere through complex interactions with attractive and repulsive forces operating near substrata. Adhesion of streptococci to the salivary pellicle has long been recognized to be a complicated process. From the time of their eruption, teeth are constantly covered with a coating of salivary glycoproteins and proteins (pellicle). This pellicle is the substratum for oral bacterial attachment. *Streptococcus sanguis* seems to possess some specificity for the pellicle components since it reaches an equilibrium with the substratum. However, that specificity has been difficult to define, and it is believed that multiple forces govern adhesion (10–12). Kinetic studies suggest that *S. sanguis* passes through two distinct binding transitions (8). The first stage is a reversible one described by pseudofirst-order rate constants for adsorption and desorption. The cells bound in this equilibrium may then become more firmly attached and have a decreased propensity to desorb in the second phase of the reaction. The proposed mechanism can be represented as

$$\text{Cell} + \text{pellicle} \rightleftarrows \text{CP}^* \rightarrow \text{CP}$$

in which CP* represents the first, lower-affinity association between cell and pellicle. The rate constants governing this phase of the reaction are low, suggesting that the initial association is the rate-limiting one. Cells often adhere to solid surfaces in this general sequence. Marine pseudomonads (18), thiobacilli (3), and human platelets (23) have all been shown to approach their substratum slowly and bind reversibly before becoming more firmly bound in a second stage. Recently, Busscher et al. (6) studied the surface thermodynamics of adhesion of *S. sanguis* to a variety of polymers over time and found the reaction to take place in two distinct stages.

In an effort to define more clearly the mechanisms responsible for the early stages of adhesion, the dependence of rate constants on temperature was studied. Thermodynamic descriptions are dependent only on initial and final states, and the quantities derived from such studies are not expected to define the adsorption mechanism, but it is possible to derive concepts that are qualitatively compatible with the energetic parameters (5, 15, 19). The rate constants for the first phase of streptococcal adhesion are low, probably reflecting a lower-affinity association mediated by the large number of forces that may act on a bacterial cell. Thermodynamic studies could help to identify these forces by suggesting whether the adhesion process is driven by entropy or enthalpy.

MATERIALS AND METHODS

Culture conditions. *S. sanguis* ATCC 10556 was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and incubated at 37°C in 5.0% CO_2. Cells were radiolabeled by growth in the presence of 6 μCi of [^{3}H]thymidine per ml (specific activity, 68 Ci/mmol) (ICN Pharmaceuticals, Inc., Irvine, Calif.). Organisms from overnight cultures were harvested by centrifugation at 10,000 × *g* for 10 min, washed twice, and suspended in a buffer containing 50 mM phosphate (pH 6.0), 1.0 mM $CaCl_2$, 0.1 mM KCl, and 0.04% (wt/vol) sodium azide at a cell density of 2×10^7 to 10×10^7 per ml.

Cells were sonicated with a Vibra-Cell model VC 40 sonicator (Sonics and Materials, Danbury, Conn.) at a power output of 50 W to break chains of streptococci. Gram staining was used to confirm the presence of singlets and doublets exclusively. After sonication and immediately be-

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fore each experiment, cells were washed with KCl buffer and centrifuged to remove any free DNA in the supernatant. The mean total from three independent counts of the cell suspension was equated with the radioactivity associated with it, as determined through scintillation spectroscopy.

Saliva collection. Whole saliva was collected with Parafilm stimulation from a single donor. Sodium azide was added to a final concentration of 0.04% (wt/vol), and the saliva was centrifuged at 12,000 × *g* for 10 min at 4°C. Supernatant was saved and kept for no longer than 2 days at −20°C.

Bead preparation. Beads were prepared for the adsorption and desorption experiments as described previously (8). Spheroidal hydroxylapatite beads (25 mg) were rinsed in distilled water and then rotated in buffered KCl. In some cases, beads were incubated in centrifuged whole saliva and then washed in buffered KCl.

Adsorption and desorption assays. A detailed description of the conditions for adsorption and desorption is provided elsewhere (8). For adsorption experiments, a radiolabeled suspension of *S. sanguis* cells was added to a series of vials containing bare hydroxylapatite (HA) or saliva-coated hydroxylapatite (SHA). Samples of supernatant were removed at intervals during the adsorption for determination of numbers of unbound cells, and bound cells were counted by scintillation spectroscopy of beads from one of the reaction vessels.

Desorption reactions were studied by allowing the cells to adsorb for 4 min and then transferring the cells to 30 ml of KCl buffer in a shaking flask. Samples of the supernatant were removed at intervals to determine numbers of cells that became unbound with time. The adsorptions were only allowed to occur for 4 min since desorptions begun at that point in the adsorption were found to yield the initial rate constant for desorption. At the end of the desorption experiment, beads were harvested and counted by scintillation spectroscopy for determination of numbers of cells remaining bound.

Both adsorptions and desorptions were conducted at various temperatures between 18 and 37°C. All components of the experiments were brought to the appropriate temperature before commencement of binding.

In some adsorption experiments, the KCl buffer in which cells were suspended was supplemented with either 10% (vol/vol) *p*-dioxane or 100 mM $(NH_4)_2SO_4$. Control experiments were conducted in which cells or SHA beads were incubated with this buffer for 30 min, washed three times, suspended in KCl buffer, and used in the adsorption assay.

Calculation of rate constants. A differential form of the Langmuir equation was used to determine adsorption and desorption rate constants (k_1 and k_{-1}, respectively). A full explanation of that equation and its application to the streptococcus-SHA system is given in reference 8.

The rate equation $dU_t/dt = -k_1U_tN(1 - \theta) + k_{-1}N(U_0 - U_t)$ yields a rate plot whereby the slope of the line, m, is related to the adsorption rate constant by $m = k_1N[(U_0/U_0 - U_f) - \theta]$ and the desorption rate constant is obtained by $k_{-1} = k_1(U_0 - B_f)(1 - \theta)/B_f$, where U_0 = unbound cells at time zero, U_t = unbound cells at time t, B_f = bound cells at equilibrium, θ = fractional saturation, and N = number of hydroxylapatite beads.

The desorption rate constant was also obtained empirically and calculated as $t_{1/2} = 0.693/k_{-1}$, where $t_{1/2}$ = time required for number of adsorbed cells to decrease by one-half.

Calculation of thermodynamic parameters. Rate constants for adsorption and desorption at all temperatures were combined on Arrhenius plots of ln k versus $1/T$ (absolute temperature). The data were best described by a second-degree polynomial curve, and a quadratic equation was used to calculate the slopes of tangent lines to each of the points on the curve. These slopes were used to calculate an estimate of the activation energy, E_a, which is related to the enthalpy of activation by $\Delta H = E_a - RT$, where R is the gas constant. A quantity known as the preexponential factor, ln A, is used to obtain the entropy of activation by ln A = ln k + Ea/RT and $\Delta S = R(\ln A - kT/h)$, where k = the Boltzmann constant and h = Planck's constant. The free energy of activation, ΔG, becomes available from $\Delta G = \Delta H - T\Delta S$ (25, 26).

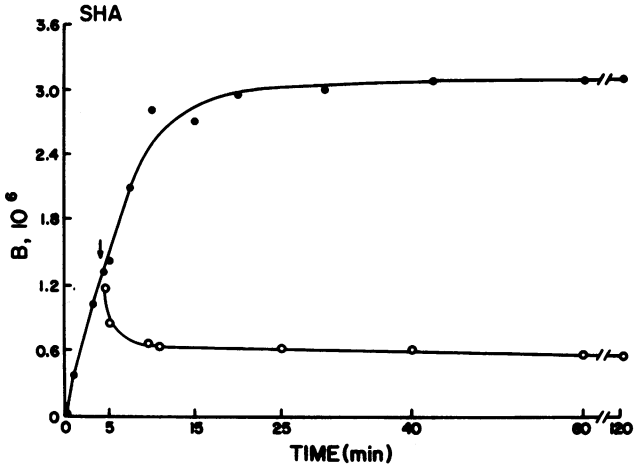

FIG. 1. Adsorption and desorption of *S. sanguis* 10556 with SHA. Symbols: ●, adsorption, 0 to 120 min (adsorption rate constant [k_1] = 1.7×10^{-3} min^{-1}); ○, desorption, begun after 4 min of adsorption (desorption rate constant [k_{-1}] = 2.3×10^{-2} min^{-1}). Total, 10^8 cells per 25 mg of SHA.

These quantities were determined at each temperature for adsorption and desorption experiments for the SHA and HA systems. Those determined for desorption were subtracted from those found for the adsorption process for each of the systems to yield net thermodynamic parameters for the reactions.

The units of all thermodynamic quantities except entropy, ΔS, are kilojoules per 25 mg of hydroxylapatite beads. Entropy units are joules per 25 mg of beads × degrees Kelvin.

RESULTS

Energetics of adsorption and desorption. Adsorption and desorption plots for SHA and HA at 25°C are shown in Fig. 1 and 2. The rate constants calculated from the kinetic plots for several temperatures from 18 to 37°C are graphed in Arrhenius plots for SHA (Fig. 3) and HA (Fig. 4). Temperature dependence was nonlinear, as is often the case with adsorption systems of macromolecular dimensions (24). The thermodynamic quantities are therefore also dependent on temperature and must be calculated at each experimental temperature. Quantities obtained from desorption were subtracted from quantities calculated for adsorption to generate parameters describing the net binding reaction. It must also be noted that thermodynamic quantities calculated for the *S. sanguis* adhesion reaction describe binding under the conditions specified, i.e., with 10^8 cells and 25 mg of hydroxylapatite beads. Calculation of net entropies, enthalpies, and free

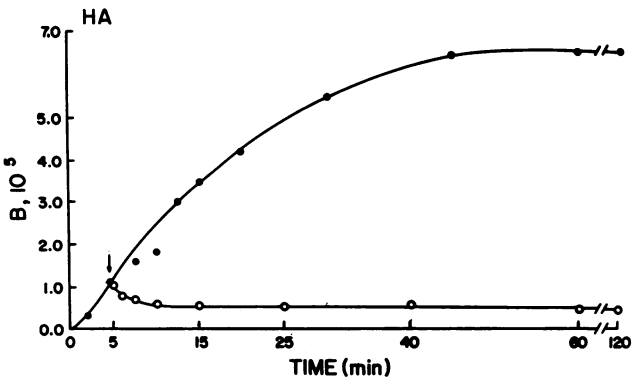

FIG. 2. Adsorption and desorption of *S. sanguis* with HA. Symbols: ●, adsorption, 0 to 120 min (adsorption rate constant [k_1] = 2.4×10^{-3} min^{-1}; ○, desorption, begun after 4 min of adsorption (desorption rate constant [k_{-1}] = 4.0×10^{-2} min^{-1}). Total, = 10^8 cells per 25 mg of HA.

energies of activation for SHA (Table 1) revealed a positive (favorable) entropy contribution at all temperatures tested, accompanied by positive (unfavorable) enthalpy terms for all but the highest temperature. Through a phenomenon known as entropy-enthalpy compensation (12), ΔG remains approximately constant. This is graphically demonstrated in Fig. 5b, which depicts the dependencies of ΔH, ΔG, and $-T\Delta S$ on temperature.

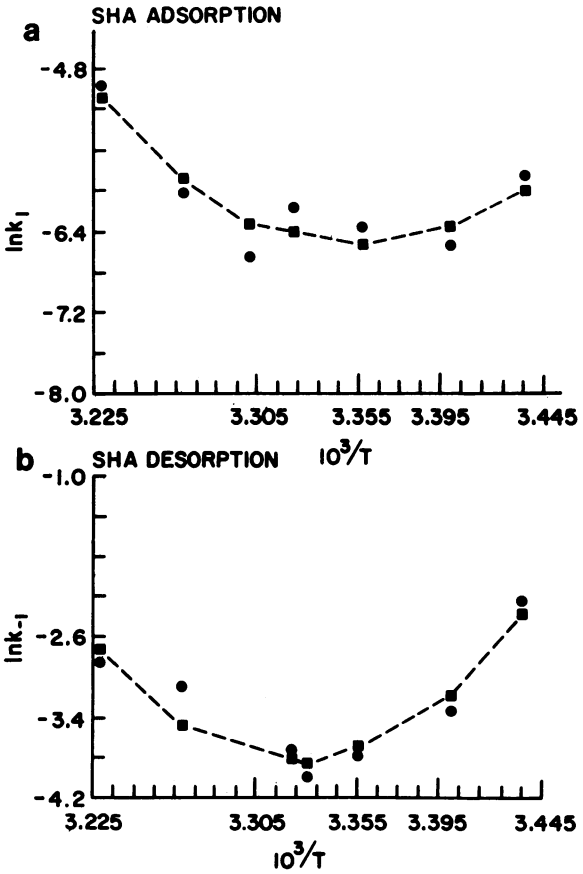

FIG. 3. Arrhenius plots for *S. sanguis* adsorption to (a) and desorption from (b) SHA. Symbols: ●, mean of two experiments; ■, second-degree polynomial curve.

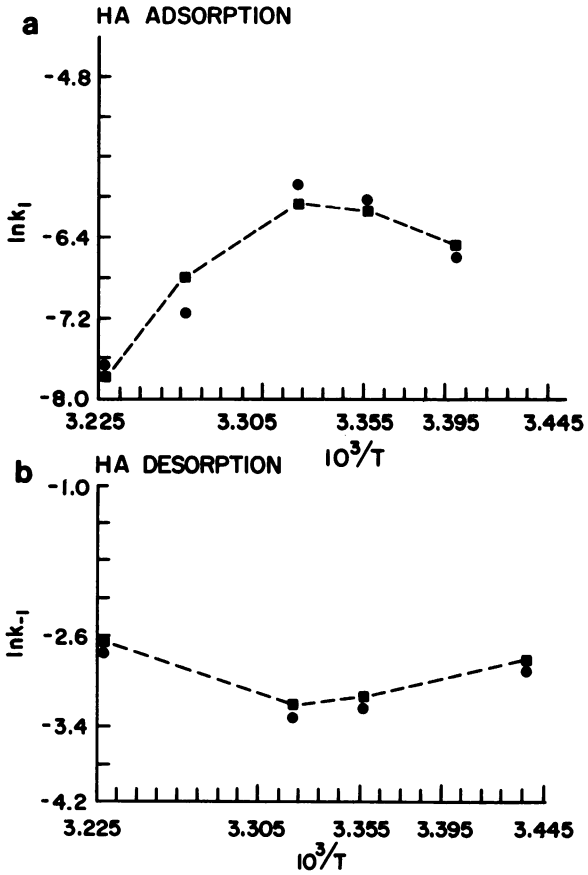

FIG. 4. Arrhenius plots for *S. sanguis* adsorption to (a) and desorption from (b) HA. Symbols: ●, mean of two experiments; ■, second-degree polynomial curve.

The thermodynamic quantities calculated for HA are presented in Table 2. Here temperature appears to play a more dominant role in the adsorption process, since both ΔS and ΔH change signs (and relative importance in binding). The compensation plot (Fig. 5a) depicts the wide variation in entropy of activation with temperature. This marked deviation springs from the difference in Arrhenius plots for adsorption and desorption in the HA system. Adsorption to HA displays its highest rates at moderate temperatures (approximately 25°C), while these are the temperatures at which the least desorption occurs (Fig. 4a and 4b). Thus, unlike with the SHA system, low adsorption is not tempered by low desorption, and a steep dependence on temperature is realized.

TABLE 1. Thermodynamic quantities for adsorption of cells to SHA

Temp (K)	ΔH (kJ/25 mg)	ΔS (J/25 mg × temp [K])	ΔG (kJ/25 mg)
291	+100.9	+311.8	+10.2
294	+83.8	+258.2	+7.9
298	+63.4	+192.0	+6.1
301	+42.1	+119.9	+6.0
303	+30.7	+80.1	+6.5
306	+14.2	+22.3	+7.4
310	−7.5	+7.9	+9.4

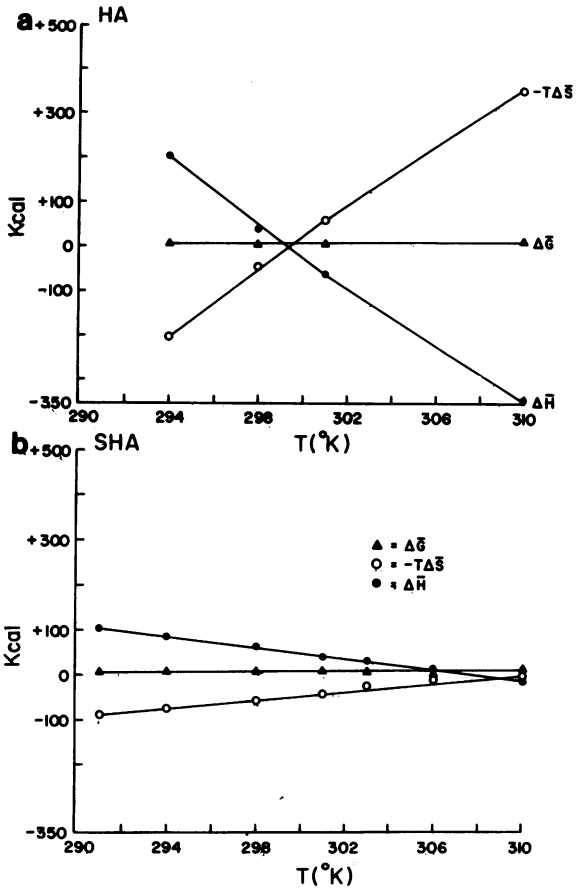

FIG. 5. Entropy-enthalpy compensation plots for net adsorption to substrata. (a) Adsorption to HA; $\Delta Cp = dH/dt = -35.15$ J. (b) Adsorption to SHA; $\Delta Cp = -5.75$ J.

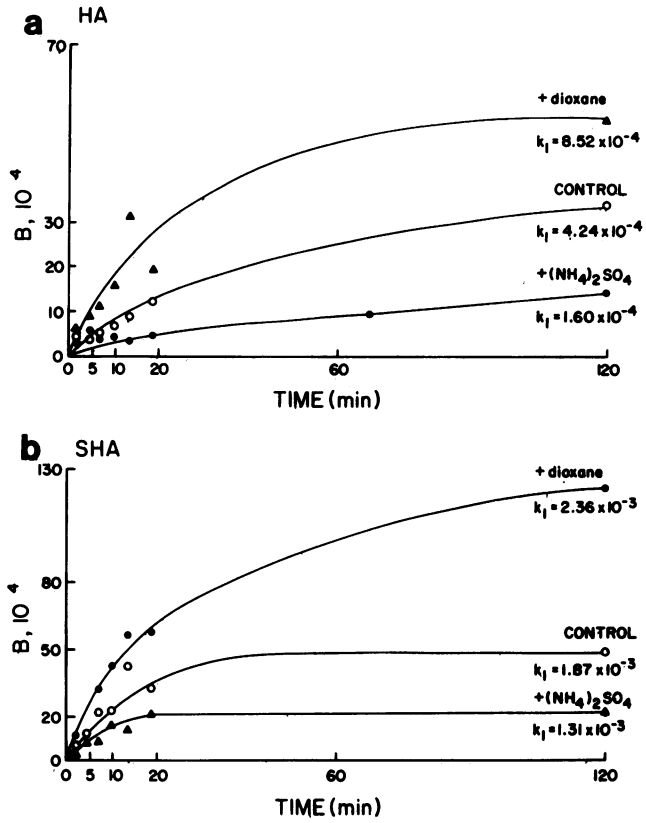

FIG. 6. Effects of solvent perturbants on kinetics of adsorption of *S. sanguis* to HA (a) and SHA (b).

Adsorption in the presence of perturbants. Adsorption of streptococci to bare HA and to SHA is accompanied by positive entropy in the face of negative enthalpy. These conditions are operative in two major types of adhesion interactions: salt linkages and hydrophobic bonding (14). To test the nature of the attraction in the two systems, adsorptions were carried out in the presence of either 10% *p*-dioxane or 100 mM ammonium sulfate in KCl buffer. Adsorption occurred at a higher rate and to a greater extent in the presence of *p*-dioxane for both the HA and SHA adsorbents (Fig. 6). Ammonium sulfate interfered with the adsorption process in both cases. This suggests a critical role for salt linkages in *S. sanguis* adhesion to both SHA and HA.

DISCUSSION

Evidence has recently been presented that *S. sanguis* 10556 adheres to artificial pellicle in a two-step process (6, 8). The first step is the rate-limiting one, represented by rate constants on the order of 10^{-3} min^{-1}. This suggests that the initial encounter with the pellicle substratum is slow enough to be studied and slow enough to be inhibited. Whereas sialic acid probably has a role in the binding reaction (12), the initial binding equilibrium seems to be largely independent of a specific high-affinity receptor (8). The rate constants for adsorption and desorption in this initial equilibrium show a marked dependence on temperature. Thus, thermodynamic studies will likely yield insight to the nature of the interaction.

TABLE 2. Thermodynamic quantities for adsorption of cells to HA

Temp (K)	ΔH (kJ/25 mg)	ΔS (J/25 mg × temp [K])	ΔG (kJ/25 mg)
294	+202.2	+657.0	+9.0
298	+41.9	+117.0	+7.0
301	−59.4	−218.1	+6.3
310	−345.1	−1155.2	+12.9

In both the SHA and HA systems, the thermodynamic quantities obtained from subtraction of desorption data from adsorption data revealed a linear temperature dependence for both entropy and enthalpy. Similar results have been found in many biopolymer and protein adsorption studies (13, 15, 24), in which entropy values decrease (become less favorable) with increasing temperature, while enthalpy values decrease (favorably), so that ΔG remains constant over the range of temperatures. The positive value for the free energy of the reaction is consistent with the observation that binding is reversible (7). Positive values for ΔG seem to indicate that the reaction will not take place. However, bacteria and mammalian cell adhesion reactions have been found to take place while displaying an unfavorable free-energy value (1, 2, 7). The reasons for this are unclear, although it must be remembered that for bacterial systems the calculated apparent ΔG does not describe a standard state. In addition, Absolom et al. (2) found that lowering the ionic content of a granulocyte suspension was shown to eliminate binding of the cells when ΔG was positive. Bus-

scher et al. (7) also conducted studies on the free energy of adhesion (ΔF) for various bacteria and substrata and found that as the calculated free energy passed from a negative value to a positive (unfavorable) value, the adhesion reactions changed from irreversible to reversible association.

The thermodynamic quantities for streptococcal binding to hydroxylapatite (Table 2) display a marked linear dependence on temperature. The compensation plot (Fig. 5a) reveals the extent of this dependence. Steep slopes are the mathematical consequence of the opposing curvatures in the Arrhenius plots for adsorption and desorption. *S. sanguis* adsorption to HA is most rapid at moderate temperatures, where desorption proceeds slowly. Thus, there is no compensation as there is in the SHA system, and whereas the free energy of activation remains constant as expected, it is due to wide complementary variations in enthalpy and entropy contributions.

The temperature dependence of extensive thermodynamic properties, such as enthalpy and entropy, is described by the heat capacity term $\Delta Cp = dH/dt$ (13, 22). Negative heat capacities are often associated with interactions that display favorable entropies. The two most important of these are hydrophobic and electrostatic interactions. In the bare HA system, the value for ΔCp is large and negative (−35.15 kJ), indicating that the enthalpy of the reaction becomes more favorable at higher temperatures. Adsorption to HA is accompanied by entropy values that decrease with temperature and quickly become negative. This could represent a decrease in radomness caused by increased constraining of bound groups. Loss of internal degrees of freedom, conformational changes among subunits, and proton translocation have been found to temper favorable entropy as the temperature is raised. These effects are most often seen with electrostatic linkages (21, 23). The large negative heat capacity is also characteristic of electrostatic interactions (22). These values have been seen in one of the steps of the self-assembly of the tobacco mosaic virus, which is thought to be purely electrostatic (17).

The heat capacity associated with streptococcal adhesion to SHA is small (−5.75 kJ). Since it is negative, electrostatic forces are again suspected. In this case, the entropy of activation is positive for every temperature studied. This is accompanied by unfavorable enthalpy values at all but the highest temperature (Table 1). Entropy almost certainly drives adhesion, suggesting that hydrophobic or electrostatic interactions are involved. The studies initiated with solvent perturbants (*p*-dioxane and ammonium sulfate) were designed to clarify the forces responsible for the thermodynamic quantities.

For both the HA and SHA systems, added electrolyte decreased the rate and extent of adhesion. Nonpolar *p*-dioxane enhanced adhesion in both cases (Fig. 6). Electrostatic linkages are therefore implicated in the bonding of *S. sanguis* to both substrata. The differences in magnitude of ΔCp bear investigation, however. For this we examined the origins of positive entropy and negative heat capacity. The compensation plot for the HA system exhibits a large degree of variation with temperature. The compensation plot for the SHA system (Fig. 5b) shows the temperature coefficients to be small. The thermodynamic quantities and the perturbant studies support a role for electrostatic linkages, but the marked temperature dependence of these interactions that were seen with HA appear to be mitigated by other forces in the SHA system. The positive entropy seen at all temperatures suggests that other forces that are entropy driven are imposed upon the entropy-controlled but temperature-variable electrostatic bond. Hydrophobic bonds are the other major significant linkages driven by entropy (14, 16), and in fact the values of ΔH and ΔS associated with hydrophobic interactions are not very temperature dependent (4). Previous studies implicate hydrophobic interactions in the adhesion of *S. sanguis* (20). Both the cell and the pellicle have hydrophobic surfaces (9, 27), and *S. sanguis* mutants exhibiting a hydrophilic phenotype are also adhesion deficient (11). Thus, hydrophobicity probably stabilizes the initial association mediated through electrostatic interactions and tempers the marked heat capacity found in purely electrostatic adsorption systems.

The slow initial adhesion of *S. sanguis* to saliva-coated hydroxylapatite may be the result of many factors, including the colloidal behavior of the ligand (cell) as it approaches the complex pellicle surface and the net repulsion or attraction determined by dispersion forces close to the interface. Consideration of the thermodynamic parameters associated with initial rate constants yields evidence that the cell-SHA interaction is mediated by a combination of electrostatic and hydrophobic linkages.

ACKNOWLEDGMENT

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