Biospecific Binding of Carbonic Anhydrase to Mixed SAMs Presenting Benzenesulfonamide Ligands: A Model System for Studying Lateral Steric Effects

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This work describes the binding of carbonic anhydrase (CA) to mixed self-assembled monolayers (SAMs) presenting benzenesulfonamide ligands at a surface consisting primarily of tri(ethylene glycol) [((EG)_3OH] groups. Surface plasmon resonance (SPR) quantified the binding of CA to the benzenesulfonamide groups. Two factors influenced the binding of CA: (a) the density of benzenesulfonamide groups at the surface, and (b) the coverage of the surface with molecules of CA adsorbed to these benzenesulfonamide groups. At low mole-fractions of benzenesulfonamide groups in the mixed SAM where the binding of CA is highly (>90%) reversible, we observe: (a) an approximately 10-fold decrease in the observed bimolecular rate constant for association, \( k_{\text{on,obs}} \), during the binding of CA (as the fraction of the surface covered by adsorbed CA increases from ~0.15 to ~0.35, the value of \( k_{\text{on,obs}} \) decreases from ~40 \( \times 10^3 \) M\(^{-1}\) s\(^{-1}\) to ~4 \( \times 10^3 \) M\(^{-1}\) s\(^{-1}\)); (b) almost no corresponding changes in the observed unimolecular rate constant for dissociation (\( k_{\text{off,obs}} \approx 0.005 \) s\(^{-1}\)) during the dissociation of CA from the surface. These observations establish that \( k_{\text{on,obs}} \) is influenced by the extent of coverage of the surface with CA, but that \( k_{\text{off,obs}} \) is not. At low surface densities of arylsulfonamide groups, one hypothesis that rationalizes these data is that the decrease in \( k_{\text{on,obs}} \) reflects repulsive steric interactions between molecules of CA near the surface and those already adsorbed. Each molecule of biospecifically adsorbed CA shields proximal benzenesulfonamide ligands from binding to incoming molecules of CA, and decreases the surface density of these ligands that are accessible to CA, at a rate that increases nonlinearly with the quantity of CA already adsorbed.

Introduction

Our objective in this work was to investigate lateral steric interactions—that is, interactions between molecules of protein associated with the surface, or in solution but close enough to the surface to interact with associated molecules—during binding of proteins to ligands immobilized at surfaces. These surfaces consisted of self-assembled monolayers (SAMs) of alkanethiols on gold-presenting benzenesulfonamide (ASA, arylsulfonamide)

1 H₂₆C₃₃S₃₃N₃O₃
2 H₂₆C₃₃S₃₃N₃O₃
3 H₂₆C₃₃S₃₃N₃O₃
4 H₂₆C₃₃S₃₃N₃O₃
5 H₂₆C₃₃S₃₃N₃O₃
6 H₂₆C₃₃S₃₃N₃O₃

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Binding of Carbonic Anhydrase to Mixed SAMs

**Scheme 1. Binding of CA to Mixed SAMs Presenting Benzenesulfonamide Ligands (4)**

![Diagram of CA binding to mixed SAMs]

The ligand is covalently immobilized to the surface by amide bond-forming reactions between its terminal amino group and active (NHS) esters on the SAM. The components of the system are drawn to scale.

**Table 1. Thermodynamic and Kinetic Constants for Binding of CA to ASA Ligands**

<table>
<thead>
<tr>
<th>R</th>
<th>technique</th>
<th>( k_{on} ) (M(^{-1}) s(^{-1}))</th>
<th>( k_{off} ) (s(^{-1}))</th>
<th>( K_d ) (M)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 R = -COO(^-)</td>
<td>ACE</td>
<td>2.2</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 R = -CONHCH(_2)CH(_3)</td>
<td>fluorescence</td>
<td>86</td>
<td>25</td>
<td>0.029</td>
<td>13</td>
</tr>
<tr>
<td>3 R = -CONH(_2)CHOO(^-)</td>
<td>fluorescence</td>
<td>220</td>
<td>11</td>
<td>0.0050</td>
<td>12</td>
</tr>
<tr>
<td>4 R = -CONHCH(_2)COO(^-)</td>
<td>fluorescence</td>
<td>36</td>
<td>120</td>
<td>0.33</td>
<td>12</td>
</tr>
<tr>
<td>5 R = -H</td>
<td>ACE</td>
<td>11</td>
<td>100</td>
<td>0.91</td>
<td>42</td>
</tr>
<tr>
<td>6 R = -CONH(CH(_2)NCO(EG)(_6)) ( ^b )</td>
<td>SPR</td>
<td>1.9</td>
<td>5.4</td>
<td>0.26</td>
<td>1</td>
</tr>
<tr>
<td>7(^a) R = -CONH(CH(_2)NCO(EG)(_6)) ( ^b )</td>
<td>SPR</td>
<td>3.5(^a)</td>
<td>5.3</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>( \chi(2/4) \approx 0.02 )</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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\(^a\) Entry 7 corresponds to values of \( k_{on} \) and \( k_{off} \) determined by SPR in this paper. \(^b\) The hexa(ethylene glycol) moiety was attached to SAMs of alkanethiolates on gold.

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**Composed of SAMs of alkanethiolates on gold**

The carbonic anhydrases are a well-characterized group of enzymes that bind para-substituted ASAs with dissociation constants \( K_d \) in the range \( 10^{-6} - 10^{-9} \) M. The reported values (from studies in solution) of \( K_d \) are \( 10^{-6} - 10^{-7} \) M\(^{-1}\) s\(^{-1}\); values for \( k_{off} \) are \( 10^{-2} - 10^{-5} \) s\(^{-1}\) (see Table 1 in Discussion). ASAs bind to CA by coordination of the sulfonamide (SO\(_2\)NH\(^-\)) moiety as an anion (SO\(_2\)NH\(^-\)) to a zinc ion located at the bottom of a conical cleft in the enzyme that is roughly 15 Å deep. We have demonstrated that bovine CA undergoes bioprecipitous adsorption to mixed SAMs presenting ASA ligands (5) at a surface composed primarily of tri(ethylene glycol) groups, and monitored in situ by SPR. At the low mole fractions of ASA used in the reported study \( \chi_{ASA} \leq 0.05 \), the adsorption of CA was \( \approx 90\% \) reversible. The reversibility of this association makes the ASA-CA interaction an attractive model system with which to study binding of proteins at surfaces. The kinetic constants reported (using SPR) by Mrksich et al. for binding of bovine CA (from bovine erythrocytes, containing A and B subunits) to SAMs of alkanethiolates on gold satisfy three requirements: (1) they resist nonspecific adsorption of biomolecules, (2) they are easily modified synthetically to include delicate and complex functional groups, and (3) they can be prepared in ways that control the average surface density of ligands used for bioprecipitous binding.

**CA-ASA Interaction.**

The carbonic anhydrases are composed of SAMs of alkanethiolates on gold, the CA-ASA interaction, and SPR.

**Mixed SAMs of Alkanethiolates on Gold as Model Surfaces for Studies of Biomolecular Recognition.**

Mixed SAMs of alkanethiolates on gold presenting well-defined amounts of a ligand at an interface that otherwise consists of tri(ethylene glycol) groups satisfy three requirements: (1) they resist nonspecific adsorption of biomolecules, (2) they are easily modified synthetically to include delicate and complex functional groups, and (3) they can be prepared in ways that control the average surface density of ligands used for bioprecipitous binding.

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isozymes) to mixed SAMs consisting of 1 and 5 were: $k_{on}$ $\approx 1.9 \times 10^4$ M$^{-1}$ s$^{-1}$, $K_{off}$ $\approx 5 \times 10^{-3}$ s$^{-1}$, $K_d$ $\approx 3 \times 10^{-2}$ M$^{-1}$.

Taylor et al. proposed a two-step mechanism for binding of ASA to CA: a partitioning of the ASA group into the active site, driven primarily by hydrophobic interactions, followed by coordination of the weakly associated ASA group to the zinc ion in the active site to give the final complex (eq 1). This mechanism is based on four observations: (a) there is a strong correlation between $k_{on}$ and the octanol–water partition coefficients of substituted ASAs;13 this correlation is also observed for binding of substituted ASAs to apocarbonic anhydrase [apo-CA, the protein lacking an active site Zn(II)];13 (b) the rate of binding of ASA to apo-CA is not influenced by pH but the rate of binding of ASA to CA is influenced by pH;13 (c) the rate of binding of ASA to CA is well-described by single observable rate constants for association ($k_{on}$) and dissociation ($k_{off}$); this observation indicates that the partitioning of ASA (between aqueous solvent and the hydrophobic active site of CA) is fast compared with formation and cleavage of the sulfonamide–zinc bond; that is, $k_{on}$ $\approx K_{1}K_{2}K_{CA}$ and $k_{off}$ $\approx K_{-2}$. (d) the total charge of the CA-ASA complex is the same as that of CA in the absence of ASA (see Discussion); therefore the water molecule coordinated to the Zn(II) ion exists primarily as a hydroxide ion.

Surface Plasmon Resonance. SPR measures the intensity of reflection of monochromatic p-polarized light incident on the back side of a gold-coated glass slide. Changes in the angle $\theta$ at which the intensity of reflected light is at a minimum are related to changes in index of refraction in the region near the gold–solution interface [within approximately one-quarter wavelength ($\approx 200$ nm) of the incident light].14,15 Within a family of similar compounds (e.g., proteins), changes in $\theta$ correlate linearly with the mass per unit area of protein adsorbed.14,15 The BioCore instrument (used in this study) reports changes in $\theta$ in resonance units (RU; 10 000 RU $= 1$°). For most proteins, a change in $\theta$ of 1000 RU corresponds to a change of about 1 ng/mm$^2$ in the quantity of protein adsorbed at the surface.16 A typical binding experiment using SPR has three phases (Figure 1): (a) a buffer (e.g., phosphate buffered saline, PBS) is passed over the sensing surface; (b) the buffer is changed to a solution containing the analyte; and (c) the solution is then replaced by the original buffer. There is a rapid increase in response (as observed by an increase in RU) upon introduction of the solution of the protein; this immediate response (within $\approx 2$ s, at a flow rate of 10 $\mu$L min$^{-1}$) reflects the change in the bulk refractive index (labeled $\Delta$ RI in Figure 1) between the buffer and the solution of protein. This fast change is followed by a slower increase in response due to adsorption of the protein at the interface. At equilibrium, when the net rate of binding to the surface is zero, the response reaches a plateau. It is possible to estimate the amount of protein adsorbed at equilibrium ($R_{eq}$) by subtracting the response due to the refractive index change ($\Delta$RI) from the response at equilibrium. When the protein solution is replaced by the original PBS buffer, there is an initial fast drop in response resulting from the differences in the index of refraction of the two solutions, followed by a slower decrease in the response due to desorption of the analyte. If the response during the desorption phase does not reach the value preceding injection of the protein, there is still some protein adsorbed to the surface; this residual protein could be reversibly adsorbed or undergo slow dissociation that is incomplete in the time scale of the SPR experiment. We refer to the adsorption of the analyte (CA, in this paper) to ligands (ASA groups, in this paper) presented at the surface as the “association phase” and the desorption of the analyte from the surface as the “dissociation phase”.

The BIACore 1000 is well-suited for binding studies in which the rate constants of binding are in the range described in this report ($K_{on,obs}$ $\approx 10^{4}$–$10^{5}$ M$^{-1}$ s$^{-1}$, $K_{off,obs}$ $\approx 0.005$ s$^{-1}$). The range of relevant rates was an important factor in choosing the CA-ASA interaction as a model system to investigate the effects of lateral interactions on processes occurring at interfaces.

**Previous Work on Lateral Steric Effects.** The influence of steric effects during adsorption phenomena involving macromolecules (e.g., protein, DNA) or self-assembled structures (e.g., vesicles) has been described. Biospecific binding of proteins to ligands at surfaces has been investigated by several researchers, for example, by Tamm and Bartoldus in phosphatidylcholine vesicles, by Edwards et al. in dextran, and by Spinke et al. in SAMs of alkanethiolates on gold.19 Tamm and Bartoldus
and Edwards et al. studied the binding of antibodies to antigens. In both these studies, the binding was not adequately described by simple kinetic models and deviations were attributed to lateral steric effects. There are additional complications in studies of the kinetics of binding of antibodies to surfaces because of the influence of bivalency, and because the rapid binding of antibodies to antigen (\(k_{\text{on}} \sim 10^6\)–10 M\(^{-1}\) s\(^{-1}\)) makes the overall rate of association limited by the rate of mass transport of protein molecules from the bulk solution through the unstirred boundary layer to the surface. Spinke et al. studied the binding of streptavidin to SAMs presenting biotin groups. In this study, they observed that maximal binding of streptavidin occurred in mixed monolayers in which the biotin ligand had an appropriate spacer and was present at low surface densities. The interaction of streptavidin to biotin is essentially irreversible and therefore not amenable to estimations of \(k_{\text{on}}\) and \(k_{\text{off}}\). In this report, we describe steric effects during the reversible and monovalent binding of CA to ASA groups on a surface that is resistant to nonspecific adsorption.

A central observation of this paper is that \(k_{\text{on,obs}}\) (but not \(k_{\text{off,obs}}\)) decreases as the coverage of the surface by molecules of CA increases. An analysis of this observation requires an understanding of the system (Scheme 2). Some of the issues that are relevant to biospecific binding of proteins to SAMs are shown schematically in Scheme 2, and a discussion of lateral effects and alternative explanations that might account for the observations described in the paper are presented in the Discussion.

Conducted inside the SPR instrument by sequential partition into the dextran gel, and equilibrate by uncomplicated. In the gel, bulky molecules of analyte must at the surface of the SAM; although mass transport may SAMs consisting of auxiliary nucleophiles (e.g., ethanolamine); (c) in mixed density of ligands at the surface in chips made from 2, which can be easily manipulated by varying the ratio of 1 and 2, control of the density of ligands in the dextran gel requires manipulation of the pH of coupling, and the use of auxiliary nucleophiles (e.g., ethanolamine); (c) in mixed SAMs consisting of 1 and derivatives of 2, binding occurs at the surface of the SAM; although mass transport may influence the kinetics of binding, the system is otherwise uncomplicated. In the gel, bulky molecules of analyte must first partition into the dextran gel, and equilibrate by diffusing through the relatively thick cross-linked gel layer, before binding to ligands inside the gel.

The couplings of the ASA ligands to the mixed SAM were conducted inside the SPR instrument by sequential injections of an aqueous solution containing N-hydroxysuccinimide (NHS) (0.05 M) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (0.20 M) to transform the surface carboxylic acid groups into amino groups. Preparative thin-layer chromatography (TLC) was used to purify the NHS-esterified carboxylic acid groups. The NHS-esterified carboxylic acid groups were then coupled to ammonia-functionalized 2. As shown in (b), the formation of mixed SAMs containing 1 and 2 (the amount of nonspecific adsorption on the carboxylated dextran gel is significant); (b) the density of ligands at the surface in chips made from 1 and 2 can be easily manipulated by varying the ratio of 1 to 2, control of the density of ligands in the dextran gel requires manipulation of the pH of coupling, and the use of auxiliary nucleophiles (e.g., ethanolamine); (c) in mixed SAMs consisting of 1 and derivatives of 2, binding occurs at the surface of the SAM; although mass transport may influence the kinetics of binding, the system is otherwise uncomplicated. In the gel, bulky molecules of analyte must first partition into the dextran gel, and equilibrate by diffusing through the relatively thick cross-linked gel layer, before binding to ligands inside the gel.

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We used polarized infrared external reflectance spectroscopy (PIERS) to study the coupling of 4 to the mixed SAMs. PIERS provides information about the presence of functional groups that are detectable by IR, and also about the order and orientation of the hydrocarbon chains within a SAM. In PIERS, vibrational modes with transition dipole moments perpendicular to the surface show maximal absorbances; vibrational modes with transition dipole moments parallel or nearly parallel to the surface show minimal absorbances. Figure 3 shows PIERS spectra of the mixed SAMs [χ(2) = 0.10] in the carbonyl (C=O) stretching region before activation with NHS/EDC (a), after activation with NHS/EDC (b), and after coupling to 4 (c). We assign the band centered at 1739 cm⁻¹ to the C=O stretch of the carboxylic acid groups in 2. After treatment of the SAM with NHS/EDC, three new bands at 1748, 1789, and 1821 cm⁻¹ appear in the spectrum. On the basis of assignments by Frey and Corn for NHS esters of SAMs, we assign the peak at 1748 cm⁻¹ to an asymmetric stretch of the NHS carboxyls, the peak at 1789 cm⁻¹ to

Results

Preparation of SAMs Presenting ASA Ligands. The commercially available sensor chip for SPR consists of a layer of carboxymethyl dextran (~100 nm thick) covalently attached to a hydroxy-terminated alkane thiol monolayer on gold. To avoid kinetic and thermodynamic problems resulting from the requirement that the protein must partition into the gel layer to reach the ligand, we used a surface consisting only of mixed SAMs of alkane thiols of 1 and 2 on gold, and coupled ligands to the carboxylic acid groups of 2 by peptide bond-forming reactions (Scheme 1). These purely SAM-based chips have several advantages over the dextran gel-based chips: (a) there is almost no detectable nonspecific adsorption of proteins on SAMs consisting of 1, 2, and 4; (b) the relative amounts of immobilization (of 4) are significant); (c) in mixed SAMs consisting of 1 and 2, binding occurs with χ(2) = 0.10 (1058 RU), i.e., 1.058 ng mm⁻² and χ(2) = 0.02 (190 RU).
a symmetric stretch of the NHS carbonyls, and the peak at 1821 cm\(^{-1}\) to the carboxyl stretch of the NHS ester of 2. Upon treatment of the activated SAM with a solution containing 4 (2 mg mL\(^{-1}\)), the bands due to the NHS esters disappear, and three bands centered at 1550, 1660, and 1733 cm\(^{-1}\) appear. A mixed SAM consisting of 1 and 5 also shows bands of approximately equal intensity at 1550 and 1660 cm\(^{-1}\); on the basis of these observations, we conclude that coupling of the ligand (4) is incorporated in the SAM. We assign the band at 1550 cm\(^{-1}\) to the two NH bends, and the band at 1660 cm\(^{-1}\) to the amide C=O stretches in the coupled ligand, and the band at 1733 cm\(^{-1}\) to the C=O stretch of residual carboxylic acid groups in 2.

We have measured the increase in thickness ellipsometrically after coupling of 4 to the NHS esters of 2 in mixed SAMs (consisting of 1 and 2). We can estimate the coupling yields (% yield) by dividing this observed increase in ellipsometric thickness (\(\Delta T_{\text{obs}}\)) by the predicted increase in ellipsometric thickness (corresponding to quantitative coupling) (\(\Delta T_{\text{pred}}\)): i.e., % yield = (\(\Delta T_{\text{obs}}/\Delta T_{\text{pred}}\))\(\times\)100. The value of \(\Delta T_{\text{pred}}\) can be estimated using eqs 2 and 3, where \(\chi(2)_{\text{SAM}}\) is the mole fraction of 2 in a mixed SAM with thickness \(T_{\text{mixedSAM}}\), \(\Delta T_{2-1}\) is the difference in ellipsometric thickness (~21 Å) between a SAM

\[
\Delta T_{\text{pred}} = \chi(2)_{\text{SAM}} \Delta T_{5-2}
\]

\[
\chi(2)_{\text{SAM}} = \frac{T_{\text{mixedSAM}} - T_{1}}{\Delta T_{2-1}}
\]

consisting only of 2 (~41 Å) and a SAM consisting only of 1 (T1 ~20 Å), and \(\Delta T_{5-2}\) is the difference in ellipsometric thickness (~20 Å) between a SAM consisting only of 5 (61 Å) and a SAM consisting only of 2. At values of \(\chi(2)_{\text{SAM}} < 0.25\), the values of % yield are >80; on the basis of these observations we infer that coupling of 4 to 2 occurs in high yield.\(^6\) This estimate is qualitatively compatible with the PIES spectra (Figure 3).

We have previously described the binding of CA to SAMs of alkanethiols on gold in which the thiol presenting the ligand (5) was synthesized separately.\(^1\) The strategy of coupling of ligands to preformed SAMs made from 1 and 2 that present a common intermediate (the NHS ester of 2) for coupling of ligands\(^6\) offers a number of advantages over these in situ, independent synthesis of thiols presenting ligands: (a) organic synthesis is minimized—the only requirement is that the ligand contain at least one nucleophilic amino group; (b) the strategy permits the immobilization of delicate functional groups that might not be compatible with the manipulations required to isolate and purify thiols; (c) problems related to phase separation\(^30\) of the ligand-presenting chains are reduced relative to mixed SAMs made from thiols with different hydrophobicities. The headgroup structures (ethylene glycol groups) of 1 and 2 are similar; this similarity tends to minimize phase separation.

We shall refer to the mole fraction of ligand in the SAM by \(\chi(2)_{\text{SAM}}\) rather than \(\chi(2)_{\text{ASA}}\) because the mole fraction of the ASA groups on the surface is not explicitly measured and because the yield on coupling the ASA groups to carboxylic groups at the surface is only an estimate. We assume that \(\chi(2)_{\text{SAM}} \approx \chi(2)_{\text{ASA}}\) since experimental evidence\(^31\)–34 indicates that the composition of

\(\text{Structure and Choice of ASA Ligand.}\) To ensure that binding of CA to ASA was not hindered by proximity of the ligand to the “surface” of the SAM, we designed 2 to incorporate three more ethylene glycol groups than 1. In initial experiments, we immobilized p-aminomethylbenzenesulfonyl (3) on the mixed SAMs consisting of 1 and activated 2. At mole fractions of ASA groups that were comparable with the published report (using 5),\(^1\) we observed equilibrium response levels (\(R_{eq}\)) that were only 50% of those described earlier. Because the only difference between 3 (coupled to 2) and 5 was the number of carbon atoms in the linker (methylene versus hexamethylene), we inferred that a longer linker would be preferable. The binding of CA to mixed SAMs presenting 4 (coupled to 2 by an amide bond) gave equilibrium response levels that were similar to that observed with mixed SAMs consisting of 1 and 5 (Figure 4).\(^3\) These data suggest that binding of CA to ASA groups at a surface is dependent on the spacer length; the influence of spacer length on the binding of


proteins to ligands at surfaces has been demonstrated previously.21,35,36

Influence of Density of ASA Groups on Binding Capacity of the Surface. We examined the binding of CA to mixed SAMs consisting of 1 and 2/4 as a function of $\chi(2/4)$. At values of $\chi(2/4)$ between 0 and 0.10, increasing $\chi(2/4)$ increased the steady-state response ($R_{eq}$) due to the binding of CA (Figure 4A, B). An increase in the density of ASA groups also resulted in an increase in the amount of CA that remained adsorbed at the end of the SPR experiment (at $\sim$1450 s, see Figure 4A). The maximum value of $R_{eq}$ ($\sim$1820 RU, [CA] $\approx$ 16.6 $\mu$M) was obtained at $\chi(2/4) \approx$ 0.10. Increasing $\chi(2/4)$ further resulted in a decrease in the value of $R_{eq}$, at $\chi(2/4) \approx$ 0.25, $R_{eq} \approx$ 1500 RU (Figure 4B).

Figure 5 is a cartoon showing the binding of CA to surfaces presenting ASA groups, at $\chi(2/4) \approx$ 0.005 (Figure 5, top) and 0.10 (Figure 5, bottom). The figure is a view from the top that emphasizes the potential for adsorbed molecules of CA to shield ASA groups on the surface sterically at higher densities of ligand. The thiol molecules are shown arranged in a hexagonal pattern; the small open circles represent molecules of 1, and the closed circles represent sites occupied by 2/4. One adsorbed molecule of CA (radius $\approx$ 2.1 nm$^2$)37 covers approximately 61 molecules of coordinated alkanethiolates (see Discussion). When $\chi$- (2/4) is increased from 0.005 to 0.10 (a factor of 20), the number of molecules of CA that can be packed on the surface (without bumping into adjacent molecules of CA) is increased only two- to threefold. On the basis of the assumed cross-sectional area of CA ($\approx$ 12.5 nm$^2$), hexagonal packing, and a uniform distribution of ligands, the mole fraction of ASA required to observe the maximal amount of binding of CA is $\chi(2/4) \approx$ 0.02. We observe an increase of $\sim$400 RU ($\sim$25%) in the value of $R_{eq}$ as $\chi(2/4)$ is increased from $\sim$0.02 to 0.10; we attribute this increase to an underestimate of the amount of 2/4 on the surface, rather than to a surface density of protein higher than that predicted theoretically. The less-than-quantitative (~80%) yield in coupling of the ASA groups to the SAM is the major reason $\chi(2/4)$ might be lower than the values estimated from $\chi(2)$ in solution used to prepare the SAMs. The increased binding at $\chi(2/4) \approx$ 0.10 could also be due to nonspecific adsorption of CA to the hydrophobic ASA groups, which could influence the kinetics of dissociation; the binding is $\sim$60% and $\sim$90% reversible at $\chi(2/4) \approx$ 0.10 and $\chi(2/4) \approx$ 0.02 respectively (Figure 4A).

Kinetics of Binding of CA to ASA. We describe the kinetics of binding in three sections. We limit our discussion of kinetics to low mole fractions of ASA groups [$\chi(2/4) \leq 0.05$], where the binding of CA is highly reversible. The first section briefly summarizes general aspects of the kinetics of binding and the equations used to estimate $k_{on,obs}$ and $k_{off,obs}$. The second section discusses the influence of coverage of the surface by CA and density of ligand on $k_{off,obs}$; the third section describes the influence of these factors on $k_{on,obs}$.

(1) Equations of Rates of Dissociation and Association. We use $k_{on}$ to refer to the rate constant that describes the second-order formation of the complex by association of CA and ASA, and $k_{off}$ to describe its first-order dissociation; we use $k_{on,obs}$ and $k_{off,obs}$ in these rate equations) when we express the surface density of the complex (CA-ASA) in terms of the SPR response, $R_t$.

We first discuss kinetics during the dissociation phase. The binding of CA to ASA was reversible at low densities of ligand [$\chi(2/4) \leq 0.05$]. The rate of dissociation of the ASA-CA complex, (CA-ASA)*, on the surface into free CA can be expressed by a first-order rate equation (eq 4): we calculated the value of $k_{off,obs}$ over the entire dissociation phase by fitting the exponential decay in the response to

$$d[CA\cdot ASA]/dt = dR_t/dt = -k_{off,obs} R_t$$ (4)

versus Density of ASA Groups.

Extent of Coverage of the Surface by CA and on the injection of CA over the surface. Even at the low density of ligand, there is a change in the surface coverage by CA. At very low values of $\chi(2/4) \sim 0.005$, the value of $k_{on,obs}$ stays approximately constant at $\sim 35 \times 10^3 \text{M}^{-1}\text{s}^{-1}$, with the value decreasing with increasing coverage of the surface by CA.

We define $P_i$ as the amount of protein adsorbed in nanograms per square millimeter; $P_i$ can be estimated by dividing the observed response, $R_i$, by 1000. The fractional coverage of the surface by adsorbed CA can then be estimated from $P_i/R_{max}$ where $P_{max}$ is the maximum amount of protein that can be packed per square millimeter.
on the surface of the monolayer. Equation 10 estimates the value of \( P_{\text{max}} \) (\( P_{\text{max}}^{\text{est}} \)): here, \( r \) is the radius

\[ P_{\text{max}}^{\text{est}} \approx \frac{10^{21}}{\pi r^2 M_W} \]  

(2.1 nm) of CA with a projected area of \( 2 \pi r^2 \) in the plane of the monolayer, \( N_A \) is Avogadro’s number, and \( M_W \) is the molecular weight of CA (30 000). For CA, \( P_{\text{max}}^{\text{est}} \approx 3.6 \) ng mm\(^{-2} \) (equivalent to a response of \( \approx 3600 \) RU).  

Figure 7B is a plot of \( k_{\text{on,obs}} \) versus \( P_{\text{max}}^{\text{est}} \); the value of \( k_{\text{on,obs}} \) decreases from \( \approx 3.5 \times 10^9 \) M\(^{-1}\) s\(^{-1} \) to \( \approx 4 \times 10^7 \) M\(^{-1}\) s\(^{-1} \) as \( P_{\text{max}}^{\text{est}} \) increases from \( \approx 0.15 \) to \( \approx 0.35 \). These data establish that the value of the observed rate constant of association decreases with the amount of protein adsorbed on the surface.

**Discussion**

We have used an experimental model system based on CA and immobilized ASA groups to explore deviations from ideal kinetic behavior. The most important observation is that at low densities of ASA groups where the binding of CA is highly reversible [e.g., at \( \gamma(2/4) \approx 0.02 \)], values of \( k_{\text{on,obs}} \) but not \( k_{\text{off,obs}} \) decrease as the amount of CA adsorbed on the surface increases. One hypothesis that explains this result is that the decrease in \( k_{\text{on,obs}} \) reflects lateral steric interactions between molecules in solution and those on the surface, and among molecules of CA adsorbed on the surface (Scheme 2, i, j).

The number of lattice sites, \( n \), covered by a spherical protein molecule of radius \( r_{\text{protein}} \), adsorbed on a hexagonal lattice characterized by a lattice vector of length \( a \), is given by eq 11,\(^{39} \) in which \( k = r_{\text{protein}}/a \). For CA (radius 2.1 nm) adsorbed on a surface having lattice sites arranged with the geometry of an alkanethiolate SAM adsorbed on planar Au (111), \( a \approx 5 \) Å, \( k \approx 4 \), and \( n \approx 61 \). Although each molecule of CA physically covers

\[ n = 3k(k + 1) + 1 \]  

\( \approx 60 \) lattice sites, there are additional excluded sites (shaded area in Figure 8) within a circle of radius 2k; we refer to these sites as excluded (that is, not available to a molecule of CA in solution) because they would require the centers (but not the peripheries) of two molecules of adsorbed CA to approach distances less than their van der Waals surfaces. Taking this additional surface into account, an isolated adsorbed molecule of CA “shields” approximately 217 lattice sites.

A graph that shows the qualitative dependence of the fraction of unshielded ASA groups on the fraction of lattice sites with molecules of coordinated CA is shown in Figure 8. At low coverage of the surface with CA, the excluded areas of different molecules of protein on average do not overlap, and each molecule of adsorbed CA shields an area \( A(2k) \) (or \( \approx 217 \) lattice sites). For uniformly distributed ASA ligands at \( \gamma(2/4) \approx 1/217 \approx 0.005 \), the ASA ligands are sufficiently far apart that each molecule of adsorbed CA shields no more than one ASA ligand and [216 (EG)]OH groups. Under these circumstances, the measured rate of association should be free of artifacts due to lateral steric effects, and therefore (in the absence of other effects) accurately represent the kinetics of the adsorption reaction at the interface. For binding of CA to mixed SAMs with \( \gamma(2/4) \approx 0.005 \), the value of \( k_{\text{on,obs}} \) remains approximately constant (Figure 7A, inset). At intermediate coverages, the adsorbed molecules of CA are closer together and share their excluded areas: therefore, each protein shields on average less than \( A(2k) \) lattice sites. The maximum coverage of CA at the surface (corresponding to no additional shielded area except that physically covered by CA) is achieved when molecules of CA are arranged in a close-packed hexagonal manner with their centers 2k away from each other. At this coverage, the fraction of lattice sites with molecules of coordinated CA is only 0.0016 (Figure 8). Experimentally, the maximum fractional coverage we observe for binding of CA to ASA groups (at \( \gamma(2/4) \approx 0.10; [CA] = 16.6 \mu M \)) is \( \sim 0.5 \). The maximum coverage achieved for an interaction depends on the value of \( K_d \) and the values of \( k_{\text{on}} \) and \( k_{\text{off}} \) relative to the time scale of the experiment; fast on/off rates lead to greater lateral shuffling and therefore more efficient packing than binding that is irreversible or is characterized by slow on/off rates.\(^{38} \)

In Table 1 we list some reported values of \( K_d \), \( k_{\text{on}} \), and \( k_{\text{off}} \) for the interaction between CA and derivatives of benzenesulfonamide. Measurements based on affinity

\(^{38} \) The maximum SPR signal we observe for binding of CA to ASA groups is only \( \sim 1800 \) RU (Figure 4). The maximum coverage that is expected with a mechanism for adsorption based on irreversible random adsorption is \( \sim 35\% \); for reversible adsorption the maximum coverage is determined by the surface density of ligand and the dissociation constant (see Jin, X.; Talbot, J.; Wang, N.-H. L. AIChE J. 1994, 40, 1685–1696).

capillary electrophoresis, \cite{40-42} stopped flow fluorescence quenching, \cite{13} and the inhibition of enzymatic activity (CO2 hydration) \cite{43} are all solution-based and therefore free of some of the artifacts (e.g., lateral effects) inherent to measurements of binding of biomolecules to surfaces. Analysis of SPR data is complicated (see below) but offers the convenience of direct monitoring of rate constants for both association and dissociation, and for equilibrium binding.

We have hypothesized that lateral interactions between molecules of CA and ASA groups and among molecules of CA are responsible for the decrease we observed in $k_{\text{on,obs}}$. We also need to consider other possible origins for this decrease and discuss some possibilities below, as outlined before in Scheme 2. The arguments we present pertain only to low mole-fractions of $\gamma$; binding of CA to these surfaces is reversible within the time scale of the experiment and is described by a single observed rate constant of dissociation. For many of the reasons described below, it would be difficult to distinguish between these mechanisms and lateral steric effects at high surface densities of ASA groups.

(1) Partitioning of Ligand (b) and Adsorbed CA (g) Between Solution and Ethylene Glycol Layer.

The (EG)$_3$ groups at the termini of the SAM form a packed layer \cite{44} that is sufficiently dense that molecules of protein would not be able to penetrate it; although interaction of protein with the outer layer is, in principle, possible, it is unlikely because proteins do not adsorb on (EG)$_3$ layers. \cite{8} There is, however, probably enough free volume in the (EG)$_3$ layer that a small group such as ASA might partition into it. If adsorption of CA on the surface of the SAM resulted in a change in the structure and the free volume of the (EG)$_3$ layer, this change might be reflected in a change in the availability of remaining ASA groups. Because the density of ASA groups at the surface influences $k_{\text{on,obs}}$ perturbations in the availability of ASA groups for binding could lead to a change in $k_{\text{off,obs}}$. In the absence of any experimental evidence regarding differences in the partitioning of ASA groups into the (EG)$_3$ layer with and without adsorbed CA, we cannot rule out the possible influence of this mechanism on $k_{\text{on,obs}}$.

(2) Nonbiospecific Interaction of CA with ASA Ligands at the Surface (e). Figure 4 shows that binding is less reversible (or is kinetically slower) at higher densities of ASA ligands relative to binding at lower densities. We assume that increasing irreversibility reflects secondary, nonbiospecific binding between CA and proximal hydrophobic ASA ligands. Lateral interactions between CA and ASA groups would lower the fraction of those ASA groups available for biospecific interaction with additional molecules of CA, or increase the activation

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Figure 8. A curve showing the qualitative dependence of the fraction of ASA groups available for binding on the fraction of lattice sites (occupied by 1 and 2/4) that contain coordinated molecules of CA (ASA-CA). Schematic views from the top showing adsorption of molecules of CA to SAMs presenting ASA groups, with the alkanethiolate molecules (\textbf{1} = small open circles; \textbf{2/4} = small closed circles) arranged on a hexagonal lattice, with a lattice vector of length $a$. We define: $k = r_{\text{protein}} / r_{\text{CA}}$, where $r_{\text{protein}}$ is the radius of CA; $k = 4$. Each molecule of adsorbed CA physically covers an area equal to $A(k)$, and "excludes" an additional area (shown as the shaded area) within a circle with area $A(2k)$. The consideration of this virtual shielded area helps define the limiting value of $\gamma$ below which no lateral effects should be observed. There are three basic regions in the curve. The first region (A) occurs at very low coverages of the surface by CA, in which on average there is no overlap of excluded areas by molecules of adsorbed CA (each molecule of CA shields an area equal to $A(2k)$, corresponding to 217 lattice sites). The second region (B) occurs at intermediate coverages, in which molecules of adsorbed CA share their excluded areas (molecules of CA shield areas between $A(2k)$ and $A(k)$, corresponding to 217 and 61 lattice sites respectively). The third region (C) of the curve refers to maximum coverage in which molecules of CA are adsorbed in a hexagonal close-packed manner (molecules of CA are separated by a center-to-center distance equal to 2$k$, and shield an area equal to $A(k)$, corresponding to 61 lattice sites); this arrangement is probably not achieved experimentally (see ref 38).
energy (or lower the free energy of association) for biospecific binding by requiring the initial dissociation of nonspecific complexes. Because this type of lateral interaction will parallel lateral interactions between molecules of CA, it is difficult to distinguish one from the other; therefore we limit our kinetic analysis only to surfaces presenting low surface densities of ASA groups where $k_{off,obs}$ remains constant over the dissociation phase.

(3) Changes in $pK_a$ of ASA Group Due to Changes in Local Environment during Binding of CA (f). The binding of CA to the ASA groups results in the expulsion of water molecules from the surface of the SAM; this exchange of water molecules for molecules of protein may result in a local decrease in the dielectric constant of the medium at the interface. On the basis of the Born model of solvation of ions, an ion has a more favorable free energy of solvation in a medium of high dielectric constant than in a medium of low dielectric constant; thus, in principle, the ASA group should be less prone to ionization (deprotonation) in a medium of low dielectric consisting of molecules of adsorbed protein than it is in aqueous buffer (f). To determine if the total charge of the CA-ASA complex is the same in the absence of ASA, we measured the electrophoretic mobility of CA by capillary electrophoresis. We observed no change in the electrophoretic mobility of CA (relative to 4-methoxybenzyl alcohol as a neutral marker) when the buffer also contained the ASA ligand 6 (at $\sim 700$ $\mu M$). Therefore, the total charge of the CA-ASA complex is the same as that of CA in the absence of ASA, which implies that the change in the free energy of deprotonation of the ASA group is irrelevant; the change in free energy of deprotonation of the ASA group is compensated by the change in the free energy of protonation of the hydroxyl ion that is displaced (eq 1). Moreover, if even there was a change in the charge of CA upon binding to ASA, changes in the $pK_a$ of the ASA group would also affect $k_{off}$, which is contrary to experimental observation.

(4) Mass Transport Limited Association and Dissociation (d). An increase in $k_{off,obs}$ at lower densities of ligand on the surface can indicate that binding is limited by mass transport. We first consider a theoretical analysis of the influence of mass transport on the observed rates of association and dissociation using an approach adapted from Glaser. The driving force for diffusion, and therefore the flux $j_b$ (in moles per second per square meter), is proportional to the difference in the concentration of the receptor in the bulk solution, $c_b$ (in moles per liter) and the concentration of the free receptor at the surface, $c_s$ (in moles per liter) (eq 12). Mass transport will influence the observed rates of association and dissociation when $c_s$ is lower than $c_b$. The driving force for binding, and therefore the flux $j_b$ (in moles per second per square meter), is proportional to the difference between $c_b$ and the concentration of receptor in solution that is in equilibrium with ligand on the surface, $c_{eq}$ (in moles per liter) (eq 13). The proportionality constants in eqs 12 and 13 that relate fluxes to differences in concentrations are referred to as Onsager coefficients: $L_m$ (in meters per second) is the Onsager coefficient of mass transport; $L_r$ (in meters per second) is the Onsager coefficient of reaction flux. Conservation of mass requires that $j_b = -j_d$: when $L_m \gg L_r$, the flux is limited by $j_d$, and the observed rates of association and dissociation reflect the intrinsic kinetics of binding; when $L_r \gg L_m$ the flux is limited by $j_b$, and the observed rates reflect the effects of mass transport.

$$j_d = -L_m(c_b - c_{fs})$$
$$j_b = L_r(c_{fs} - c_{eq})$$

An estimate of the effects of mass transport requires an estimate of the values of the Onsager coefficients, $L_m$ and $L_r$. The value of $L_m$ can be estimated from Fick's first law, given in eq 14, where $D$ is the coefficient of diffusion ($\sim 1 \times 10^{-10}$ m$^2$ s$^{-1}$, for CA) and $\partial c/\partial z$ is the value of the gradient of concentration, which, in turn, may be estimated from the values of $c_b$, $c_s$, and the thickness of the diffusion boundary layer, $d$. One estimate of $d$, on the basis of the work of Leveque (ref 16 of the paper by Glaser), is given in eq 15, where $f$ is the rate of flow (0.3 $\mu L$ s$^{-1}$); $h_0$ and $w$ are the height (50 $\mu m$), width (300 $\mu m$), and length (800 $\mu m$), respectively, of the flow cell. The value of $L_m$ using this estimate of $d$ is expressed in eq 16. We estimate $L_m \approx 1.5 \times 10^{-5}$ m s$^{-1}$.

$$j_d = -D \frac{\partial c}{\partial z} \approx -D \frac{(c_b - c_{fs})}{d}$$
$$d \approx \sqrt{\frac{D h_0 w}{f}}$$
$$L_m \approx \frac{3}{2} \sqrt{\frac{D f}{h_0 w}}$$

The value of $L_r$ is estimated from the expression for $j_b$, given in eq 17, where $c_{fs}$ (in moles per square meter) is the density of ligands on the surface that are bound to receptors, $g_s$ (in moles per square meter) is the density of free ligands on the surface, and $L_m$ and $L_r$ are the rate constants for association and dissociation, respectively. Factoring out the term $k_{off}$, $g_s$, and recognizing that $k_{off} g_s / k_{off} g_s = c_{eq}$, gives eq 18, yielding the expression for $L_r$ (eq 19).

$$j_b = \frac{\partial g_s}{\partial t} = k_{on} c_{fs} g_r - k_{off} g_s$$
$$j_b = k_{on} g_s (c_{fs} - k_{off} g_s / k_{on} g_r) = k_{on} g_s (c_{fs} - c_{eq})$$

Equation 19 implies that mass transport is most prominent at the beginning of the association phase and toward the end of the dissociation phase when the density of unoccupied sites ($g_r$) is highest. We next estimate $L_r$ at the beginning of the association phase when the value of $L_r$ is a maximum. At this stage, the observed rate of binding is most limited by mass transport; the estimation of the maximum value of $L_r$ provides the lower limit of $L_m L_r$.

We first estimate the density of ASA groups at the surface on the basis of the observed amount of CA that adsorbs. When the mole fraction of ASA groups at the surface is low, it is possible to estimate the molar density of ASA groups (in moles per square meter) at the surface by calculating the molar density of adsorbed CA (in moles per square meter): that is, $1$ RU $\sim 10^{-6}$/MW of CA (in moles per square meter); $MW$ is the molecular weight of

CA, ~ 30 000). For example, at \( \chi(2/4) \approx 0.005 \), the maximal binding capacity of the surface is \( \approx 400 \) RU; this number corresponds to a molar density (for CA) of \( 1.3 \times 10^{-8} \) mol m\(^{-2}\). If we assume that maximal binding corresponds to binding of one molecule of CA to every ASA ligand (1:1 binding), a reasonable assumption at \( \chi(2/4) \approx 0.005 \), the total density of ASA ligands at the surface is approximately \( 1.3 \times 10^{-8} \) mol m\(^{-2}\). Substituting this number and the value of \( k_{\text{on,obs}} \) (\( \approx 3.5 \times 10^{4} \) M\(^{-1}\) s\(^{-1}\)) in eq 19 allows us to calculate the maximum value of \( L_s \) at any mole fraction of ligand. Assuming that the molar density of ligands (in moles per square meter) is proportional to the mole fraction of the ligand, we calculate: \( L_s \approx 4.55 \times 10^{-7} \) mol m\(^{-2}\) at \( \chi(2/4) \approx 0.005 \); \( L_s \approx 18.2 \times 10^{-7} \) mol m\(^{-2}\) at \( \chi(2/4) \approx 0.02 \).

At the beginning of the association, at \( \chi(2/4) \approx 0.02 \), the value of \( L_s \) is \( \approx 0.02 \), and hence the observed kinetics is not limited by mass transport. The value of \( k_{\text{on,obs}} \) decreases with coverage of the surface by CA at this mole fraction (Figure 7). We conclude that this decrease is not due to the effects of mass transport. We also note that a decrease in \( k_{\text{on,obs}} \) with coverage of the surface by CA is opposite to the expected increase in \( k_{\text{on,obs}} \) with coverage that is expected if mass transport effects were dominant (eq 7, 19).

(i) Variations in Flow Rate. We do not observe an increase in the rates of association and dissociation by increasing the flow rate (between 0.08 and 1.7 \( \mu L \) s\(^{-1}\), which results in a change in \( L_s \), of \( 9.6 \times 10^{-6} \) to \( 2.7 \times 10^{-5} \) ms\(^{-1}\) (eq 16).

(ii) Analysis of Dissociation Phase. We do not observe any significant decrease in \( k_{\text{off,obs}} \) during the course of the dissociation. If the interaction were limited by mass transport, we would have expected a decrease in the observed value of \( k_{\text{off,obs}} \) over the course of the dissociation due to rebinding of molecules of CA to the increasing number of free ASA groups at the surface. Plots of \( k_{\text{off,obs}} \) versus time (Figure 6) show less than a 5% decrease in its value.

(iii) Analysis of Dissociation Phase. Addition of Soluble Ligand. It is possible to infer effects due to mass transfer experimentally by the addition of soluble ligand during the dissociation phase.24 This experimental technique is the most commonly used method for detecting interactions limited by mass transport, because it is more sensitive to the effects of mass transport than the method of variations in flow rate (L\(_m\) has cube root dependence on f, eq 16).24,25 If the interaction is limited by mass transport, the presence of soluble ligand leads to faster rates of dissociation. The increase is due to the capture of the dissociated molecules of analyte near the surface; this competitive interaction prevents rebinding to the surface. Figure 9 shows the dissociation phases from sensorgrams at \( \chi(2/4) \approx 0.02 \) obtained in the presence and absence of soluble ligand (100 \( \mu M \) p-carboxybenzenesulfonamide, \( K_d \approx 2.2 \mu M \)). We observe that the rate of dissociation of CA is unaffected by the presence of p-carboxybenzenesulfonamide in the buffer. These data establish that the interaction of CA and ASA is not limited by mass transport at low surface densities of ASA groups.

Conclusion

The binding of CA to mixed SAMs presenting ASA groups was chosen as a model system to study lateral steric effects. At low surface densities of ASA groups, this model system is not complicated by issues such as nonspecific binding, divalency, irreversible adsorption, and mass transport. We observed that the value of the association constant \( (k_{\text{on,obs}}) \) decreased ~10-fold as the fractional coverage of the monolayer by CA increased from 0.15 to 0.35; we did not observe any corresponding decrease in the value of the dissociation constant \( (k_{\text{off,obs}}) \). We hypothesize that these observations reflect lateral steric effects.

One explanation of this effect is that each molecule of biospecifically adsorbed CA shields proximal ASA ligands and prevents their binding to incoming molecules of CA; this shielding decreases the concentration of free ASA groups and results in an apparent decrease in the value of the association constant. This effect would thus be a statistical effect: the available surface density of ASA ligands decreases during adsorption of CA substantially more rapidly than the increase in the surface density of CA, provided the surface density of ASA groups is high (but not too high to lead to additional complications). Although this explanation is compatible with the experimental data, and although we chose this system to study lateral effects because of its apparent simplicity, we cannot eliminate some other possibilities that could also account for these observations: lateral interactions of ASA groups with themselves or with bound molecules of CA, partitioning of ASA groups into the (EG)\(_n\) layer, etc.

This study illuminates some of the complications that are inherent in studies of the kinetics of biospecific binding of proteins to surfaces. The work also demonstrates the advantages of using mixed SAMs of alkanethiolates on gold-presenting ligands and (EG)\(_n\)OH groups for studies of fundamental aspects of biomolecular recognition at surfaces. Even with this structurally well-defined system, the complexities in the analysis are substantial. We suspect that a corresponding analysis with an experimental system based on dextran gels would be intractably difficult, because it introduces yet further issues in partitioning and mass transport.

Experimental Section

Materials. Bovine CA II was obtained from Worthington. Sodium dodecyl sulfate (SDS) was obtained from BioRad. NHS, EDC, and p-aminoethylbenzenesulfonamide were purchased from Aldrich. Compounds 1 and 6 were prepared as described previously.7,8,26

Preparation of Gold Substrates Presenting Mixed SAMs. Gold substrates were prepared by evaporating thin films of titanium (1.5 nm, to promote adhesion of gold to glass) and gold (40 nm) onto glass coverslips (0.2 mm, no. 2, Corning, used as
obtained. Stock solutions of thiols 1 and 2 (2 mM in ethanol) were combined in different ratios in glass scintillation vials. The gold substrates were incubated in the solutions of thiols for 8–12 h, rinsed with ethanol, dried under a stream of nitrogen, glued into cartridges (commercial CM5 cartridges from BIACore from which the entire gold-coated glass slide bearing the dextran is peeled off with a razor blade), and then docked into the BIACore instrument. These substrates were used within 24 h. For repetitions of experiments, fresh solutions of mixed thiols were made and used within 24 h.

**Buffers and Solutions.** All solutions were passed through 0.22-μm filters (Acrodisc, HT Tuffryn membrane). PBS (10 mM phosphate, 138 mM NaCl, and 2.7 mM KCl) was prepared in distilled, deionized water. Solutions of NHS (0.10 M) and EDC (0.4 M) were prepared in distilled, deionized water. p-polarized light was collected using a Beckman P/ACE 5500 system for CE. The procedures for immobilization of ASA ligands for SPR were similar to that described for the ellipsometric measurements.

**PIERS.** PIERS spectra were obtained in single reflection mode using a dry, nitrogen-purged Digilab Fourier transform infrared spectrometer (BioRad, Cambridge). The p-polarized light was incident at 80° relative to the surface normal of the substrate and a mercury–cadmium–telluride detector cooled with liquid nitrogen was used to detect the reflected light. A bare gold substrate similar to the one described for the ellipsometric measurements was used as a reference. Typically, 1024 scans were averaged to obtain spectra shown in this paper. The coupling procedure was similar to that described for the ellipsometric measurements.

**Capillary Electrophoresis (CE).** Electropherograms were collected using a Beckman PACE 5500 system for CE. The general conditions used were: operating voltage of 15 kV; temperature, 25 °C (maintained by liquid cooling); total length of capillary, 47 cm; inner diameter, 50 μm. The buffer used for electrophoresis was 25 mM Tris and 192 mM Gly (pH 7.4), with or without the ligand 6 dissolved in the buffer (694 μM). The capillary was flushed with 0.1 N NaOH, distilled water, and electrophoresis buffer for 2 min before each experiment.

**Procedures for Immobilization of ASA Ligands for Studies by SPR.** The BIACore 1000 is equipped with an autosampler and can be programmed for automatic handling of samples. All operations including mixing of solutions, activation of the surface, coupling by peptide bond-forming reactions, and quenching of unreacted active ester groups were carried out automatically and sequentially using programs for immobilization of ligands to surfaces. A flow rate of 5 μL min⁻¹ was used throughout the immobilization.

**Vials containing solutions of NHS (0.05 M in water), EDC (0.20 M in water), the ligand (3, 4) (6.5 mM in pH 8 phosphate buffer), and pH 8.6 phosphate buffer were put (in separate positions of the autosampler tray. Equal volumes of the NHS and EDC solutions were combined in an empty vial in the autosampler tray by using the DILUTE command (described in the instruction manual for the BIACore 1000).

**Measuring Binding of CA to ASA Groups by SPR.** The adsorption of CA to the surface was measured by allowing a solution of buffer (PBS) to flow through the cell for 5 min, substituting a solution of CA (at concentrations of 16.6, 8.30, 4.15, 2.08, 1.04, and 0.52 μM) in the same buffer for 5 min, and then replacing it with the original buffer for 15 min (Figure 1). After each binding experiment, residual amounts of adsorbed CA were removed by flowing a solution of buffer (PBS, 10 mg mL⁻¹ in PBS) over the surface at a flow rate of 10 μL min⁻¹ for 5 min.

**11-[19-Carboxymethyl(hexa(ethylene glycol)]lundec-1-yl-thiol (2).** A solution of CaS(CH₂)₁₁O(CH₂)₂O₂CaH₂CO₂Me (200 mg, 0.34 mmol) in MeOH (2 mL) and EDC (35 mg) in MeOH (8 mL), both separately purged with Ar for 30 min, were mixed together. After stirring under argon for 12 h at room temperature, acetic acid (180 μL) was added by syringe to neutralize the reaction mixture. The solvent was evaporated and the crude residue was purified by flash chromatography (SiO2, 88:10:2 chloroform/methanol/acetic acid), giving 2 (105 mg, 58%). 1H NMR (300 MHz, acetone-d₆): 4.00 (s, 2H), 3.7–3.5 (m, 24 H), 3.42 (t, J = 6.5 Hz, 2H), 2.50 (dt, J = 7.8, 7.0 Hz, 2H), 1.67 (t, J = 7.8 Hz, 1H), 1.6–1.3 (br s, 18 H), HRMS (FAB⁺): 549.3090 [calcd for C₂₃H₄₅O₅N₂Na (M + Na): 549.3073].

**4-Aminohexamethylene sulfamic acid (4).** The benzenesulfonamide ligand 5 was prepared by coupling the NHS ester of 4-carboxybenzenesulfonyl chloride with mono-Boc-protected 1,6-hexanediamine, followed by deprotection with 50% trifluoroacetic acid in CH₂Cl₂ (2 h, room temperature). 1H NMR (400 MHz, acetone-d₆): 8.43 (br, 2H), 8.19 (t, J = 5.4, 1H), 8.01 (d, J = 8.2, 2H), 7.94 (d, J = 8.2, 2H), 6.92 (br, 2H), 3.81 (m, 2H), 3.41 (m, 2H), 1.82 (m, 2H), 1.45 (m, 4H). MS (FAB⁺): 300 (M⁺, calcd for C₁₃H₁₂N₂O₅S).

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