Determination of Kinetic Parameters for Interfacial Enzymatic Reactions on Self-Assembled Monolayers

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This paper reports a method to characterize the kinetic constants for the action of enzymes on immobilized substrates. This example uses cutinase, a serine esterase that hydrolyzes 4-hydroxyphenyl valerate moieties that are immobilized on a self-assembled monolayer of alkanethiolates on gold. The product of the enzyme reaction is a hydroquinone, which is redox active and therefore permits the use of cyclic voltammetry to monitor the extent of reaction *in situ*. A kinetic model based on the Michaelis−Menten formalism is used to analyze the dependence of initial rates of reaction on both the substrate density and the enzyme concentration. The resulting value of k_{cat}/K_M for the interfacial reaction is comparable to that for a homogeneous phase reaction with a substrate of similar structure. This strategy of using monolayers presenting substrates for the enzyme and cyclic voltammetry to measure reaction rates provides quantitative and real-time information on reaction rates and permits a level of analysis of interfacial enzyme reactions that to date has been difficult to realize.

Introduction

The binding of proteins and the action of enzymes at interfaces is common. Several classes of enzymes, including cellulases, lysozymes, and receptor tyrosine kinases, have evolved to act on substrates that are localized at interfaces.[1−4] Many applications in bioanalytical chemistry utilize solid-phase assay formats,[5−12] including gene microarrays,[13,14] protein chips,[15−19] and biosensors,[20−22] leading to a renewed significance in understanding the fundamental aspects of biomolecular interactions at interfaces.[23,24] Recent examples have shown that interfacial reactions can differ in substantial ways from the corresponding homogeneous phase reactions, with differences in substrate specificities,[25] cooperative interactions with the surface,[26,27] and rebinding and diffusion,[28−30] all of which can affect the rates of the reactions. Unlike studies of enzymes that act on soluble substrates, for which there exist well-developed and standard methods to compare the activities of a family of enzymes, studies of enzymes acting on immobilized substrates generally do not provide quantitative measures of the microscopic constants that govern enzyme activity. Comparisons of activities, for example, are often based on the extent of reaction (or the analogous initial rate of reaction) under a limited set of conditions, providing comparisons that do not strictly reflect the intrinsic reactivities of the enzymes. This limitation is largely due to a lack of experimental systems that can give quantitative information on kinetics.

Among the significant recent work in this area is a study by Gast and co-workers that used UV−vis spectrophotometry to determine the reaction kinetics of collagenase acting on immobilized peptides.[30−32] This study found that the overall rate depends on both the intrinsic catalysis rates of the enzyme and the diffusion of the enzyme on the surface. In another study these

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(1) Bayer, E. A.; Belaich, J. P.; Shoham, Y.; Lamed, R. *Annu. Rev. Microbiol.* **2004**, *58*, 521−554.
(2) Boraston, A. B.; Bolam, D. N.; Gilbert, H. J.; Davies, G. J. *Biochem. J.* **2004**, *382*, 769−781.
(3) Ingram, J. L.; Bonner, J. C. *Curr. Mol. Med.* **2006**, *6* (4), 409−421.
(4) Watanabe, K. *Appl. Microbiol. Biotechnol.* **2004**, *63* (5), 520−526.
(5) Altreuter, D. H.; Dordick, J. S.; Clark, D. S. *Biotechnol. Bioeng.* **2003**, *81* (7), 809−817.
(6) Borchardt, A.; Still, W. C. *J. Am. Chem. Soc.* **1994**, *116*, 373−374.
(7) Humphrey, C. E.; Turner, N. J.; Easson, M. A. M.; Flitsch, S. L.; Ulijn, R. V. *J. Am. Chem. Soc.* **2003**, *125*, 13952−13953.
(8) Tolborg, J. F.; Petersen, L.; Jensen, K. J.; Mayer, C.; Jakeman, D. L.; Warren, R. A. J.; Withers, S. G. *J. Org. Chem.* **2002**, *67*, 4143−4149.
(9) Ulijn, R. V.; Baragana, B.; Halling, P. J.; Flitsch, S. L. *J. Am. Chem. Soc.* **2002**, *124*, 10988−10989.
(10) Houseman, B. T.; Mrksich, M. *J. Org. Chem.* **1998**, *63*, 7552−7555.
(11) Dressman, D.; Yan, H.; Traverso, G.; Kinzler, K. W.; Vogelstein, B. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (15), 8817−8822.
(12) Bosma, A. Y.; Ulijn, R. V.; McConnell, G.; Girkin, J.; Halling, P. J.; Flitsch, S. L. *Chem. Commun.* **2003**, No. 22, 2790−2791.
(13) DeRisi, J. L.; Iyer, V. R.; Brown, P. O. *Science* **1997**, *278* (5338), 680−686.
(14) Ramsay, G. *Nat. Biotechnol.* **1998**, *16* (1), 40−44.
(15) Kwon, Y.; Han, Z.; Karatan, E.; Mrksich, M.; Kay, B. K. *Anal. Chem.* **2004**, *76*, 5713−5720.
(16) Arenkov, P.; Kukhtin, A.; Gemmell, A.; Voloshchuk, S.; Chupeeva, V.; Mirzabekov, A. *Anal. Biochem.* **2000**, *278* (2), 123−131.
(17) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293* (5537), 2101−2105.
(18) Lee, Y.-S.; Mrksich, M. *Trends Biotechnol.* **2002**, *20* (12, Suppl.), S14−S18.
(19) Houseman, B. T.; Mrksich, M. *Trends Biotechnol.* **2002**, *20* (7), 279−281.
(20) Jena, B. K.; Raj, C. R. *Anal. Chem.* **2006**, *78*, 6332−6339.
(21) Jena, B. K.; Raj, C. R. *Chem-Eur. J.* **2006**, *12* (10), 2702−2708.
(22) Wang, Z. X.; Levy, R.; Fernig, D. G.; Brust, M. *J. Am. Chem. Soc.* **2006**, *128*, 2214−2215.
(23) Halling Peter, J.; Ulijn Rein, V.; Flitsch Sabine, L. *Curr. Opin. Biotechnol.* **2005**, *16* (4), 385−392.
(24) Gutierrez, O. A.; Chavez, M.; Lissi, E. *Anal. Chem.* **2004**, *76*, 2664−2668.
(25) Horan, N.; Yan, L.; Isobe, H.; Whitesides, G. M.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (21), 11782−11786.
(26) Cooper, M. A.; Fiorini, M. T.; Abell, C.; Williams, D. H. *Bioorg. Med. Chem.* **2000**, *8* (11), 2609−2616.
(27) Rao, J.; Yan, L.; Xu, B.; Whitesides, G. M. *J. Am. Chem. Soc.* **1999**, *121*, 2629−2630.
(28) Lagerholm, B. C.; Thompson, N. L. *J. Phys. Chem. B* **2000**, *104*, 863−868.
(29) Lieto, A. M.; Lagerholm, B. C.; Thompson, N. L. *Langmuir* **2003**, *19*, 1782−1787.
(30) Gaspers, P. B.; Robertson, C. R.; Gast, A. P. *Langmuir* **1994**, *10*, 2699−2704.
(31) Gaspers, P. B.; Gast, A. P.; Robertson, C. R. *J. Colloid. Interface Sci.* **1995**, *172* (2), 518−529.
(32) Trigiante, G.; Gast, A. P.; Robertson, C. R. *J. Colloid Interface Sci.* **1999**, *213* (1), 81−86.

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workers used surface plasmon resonance (SPR) spectroscopy and surface plasmon enhanced fluorescence (SPEF) to determine the reaction rates of several mutants of the subtilisin serine protease on immobilized bovine serum albumin (BSA).[33] Through fluorescent labeling of the BSA protein, this method could simultaneously monitor the total amount of protein at the surface (enzyme and substrate) and the cleavage of substrate, providing adsorption and reaction rates of the protease on the substrate. In a related study, Corn and co-workers used surface plasmon resonance imaging (SPRI) and SPEF to study the reaction of ribonuclease H on RNA−DNA heteroduplexes.[34] An interesting example comes from Okahata and co-workers, who used a quartz crystal microbalance (QCM)[35,36] to study the action of phosphorylase *b* on immobilized amylopectin. An inactive form of the enzyme binds to the substrate, giving an increase in mass that is detected by the QCM. On activation by adenosine monophosphate (AMP) the enzyme degrades the substrate, which results in a decrease in mass that again can be monitored using QCM, providing kinetic parameters associated with the enzyme reaction. There are several examples of the substrates having been immobilized on nonsolid supports including micelles, liposomes, and lipid membranes.[37−39] Berg and co-workers have reported extensive studies on the enzymatic reaction of pancreatic secreted phospholipase A2 (sPLA2) on phospholipid micelles, where sPLA2 hydrolyzes phospholipids.[40] They have developed models for the enzymatic reaction on the lipid−water interface, including the "scooting" model, in which an enzyme diffuses laterally on the surface of the micelle hydrolyzing the polar head groups of the phospholipids.

Our group has had a primary interest in developing model systems for measuring enzyme activities using immobilized substrates. The approach is based on self-assembled monolayers (SAMs) that present biologically active substrates against a background of oligo(ethylene glycol) groups.[19,41−49] The monolayers permit good control over the densities (and patterns) of immobilized species, are effective at preventing nonspecific interactions of proteins with the surface, and are compatible with several analytical techniques. In one example, we demonstrated an electrochemical method for obtaining kinetic information on an interfacial enzyme reaction.[43] Here we employ this approach to develop methods for determining the kinetic parameters for the interfacial reaction—here, the k_{cat}/K_M value—and that allow for a clear comparison of enzymes acting on immobilized substrates.

Experimental Section

6-Mercapto-1-hexanol and 11-mercapto-1-undecanol were purchased from Aldrich, 8-mercapto-1-octanol was purchased from Dojindo Chemicals, and 16-mercapto-1-hexadecanol was purchased from Frontier Scientific. Phosphate-buffered saline (PBS) used in electrochemical experiments was purchased from Gibco. The enzymatic substrate, 4-hydroxyphenyl valerate terminated alkanethiol, was synthesized in eight steps as described previously.[43] For solution assays, *p*-nitrophenyl butyrate and Triton X-100 were purchased from Sigma Chemical Co. Glass coverslips for gold depositions were obtained from Fisher Scientific.

Preparation of Monolayers. Gold substrates were prepared by vacuum deposition of titanium (∼100 Å) followed by gold (∼900 Å) onto glass coverslips. Monolayers were formed by immersing gold substrates in an ethanolic solution of the particular alkanethiol (1 mM) for 16 h. To obtain mixed monolayers, the gold substrates were immersed in solutions containing mixtures of the desired alkanethiols. In this study the total concentration of the alkanethiols in the solution was kept constant at 1 mM, and the amount of 4-hydroxyphenyl valerate thiol in comparison to the background molecules was varied between 1 and 60%. Before the monolayers were used for electrochemistry, the substrates were washed copiously with absolute ethanol and then dried under a stream of nitrogen gas.

Electrochemical Measurements. Cyclic voltammetry was performed with a Bioanalytical Systems potentiostat using PBS as the electrolyte. The electrochemical cell was configured with the monolayer as the working electrode, Ag/AgCl as the reference electrode, and a platinum wire as the counter electrode.[50−52] The potential was scanned from −400 to 400 mV at 50 mV/s. For each monolayer the density of hydroquinone and hence the density of the enzyme substrate on the surface was determined by integrating the area under the anodic peak in the voltammograms. The baseline of the voltammogram was extended through the peak prior to integration to integrate current associated only with the redox process.

Expression and Purification of Cutinase. Details for the construction of the plasmid and expression of cutinase can be found in a previous paper.[42] Briefly, the protein was expressed periplasmically in bacterial strain BL21 (DE3) and extracted from the cells by osmotic shock. Cutinase was separated and purified by size exclusion chromatography using a Hiprep 16/60 Sephacryl S-200 column and PBS (pH 7.4) as a solvent. The protein was stored in PBS (pH 7.4) at 4 °C. The concentration of the protein was determined by measuring the absorbance at 280 nm in 6 M guanidine−HCl (ϵ_{280} = 13370 M^{-1} cm^{-1}) at pH 6.5.

Solution Assay of Cutinase. Rate constants for the homogeneous reactions were determined by following the hydrolysis of *p*-nitrophenyl butyrate by cutinase.[53] All of the measurements were done on a Beckman Coulter DU 640 spectrophotometer. The k_{cat} and K_M values were obtained by nonlinear regression curve fitting using Sigma Plot.

Results and Discussion

Experimental Approach. We used monolayers that present the 4-hydroxyphenyl valerate group against a background of hydroxyl-terminated alkanethiolate (Figure 1). In this way, the density of the substrate can be controlled by varying the ratio

(33) Kim, J.-H.; Roy, S.; Kellis, J. T., Jr.; Poulose, A. J.; Gast, A. P.; Robertson, C. R. *Langmuir* **2002**, *18*, 6312−6318.
(34) Fang, S.; Lee, H. J.; Wark, A. W.; Kim, H. M.; Corn, R. M. *Anal. Chem.* **2005**, *77*, 6528−6534.
(35) Nishino, H.; Nihira, T.; Mori, T.; Okahata, Y. *J. Am. Chem. Soc.* **2004**, *126*, 2264−2265.
(36) Nishino, H.; Murakawa, A.; Mori, T.; Okahata, Y. *J. Am. Chem. Soc.* **2004**, *126*, 14752−14757.
(37) Ivanova, T.; Malzert, A.; Boury, F.; Proust, J. E.; Verger, R.; Panaiotov, I. *Colloid Surf. B* **2003**, *32* (4), 307−320.
(38) Burns, R. A., Jr.; El-Sayed, M. Y.; Roberts, M. F. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79* (16), 4902−4906.
(39) Ivanov, A. E.; Volchenkova, T. A.; Aha, B.; Zaitsev, S. Y. *Colloid Surf. B* **2002**, *23* (4), 349−356.
(40) Berg, O. G.; Gelb, M. H.; Tsai, M. D.; Jain, M. K. *Chem. Rev.* **2001**, *101*, 2613−2653.
(41) Houseman, B. T.; Huh, J. H.; Kron, S. J.; Mrksich, M. *Nat. Biotechnol.* **2002**, *20* (3), 270−274.
(42) Hodneland, C. D.; Lee, Y.-S.; Min, D.-H.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (8), 5048−5052.
(43) Yeo, W.-S.; Mrksich, M. *Angew. Chem. Int. Ed.* **2003**, *42* (27), 3121−3124.
(44) Collier, J. H.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (7), 2021−2025.
(45) Su, J.; Bringer, M. R.; Ismagilov, R. F.; Mrksich, M. *J. Am. Chem. Soc.* **2005**, *127*, 7280−7281.
(46) Min, D.-H.; Su, J.; Mrksich, M. *Angew. Chem. Int. Ed.* **2004**, *43* (44), 5973−5977.
(47) Min, D.-H.; Yeo, W.-S.; Mrksich, M. *Anal. Chem.* **2004**, *76*, 3923−3929.
(48) Houseman, B. T.; Mrksich, M. *Angew. Chem. Int. Ed.* **1999**, *38* (6), 782−785.
(49) Su, J.; Rajapaksha, T. W.; Peter, M. E.; Mrksich, M. *Anal. Chem.* **2006**, *78*, 4945−4951.
(50) Yousaf, M. N.; Chan, E. W. L.; Mrksich, M. *Angew. Chem. Int. Ed.* **2000**, *39* (11), 1943−1946.
(51) Gawalt, E. S.; Mrksich, M. *J. Am. Chem. Soc.* **2004**, *126*, 15613−15617.
(52) Chan, E. W. L.; Yousaf, M. N.; Mrksich, M. *J. Phys. Chem. A* **2000**, *104*, 9315−9320.
(53) Kolattukudy, P. E.; Purdy, R. E.; Maiti, I. B. *Methods Enzymol.* **1981**, *71* (Lipids, Part C), 652−664.

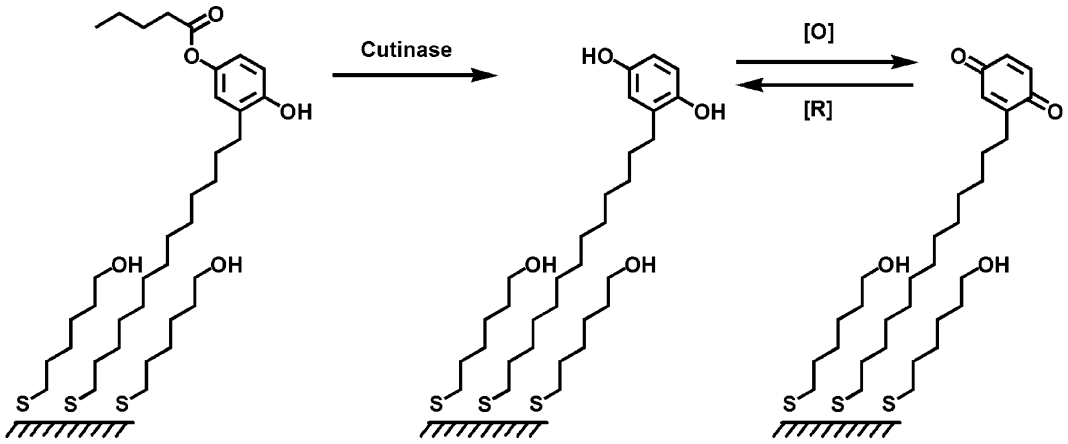

Figure 1. This work uses monolayers that present the 4-hydroxyphenyl valerate group (left). Cutinase is a serine esterase that acts on this substrate to yield the corresponding hydroquinone. Because this quinone is redox active, whereas the parent ester is not, cyclic voltammetry can be used to quantitatively measure the density of the quinone and, therefore, the rate of the enzyme reaction.

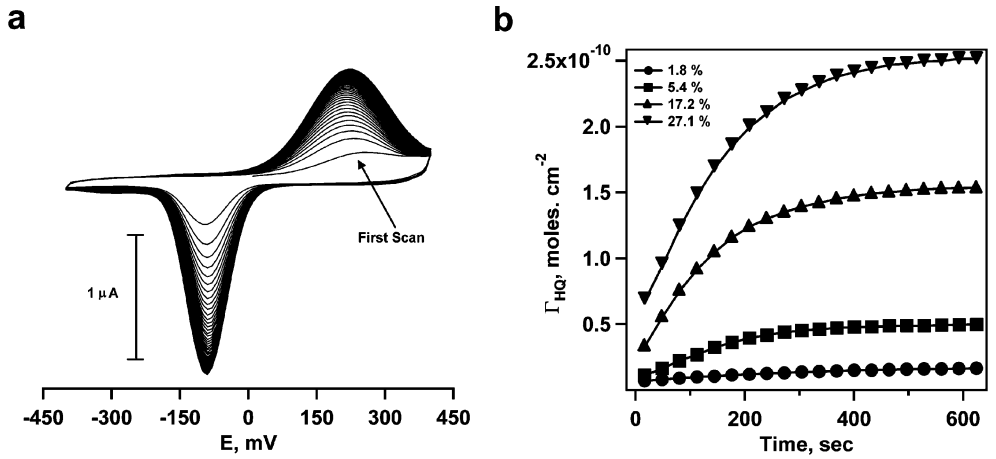

Figure 2. (a) Typical voltammograms obtained after the addition of cutinase to a monolayer presenting 4-hydroxyphenyl valerate. The increasing cathodic and anodic peaks represent the accumulation of the hydroquinone product from enzyme action. (b) Amount of hydroquinone present on the monolayer (Γ_{HQ}) as a function of time for surface densities between 1.8 and 27.1%. Values of Γ_{HQ} were determined by integrating the areas under the peaks at each time point.

of the two alkanethiolates in the solution from which the monolayer assembles. Furthermore, the environment of this group can be controlled by altering the length of the surrounding hydroxyl-terminated alkanethiolate. In all cases, the ester is hydrolyzed by cutinase to reveal a hydroquinone, which is electroactive and therefore permits the use of cyclic voltammetry to measure the kinetic profiles for the reactions. Before the addition of cutinase, we observed a basal non-faradaic current, which remained constant for several scans. Upon addition of the enzyme, the voltammetric waves for oxidation and reduction of the hydroquinone and quinone, respectively, became evident. These waves increased with time until they reached the maximum intensity corresponding to complete hydrolysis of the ester (Figure 2a). We performed this experiment for surfaces having the substrate molecule present at densities ranging from approximately 2 to 30% (relative to total alkanethiolate) and found that the amount of hydroquinone product smoothly approached the final density of the substrate (Figure 2b). The kinetic profiles for these reactions are similar to those obtained using *p*-nitrophenyl butyrate for the corresponding homogeneous phase reactions. The total substrate densities reported in Figure 2b were determined by integrating the areas under the peaks once the signal had reached a maximum stable value. We also found that the initial rate (taken from the slope of the linear region of the curves in Figure 2b) is proportional to the density of substrate on the monolayer. For higher substrate densities the rate does not strictly extrapolate to the origin, likely because the scan rate, and therefore the frequency of the measurement, is slow for these rapid reactions.

Dependence of Rate on Enzyme Concentration. We first determined the initial rates of the enzymatic reaction using monolayers that present the substrate at two different densities and in each case using a series of enzyme concentrations (Figure 3). We found that the rate of reaction increased with the enzyme concentration and reached a limiting velocity. Furthermore, we found that the monolayer having a lower density of substrate reached a maximum initial rate at lower enzyme concentrations than did the monolayer having a higher density of substrate. Hence, the maximum initial rate depends on the enzyme concentration. *An important point of these data is that they show how the initial rates alone cannot be used to compare enzymatic reactions, as the initial rate is not an intrinsic parameter but rather depends on several factors, including the density of the substrate and the concentration of the enzyme.* For the monolayers presenting substrate at lower density, the rate reaches a limiting value at concentrations higher than ~30 nM, whereas for the monolayers having a higher density of substrate (18%) the rate does not level off in the range of the enzyme concentration utilized here. On the basis of these data, we decided to employ enzyme concentrations between 0 and 20 nM because this range gives initial rates that increase linearly and do not become limited by substrate.

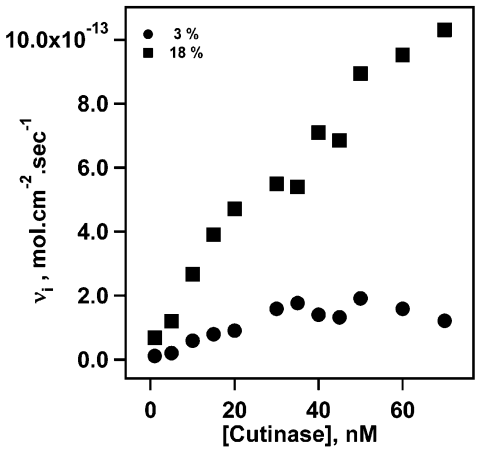

Figure 3. Initial rate (ν_i) of formation of Γ_{HQ} as a function of concentration of the enzyme for two different substrate densities.

Table 1. Comparison of Interfacial k_{cat}/K_M Values for Different Concentrations of Enzyme and the Corresponding Value for the Homogeneous Phase Reaction (Solution)

	$k_{cat}/K_M \times 10^6$ (M^{-1} s^{-1})
solution	1.22 ± 0.13^a
20 nM	0.89 ± 0.06^b
10 nM	0.94 ± 0.07^b
5 nM	1.06 ± 0.05^b

[a] k_{cat}/K_M values were obtained from the nonlinear regression curve fits. [b] k_{cat}/K_M values were obtained from Figure 4a using eq 8.

Kinetic Model. We develop a model that relates the kinetic data in Figure 3 to constants that represent the intrinsic catalytic efficiency of the enzyme. Equation 1 defines the rate constants for association (k_a) and dissociation (k_d) of the enzyme (E) and substrate (S) to give the enzyme−substrate (ES) complex. We represent the conversion of this complex to the product and free enzyme with a single first-order rate constant (k_{cat}). We note that the acyl group of the substrate is not hydrolyzed directly, but rather is transferred to an active site serine, and therefore the regeneration of enzyme is included in the k_{cat} term.

$$E + S \underset{k_d}{\overset{k_a}{\rightleftharpoons}} ES \tag{1}$$

$$ES \xrightarrow{k_{cat}} E + P \tag{2}$$

Note that E represents the concentration of enzyme in the solution (in units of mol/L) but that S, ES, and P represent surface densities of these species (in units of mol/cm^2). The rate of formation of ES and P can be represented as

$$\frac{d\Gamma_{ES}}{dt} = k_a[E]\Gamma_S - k_d\Gamma_{ES} - k_{cat}\Gamma_{ES} \tag{3}$$

$$\frac{d\Gamma_P}{dt} = k_{cat}\Gamma_{ES} \tag{4}$$

where Γ_S, Γ_{ES}, and Γ_P correspond to the surface densities of the respective species.

At steady state

$$\frac{d\Gamma_{ES}}{dt} = 0 \tag{5}$$

The total amount of enzyme E_0 in the system is the sum of free enzyme E and the enzyme bound to the substrate ES. Because the amount of substrate is very small relative to the amount of enzyme, the concentration of enzyme remains approximately constant during the reaction, and $[E] \sim [E_0]$.

Following the derivation of the Michaelis−Menten equation and replacing [E] with $[E_0]$, we obtain

$$\Gamma_{ES} = \frac{\Gamma_S[E_0]}{K_M} \tag{6}$$

K_M is the Michaelis constant for enzyme binding of the immobilized substrate.

$$K_M = \frac{k_d + k_{cat}}{k_a} \tag{7}$$

Straightforward manipulation provides the following relationship for the rate of the reaction:

$$v = \frac{d\Gamma_P}{dt} = \left(\frac{k_{cat}}{K_M}[E_0]\right)\Gamma_S \tag{8}$$

Hence, the value of k_{cat}/K_M for the interfacial reaction can be determined from the slope of the plot that relates the rate of the reaction to the density of substrate.

A study report by Gutierrez and co-workers used a slightly different line of derivation to develop a theoretical approach for describing the kinetics of heterogeneous enzyme reactions, but arrived at the same conclusion as our derivation.[24] In that paper, the authors found that under conditions where the concentrations of both enzyme and substrate are small relative to the K_M, the initial velocity of the interfacial reaction increases linearly with the total enzyme concentration and with the density of substrate on the surface. This requirement is reasonable, as low concentrations of enzyme and substrate ensure that the enzyme−substrate complex is not saturated and, therefore, provide a linear dependence of reaction rate on the concentrations of these species. The use of an immobilized substrate, however, complicates the application of this condition because a density of species and a concentration of species cannot be compared directly. To circumvent this dimensional mismatch, previous authors have converted the surface density into a volume term by making the formal assumption that the substrate is evenly distributed throughout the volume of solution in contact with the surface. This assumption is clearly less accurate when the volume of the solution is large, because only enzyme near the substrate is functional in the assay and increasing the volume (and therefore enzyme far from the substrate) has a diminishing influence on the reaction rate. In any event, in most solid-phase assays, it is likely that this condition of low enzyme concentration and substrate density is met, and it is certainly the case with cutinase acting on *p*-nitrophenyl butyrate, for which the value of K_M is 160 μM.

Determination of k_{cat}/K_M. The implementation of the kinetic model described above requires experimental data that quantitatively report the extent of reaction with a high temporal resolution. Methods that require the substrate to be removed from the reaction mixture prior to analysis generally do not provide data of sufficient temporal resolution or quantitative character. The use of cyclic voltammetry is important in this respect, as this method is quantitative and monitors the surface *in situ*, avoiding the need to continuously remove the substrate from the reaction medium or, in the case of analytical methods that damage the sample, to perform separate reactions for each time point. We used eq 8 to determine the values of k_{cat}/K_M for the interfacial

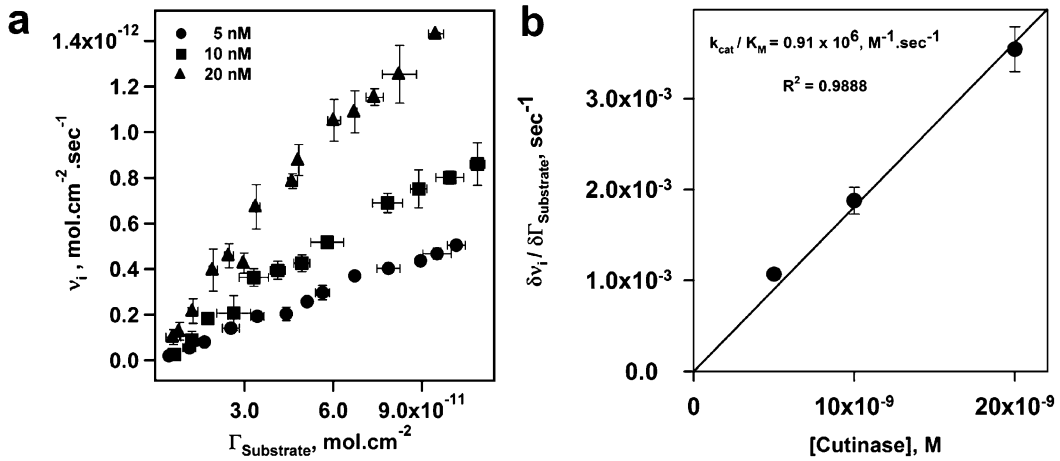

Figure 4. (a) Initial rate (ν_i) of formation of Γ_{HQ} as a function of substrate density for three different enzyme concentrations. The error bars in the *X* direction represent one standard deviation from the mean of four separate measurements of substrate densities obtained from the same thiol solution. The error bars in the *Y* direction represent one standard deviation from the mean of four separate measurements of initial rates obtained from the same thiol solution. (b) Plot of initial slopes as calculated from (a) as a function of the respective enzyme concentrations.

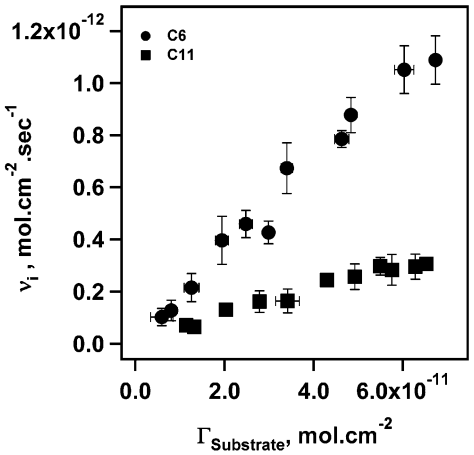

Figure 5. Initial rate (ν_i) of formation of Γ_{HQ} as a function of substrate density for monolayers having background molecules of different chain lengths (C6 and C11), where C6 is 6-mercapto-1-hexanol and C11 is 11-mercapto-1-undecanol. The total enzyme concentration was 20 nM. C6 has 6 methylene groups and an end hydroxyl group; similarly, C11 has 11 methylene units and an end hydroxyl group.

enzyme reaction. We first plotted the dependence of initial rate (ν_i) on the density of the substrate (Γ_S) for each enzyme concentration ([E_0]). We determined the best-fit slope for this relationship and divided this value by the total enzyme concentration to obtain a value for k_{cat}/K_M.

For each concentration of enzyme, we found that the initial rates increase linearly with the substrate density (Figure 4a). Furthermore, the slopes of these plots were proportional to the enzyme concentration and agreed with the model derived above. This representation of the kinetic data is analogous to that used in the Michaelis−Menten analysis of homogeneous reactions. The error bars in the *X* direction represent one standard deviation in substrate density obtained from four different monolayers prepared with the same solution of mixed thiols at a particular ratio. The magnitude of the error bars reveals that a particular solution ratio of the thiols gives quite similar surface coverage of the substrate on the monolayer. Similarly, the error bars in the *Y* direction represent one standard deviation in the initial rate of the reaction for the corresponding monolayers. Because eq 8 is valid in the limit of low substrate densities, we determined the slopes using data obtained for extents of reaction of less than $\sim 7 \times 10^{-11}$ mol cm^{-2} of substrate for calculating the k_{cat}/K_M. As stated earlier for high substrate densities, for which the initial rates are high, the technique may not be able to accurately follow the enzymatic reactions; hence, at those high values of substrate densities we see deviation from linearity. The values of k_{cat}/K_M for each of three concentrations of the enzyme were similar (Table 1). This result further shows the importance of using k_{cat}/K_M to assess the efficiency of an enzyme acting on an immobilized substrate. A direct comparison of initial rates, by contrast, would vary with the enzyme concentration used and therefore would lead to relative rates that are dependent on the choice of experimental condition. We also found that the values of k_{cat}/K_M are slightly lower than that obtained in the corresponding solution reaction using a similar substrate. This difference may be due to the modest differences in structures of the substrates used in the solid-phase and homogeneous assays or to effects of the surface. Finally, Figure 4b relates the slopes taken from Figure 4a to the enzyme concentration. The values of the slope agree and further emphasize the accuracy that can be obtained with this technique. The k_{cat}/K_M calculated from the slope of Figure 4b is 0.91×10^6 M^{-1} s^{-1}.

Dependence of Kinetics on Microenvironment. As a demonstration that the values of k_{cat}/K_M can be used to compare the relative activities of an enzyme for immobilized substrates, we prepared 4-hydroxyphenyl valerate monolayers in which the relative lengths of the substrate-terminated alkanethiolates and the background hydroxy-terminated alkanethiolates were varied. In this way, the monolayers are uniform in that they present the same substrate at similar densities, but these substrates have a varied degree of "accessibility" as determined by the length of the tether that separates the substrate from the background monolayer. Indeed, previous work has demonstrated that the length of the chain that tethers a molecule to the monolayer has an influence on the reactivity[54] or affinity for a receptor[55] of that molecule at the surface.

We prepared three additional monolayers wherein the background alkanethiolates had 8, 11, or 16 methylene units (referred to as the C8, C11, and C16 monolayers, respectively), to make comparisons to the system described above wherein the background chains were 6 methylene units in length (referred to as the C6 monolayer). We employed the method described above to characterize the enzyme kinetics on each of the monolayers. The C11 monolayer revealed a significant decrease

(54) Kwon, Y.; Mrksich, M. *J. Am. Chem. Soc.* **2002**, *124*, 806−812.
(55) Houseman, B. T.; Mrksich, M. *Biomaterials* **2001**, *22* (9), 943−955.

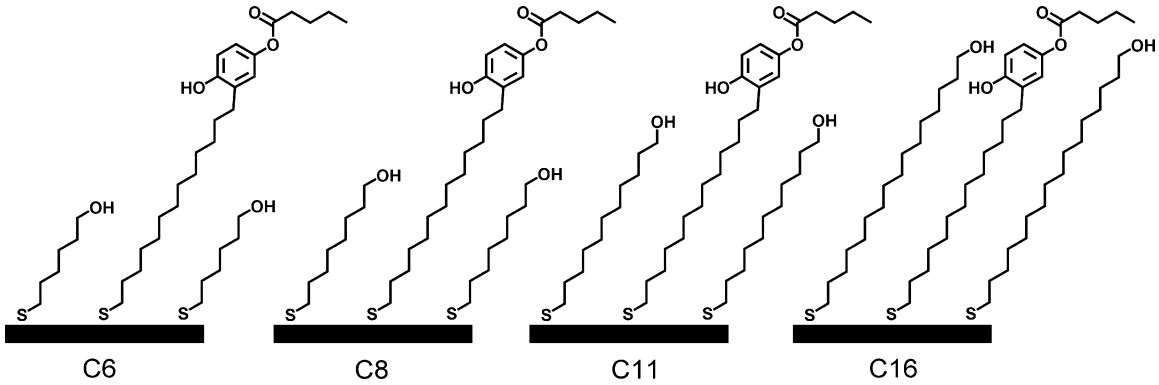

Figure 6. Comparison of various background molecules with respect to the substrate. C6 and C8 seem to offer similar hindrance to the enzyme, and hence no substantial change in the rates was observed. In the case of C11 the end groups are much closer to the substrate and hence affect the rate of reaction more drastically (Figure 5). The substrate in the C16 background shows no activity due to excessive crowding of the substrate.

in the rates of the enzyme reaction as compared to the C6 monolayers and gave a k_{cat}/K_M value of 0.26 ($\pm$0.01) $\times$ 10^6 M^{-1} s^{-1} (Figure 5). The enzymatic reaction on the C16 monolayer was too slow to obtain kinetic data, whereas the reaction on the C8 monolayer gave kinetic data that were experimentally similar to thoseof the C6 monolayer (data not shown). Hence, the four monolayers showed an expected trend in reactivity for cutinase: as the spacing between the substrate molecule and the monolayer is decreased, a threshold is reached, after which the substrate shows decreased reactivity for the enzyme. Figure 6 compares the spacing of the background molecules from the substrate for different chain lengths of the background molecules used. Note that the C6 and C8 monolayers had similar values of k_{cat}/K_M, suggesting that the spacing of the substrate from the monolayer was sufficient to permit free access to the enzyme active site. The C11 monolayer had an approximately 4-fold lower turnover number, suggesting that the spacing of substrate from the monolayer was less than optimal and gave rise to an energetic penalty that is likely reflected in the K_M term. Finally, the C16 monolayer gave little reactivity with the enzyme, which is likely due to an insufficient spacing between the substrate and the monolayer.

Conclusions

This study represents an early example to quantitatively assess the action of an enzyme at a surface. Whereas much recent work has been performed on quantitative comparisons of enzymes that act on immobilized substrates, the methods are largely based on measures that depend on the choice of experimental variables and therefore do not give intrinsic quantities by which enzymes can be compared. The combination of self-assembled monolayers and electrochemical methods that we use in this work provides for a uniform activity of immobilized substrate and quantitative time-dependent information on the extent of reaction, both of which are important in determining the Michaelis−Menten parameters for a reaction. This method will be most important in model studies to understand the mechanistic features that are unique to interfacial enzyme reactions and in more rigorous comparison of homogeneous and heterogeneous enzyme reactions.

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