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ARTICLE *in* LANGMUIR · JULY 2006

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Adsorption and Mobility of a Lipase at a Hydrophobic Surface in the Presence of Surfactants

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Received November 18, 2005. In Final Form: March 3, 2006

With the aim of being able to manipulate the processes involved in interfacial catalysis, we have studied the effects of a mixture of nonionic/anionic surfactants, $C_{12}E_6$/LAS (1:2 mol %), on the adsorption and surface mobility of a lipase obtained from *Thermomyces lanuginosus* (TLL). Surface plasmon resonance (SPR) and ellipsometry were used to analyze the competitive adsorption process between surfactants and TLL onto hydrophobic model surfaces intended to mimic an oily substrate for the lipase. We obtained the surface diffusion coefficient of a fluorescently labeled TLL variant on silica silanized with octadecyltrichlorosilane (OTS) by fluorescence recovery after photobleaching (FRAP) on a confocal laser scanning microscope. By means of ellipsometry we calibrated the fluorescence intensity with the surface density of the lipase. The TLL diffusion was measured at different surface densities of the enzyme and at two time intervals after coadsorption with different concentrations of $C_{12}E_6$/LAS. The surfactant concentrations were chosen to represent concentrations below the critical micelle concentration (CMC), in the CMC region, and above the CMC. The apparent TLL surface diffusion was extrapolated to infinite surface dilution, D_0. We found that the presence of surfactants strongly modulated the surface mobility of TLL: with $D_0 = 0.8 \times 10^{-11}$ cm^2/s without surfactants and $D_0 = 13.1 \times 10^{-11}$ cm^2/s with surfactants above the CMC. The increase in lipase mobility on passing the CMC was also accompanied by a 2-fold increase in the mobile fraction of TLL. SPR analysis revealed that surface bound TLL was displaced by $C_{12}E_6$/LAS in a concentration-dependent manner, suggesting that the observed increase in surface mobility imparts bulk-mediated diffusion and so-called rebinding of TLL to the surface. Our combined results on lipase/surfactant competitive adsorption and lipase surface mobility show how surfactants may play an important role in regulating interfacial catalysis from physiological digestion to technical applications such as detergency.

1. Introduction

Glyceride lipases catalyze in an aqueous environment the hydrolysis of the ester linkage between acyl chains and the glycerol backbone of glycerides. These enzymes are involved in lipid metabolism and are widespread in nature from simple to higher organisms. The catalytic activity of lipases is interfacially activated: the turnover of the enzyme may increase by orders of magnitude if the enzyme is presented to the substrate at an interface (e.g., if the critical micellar concentration of a lipid substrate is exceeded).[1] The origin of the interfacial activation is by no means fully understood; in part due to the complexity in describing the time evolution of the coupled binding equilibria including enzyme, substrate, and product molecules in different microenvironments.[2,3] On the molecular level of the enzyme, the surface activation of some lipases also involves a conformational change within the enzyme, in which a surface domain, the so-called lid, moves and exposes the active site region.[4] The open and active conformation of this type of lipase is more hydrophobic than the closed state of the enzyme. Another critical feature on the molecular level is the mobility of the individual enzyme at the water−lipid interface. For secreted phospholipase A_2, the surface dynamics has been analyzed in detail in terms of two modes of interactions between the enzyme and the surface: hopping and scooting.[2] If the lipase binds possessively to the surface, the enzyme is said to act in the so-called scooting mode, whereas the hopping mode involves an exchange between solution and surface. We have earlier shown that a glyceride lipase variant from *Thermomyces lanuginosus* adheres strongly to solid surfaces of varying hydrophobicity and displays a slow lateral diffusion on the order of 10^{-11} cm^2/s.[5] In this type of scooting interaction, the turnover of the lipase toward a substrate on a solid support, the surface mobility of the enzyme becomes an important parameter to understand and potentially manipulate for various medical and technical applications of lipases. In this context and in general, it would be valuable to gain insight on the effect of surface active molecules on the adsorption and diffusion properties of proteins.

Surfactants and proteins interact with each other, both in solution and at interfaces. Protein−surfactant interactions have broad significance in, for example, lipid−protein interactions in biological membranes and technical applications such as detergency formulations, gel electrophoresis, and enzyme catalysis in microemulsions.[6] Surfactants can influence the surface activity of enzymes in several ways. By accumulating at interfaces, surfactants change properties such as charge and hydrophobicity of the surface. Enzymes and surfactants can cooperatively remove oily soils from surfaces; enzymes by digesting the substrate to products easily solubilized or emulsified by surfactants.[7] The

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10.1021/la0531244 CCC: $33.50
Published on Web 05/20/2006

presence of surfactants might affect protein adsorption in a number of ways, from complete hindrance of adsorption to cooperative adsorption, leading to increased adsorbed amounts.[8] Surfactants are also able to displace preadsorbed proteins from solid surfaces. This is achieved either by replacing the protein at the surface or by binding to the protein and thereby solubilizing it.[9] Furthermore, low concentrations of ionic surfactants complex with most proteins in solution, affecting the conformational stability[7,10] and the hydrophobicity of the protein surface.[11,12] Higher surfactant concentrations might lead to partial or complete unfolding of the tertiary protein structure.[10,13]

The aim of this study was to analyze how a mixture of two standard surfactants influences the adsorption and surface mobility of a variant of *Thermomyces lanuginosus* lipase (TLL) on solid hydrophobic surfaces used as a model system for a substrate-like surface. TLL is a lipase with pronounced interfacial activation,[14] and a technical application of this enzyme is in detergency formulations.[15] Adsorption of TLL and the surfactants was measured with ellipsometry and surface plasmon resonance (SPR), which continuously monitor changes in the refractive index of a surface adsorbed layer.[16,17] TLL surface diffusion was quantified with fluorescence recovery after photobleaching (FRAP), a well-established technique for mobility studies.[18] The diffusion of TLL on solid hydrophobic surfaces has been found to be a very slow process as compared to other systems.[5] Therefore, it is interesting to find out if competition with surfactants on the surface affects the mobility of the enzyme. The present study represents to our knowledge the first systematic report on the effects of surfactants on protein mobility at solid surfaces. The modulation by surfactants of surface mobility and exchange dynamics between surfaces and bulk of a water-soluble enzyme has important bearings for parameters such as substrate turnover as well as product inhibition.

2. Materials and Methods

2.1. Materials. HCl (25%), H_2O_2 (30%), NH_3 (25%), and chloroform (>99%) were from Merck; toluene (>99.5%) was from Fluka; octadecyltrichlorosilane (OTS) (Catalog No.104817-25G) was from Sigma-Aldrich. Alexa Fluor 488 protein labeling kits were purchased from Molecular Probes Europe BV (Leiden, The Netherlands), and the lipase variant from *Thermomyces lanuginosus* (TLL) was provided by Novozymes A/S (Bagsvaerd, Denmark). Hexaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_6$) was from Nikko Chemicals CO, Ltd. (Tokyo, Japan, Lot No. 6025), and linear alkylbenzene sulfonate (LAS, technical grade with an alkyl chain length average of 12) was from Petresa (Spain). The buffer used throughout the entire study was glycine pH 9.0 (10 mM NaCl, 0.05 mM EDTA, 50 mM glycine, and 1 mM NaN_3), and all water was Milli-Q grade.

2.2. Surface Preparations. Silica wafers were cleaned for 5 min in 80 °C 5:1:1 (v/v/v) H_2O:NH_3:H_2O_2, rinsed in water, and then cleaned for 5 min in 80 °C 5:1:1 (v/v/v) H_2O:HCl:H_2O_2. The wafers were then rinsed several times in water and ethanol and blown dry with nitrogen before immersing them in an unstirred solution of 0.5% (v/v) OTS in toluene for 24 h. The wafers were then thoroughly rinsed in chloroform and put in a chloroform bath for 2 min. Finally, the wafers were rinsed with ethanol and water and blown dry with nitrogen. The OTS surfaces had a contact angle of 105° and were stored in ethanol prior to use. The hydrophobic surface used in the SPR instrument was a commercial HPA chip from Biacore made by covalent fixation of a self-assembled monolayer of alkanethiolates on a gold surface (cf. www.biacore.com).

2.3. TLL Labeling. A variant of TLL modified to give a well-defined labeling was labeled with Alexa Fluor 488 dyes. The Alexa Fluor 488 reactive dye has absorption and fluorescence emission maxima at 494 and 519 nm, respectively. Labeling involved incubating 0.5 mL of 0.33 mg/mL TLL to one vial of reactive Alexa 488 (http://www.probes.com). The reaction was left to take place for 8 h in room temperature followed by 14 h in +8 °C. Labeled TLL was separated with a Bio-Rad BioGel P-30 fine exclusion purification resin with glycine buffer as the elution buffer. To determine the protein and fluorophore concentration, the absorbance at 280 and 494 nm was measured, using $\epsilon_{\mathrm{TLL,\ 280\ nm}} = 38440\ \mathrm{cm^{-1}\ M^{-1}}$ and $\epsilon_{\mathrm{Alexa488,\ 494\ nm}} = 71000\ \mathrm{cm^{-1}\ M^{-1}}$, which led to a degree of labeling of 0.95 mol of dye/mol of enzyme. Labeled lipase was stored in aliquots at −20 °C for up to 6 months after labeling. Fractions were thawed for 1 h and diluted in glycine buffer to appropriate concentration prior to use. Thawed samples were stored at +6 °C and used for two consecutive days.

2.4. Surface Tension Measurements. Surface tension at the air−water interface was measured with a KSV Sigma 70 instrument using a du Noüy ring. Measurements were performed in a glass beaker precleaned in Deconex (Borer Chemie AG, Zuchwil, Switzerland), and addition of a 1 mM stock solution of surfactants was done with a Methrom dosimat titration unit.

2.5. SPR Adsorption Analysis. Adsorption behavior of lipases and surfactants were analyzed with SPR.[16,17] A Biacore 3000 instrument (Biacore, Uppsala, Sweden) was used operating at 25 °C. 60 μL samples were injected, and adsorption to the hydrophobic HPA surface was monitored under a constant flow of 10 μL/min for 6 min, followed by a rinse with buffer solution for 6 min. Refractive index variances of the adsorbed film were continuously measured and converted to surface excess (mg/m^2) using the built-in software. Steady-state surface excess data were collected before end-of-injection and corrected for bulk contribution.[19] Residual adsorption was recorded after rinsing with buffer 4 min after end-of-injection.

2.6. Ellipsometry Adsorption Analysis. A Rudolph thin film ellipsometer, type 436 (Rudolph Research, Fairfield, NJ), was used with a Xenon arc lamp, wavelength of 4015 Å ,and an angle of incidence of 67.7°. Adsorption of unlabeled TLL to OTS surfaces was measured in situ in a cuvette with a solution volume of 3 mL under nonflow conditions but with continuous stirring. The refractive index and thickness of the surface layer were determined numerically using the McCrackin algorithm,[20] and the adsorbed mass was determined using the de Feijter formula.[21]

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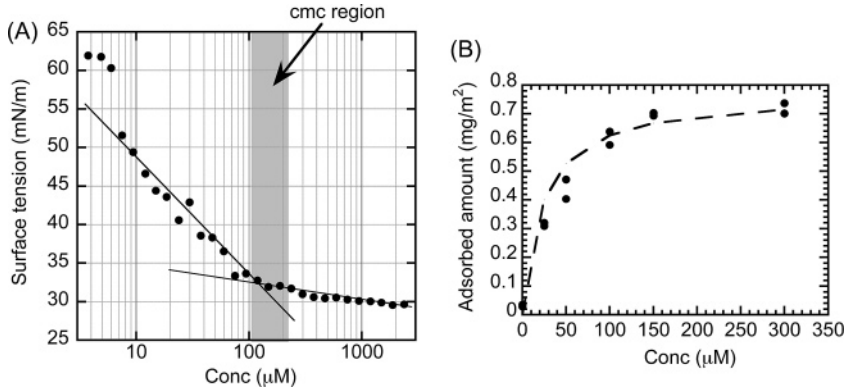

Figure 1. (A) Surface tension vs log concentration of $C_{12}E_6$/LAS (1:2 mol %) indicating a CMC at a concentration of 100−200 μM. (B) Adsorption isotherm of $C_{12}E_6$/LAS on the hydrophobic HPA surface, saturating at surfactant concentration of 150 μM. Data obtained from SPR experiments were run in duplicates. Dashed line is plotted as a guide to the eye.

A more detailed description of the instrumental setup has been published elsewhere.[22]

2.7. FRAP Studies. OTS surfaces of 13 × 18 mm were mounted in a flow cell to an Axiovert 100M microscope with a Zeiss LSM Pascal scanner and a 40 × 1.3 oil immersion objective. For excitation, the 488 nm line of an Ar-ion laser was used. A 100 μm thick silicon rubber and a coverslip glass limited the flow cell volume to 15 μL. Solution was pumped through the system with a syringe pump (Harvard Apparatus), and samples were loaded in a 50 μL loop and injected via a 6-port injection valve (Skandinaviska Genetec AB, Göteborg, Sweden). The valve allowed the use of different solutions before and after the sample injection. Before each experiment, the effect of the Ar 488 laser and the PM-tube voltage on the detected fluorescence was controlled with an InSpeck Green(505/515) Microscope Image Intensity Calibration kit with 6 μm microspheres purchased from Molecular Probes Europe BV (Leiden, The Netherlands), verifying that there was no drift between experiments.

Diffusion experiments were carried out by rinsing the OTS surface with buffer, co-injecting a surfactant−lipase solution to the flow cell followed by rinsing for 2 min with surfactant at the same concentration. This procedure led to lipase and surfactant on the surface and essentially only surfactants in the bulk solution (i.e., no bulk lipase should contribute to the detected fluorescence signal). Surfactants and lipase were mixed just prior to injection. A rectangle of about 60 × 220 μm of the surface was photobleached for 1 min, using the Ar 488 laser with 100% intensity. Recovery micrographs were sampled under no-flow conditions every fourth min for 90 min after bleaching. The pinhole was fully opened during the recovery to compensate for possible tilts and inhomogeneities of the surface. At every sampled time, a *z*-stack of pictures was recorded so that the picture in focus could be analyzed. After the first 90 min, the FRAP procedure was repeated on another spot on the same surface. The diffusion coefficient was averaged over 0−90 min after adsorption (first bleaching) and over 90−180 min after adsorption (second bleaching), denoted $D_{t=0-90}$ and $D_{t=90-180}$, respectively. For analysis of mobile fraction, a ≈10 × 10 μm square of the surface was bleached. The average intensity of the bleached square and the average intensity of the surrounding unbleached region were measured until the ratio between the two areas reached a plateau value. All measurements were performed at room temperature.

To convert the surface fluorescence after adsorption to surface density of TLL, ellipsometry adsorption data was used according to earlier protocols.[5] Mixed samples of unlabeled and labeled TLL with a total concentration of 1.0 μM were injected into the flow cell, adsorbed for 600 s, followed by a 2 min rinse with glycine buffer. The intensity at the surfaces after adsorption of each sample was measured as the average of 10 220 × 220 μm squares.

To investigate the displacement of adsorbed TLL due to surfactants, a lipase−surfactant mixture was co-injected to the surface according to the standard protocol, and the intensity from the surface was measured during 180 min as the average intensity of a 220 × 220 μm square. Every 15th minute after adsorption, the flow cell was rinsed with a short pulse of surfactants to assess if lipase displaced from the surface, still in the confocal volume, contributed to the detected signal.

2.8. Analysis of FRAP Data. The diffusion coefficient was estimated with a method developed and described in an earlier study.[5] In short, the large dimension of the bleached rectangle as compared to the slow diffusion of proteins at solid surfaces makes it possible to analyze the intensity profile $I(x, t)$ of the middle section of the border between the bleached and unbleached area as a one-dimensional diffusion from a step function. $I(x, t)$ is the sum of the contributions from the mobile fraction f and the immobile fraction $(1 - f)$ (eq 1):

$$I(x, t) = f \times F(x, t) + (1 - f) \times H(x) \qquad (1)$$

$F(x, t)$ is the solution of the diffusion equation for a one-dimensional diffusion from a step function, and $H(x)$ is the initial step function. Differentiating eq 1 and using a median filter operator to remove the contributions from the immobile fraction leads to a simple expression of the maximum derivative of the intensity profile as a function of time (t), the mobile fraction (f), and the diffusion coefficient (D) (eq 2):

$$\left|\frac{\partial I(x, t)}{\partial x}\right|_{x=0} = \frac{f}{\sqrt{4\pi D t}} \qquad (2)$$

The mobile fraction f was estimated separately (see above), and eq 2 could be used to extract D.

3. Results

3.1. Surface Tension and Adsorption of Surfactants. Surface tension as a function of log concentration of $C_{12}E_6$/LAS (1:2 mol %) in buffer can be seen in Figure 1A. The critical micellar

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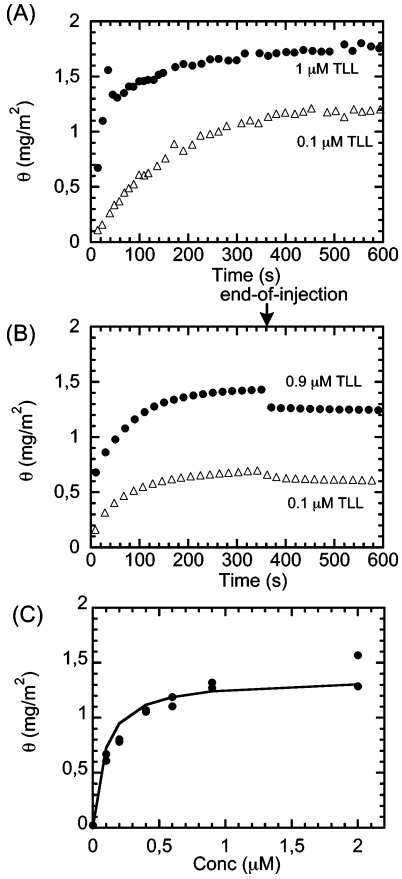

Figure 2. (A) Surface density (mg/m^2) of TLL vs time on the OTS surface using ellipsometry at different bulk concentrations, (●) = 1 μM TLL, (Δ) = 0.1 μM TLL. (B) SPR sensorgram at different bulk concentrations TLL on the HPA surface, (●) = 0.9 μM TLL, (△) = 0.1 μM TLL. This plot is not corrected for buffer mismatch and bulk enzyme contribution to refractive index variation. (C) Adsorption isotherm of TLL on the HPA surface. The solid line is plotted as a guide to the eye. All SPR measurements were done in duplicate.

concentration (CMC) was found at a $C_{12}E_6$/LAS concentration of 100−200 μM (i.e., when increased surfactant concentration did not lead to a decrease in surface tension). The SPR surfactant adsorption isotherm gave similar results (Figure 1B). Using the hydrophobic HPA chip, the surface excess before end-of-injection was obtained for the pure surfactant system in concentrations between 0 and 300 μM. The maximum adsorbed amount on the hydrophobic HPA surface, 0.7 mg/m^2, was reached with bulk concentration of 150 μM.

3.2. Adsorption of TLL. Two bulk concentrations of unlabeled TLL, 0.1 and 1 μM, were used for adsorption studies on the OTS surface in the ellipsometer (see Figure 2A). The lipase adsorbed readily on the OTS, reaching a surface density of 1.75 mg/m^2 for 1 μM. No desorption of TLL occurred during rinsing with buffer after 1500 s with any of the concentrations (data not shown). TLL adsorption to the HPA surface was measured with SPR in the concentration ranges from 0 to 2 μM. Representative SPR sensorgrams are displayed in Figure 2B, where the effect of the difference in bulk refractive index between running buffer and sample solution is seen as a sharp drop in signal at end-of-injection. The resulting adsorption isotherm, from surface excess data before end-of-injection, is seen in Figure 2C. The isotherm saturates at 1.3 mg/m^2 with a bulk concentration of 1 μM or above.

3.3. Calibration of Intensity to Surface Density. A graph of average intensity from the OTS surface versus fraction of

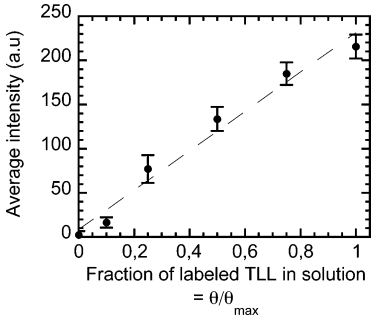

Figure 3. Calibration curve based on fluorescence CLSM experiments. The average fluorescence intensity of Alexa488-labeled TLL at the surface (± standard deviation) is plotted vs the labeled fraction of TLL in solution, corresponding to the relative surface density of TLL (θ/θ_{max}). A linear fit is plotted as a guide to the eye (dashed line).

labeled TLL in bulk solution is seen in Figure 3. All samples had the same total TLL concentration (1 μM) but different fractions of labeled lipase. At this TLL concentration and at an adsorption time of 600 s, the resulting surface density would be maximized (θ_{max}) for all samples according to the SPR isotherm (Figure 2C). In this way, surface intensity after adsorption could be translated to relative surface density, θ/θ_{max}, simply by normalizing to the maximum intensity value in Figure 3. In the graph, a linear fit is plotted as a guide to the eye. This reveals that there is a good linear relationship of surface intensity to bulk fraction of labeled TLL with only small possible deviations with 10% or 100% labeled TLL.

3.4. Mobility of TLL. FRAP experiments were performed with the Alexa488 labeled TLL on the OTS surface in the presence of varying concentrations of $C_{12}E_6$/LAS in the buffer: 0, 25, 150, or 500 μM. The surfactant concentrations were chosen to represent concentrations below, above, and in the CMC region (see Figure 1). TLL concentrations of 0.1, 0.3, or 1.0 μM and adsorption times of 1−5 min were used to achieve different relative surface densities θ/θ_{max}, calibrated from the intensity from the surface after adsorption. For every surfactant concentration, TLL surface diffusion was measured at different surface densities. The diffusion coefficient at infinite surface dilution for both periods of time after adsorption ($D_{0,t=0-90}$ and $D_{0,t=90-180}$) were extrapolated using an exponential fit of the data, which has been suggested from theoretical work on surface diffusion.[23] The fits are forced to pass through $D = 10^{-12}$ cm^2/s as θ/θ_{max} goes to infinity, which is a diffusion rate considered as immobile in this study and in our previous work.[5] The mobile fraction was measured on surfaces where θ/θ_{max} was between 0.1 and 0.15, 0−90 min after adsorption. The results are summarized in Figure 4 and Table 1.

With all $C_{12}E_6$/LAS concentrations, the TLL surface diffusion decreased with increasing θ/θ_{max}. The diffusion was heavily influenced by the surfactants, especially at low surface densities. When the CMC of the surfactants was exceeded, the estimated $D_{0,t=0-90}$ for TLL increased more than 10-fold as compared to when no surfactants were present; from 0.8×10^{-11} cm^2/s to 13.1×10^{-11} cm^2/s. The increase in diffusion coefficient was accompanied by an increase in mobile fraction, from 0.38 with 0 μM surfactants to 0.83 with 500 μM $C_{12}E_6$/LAS (Table 1). The $C_{12}E_6$/LAS concentration below CMC (25 μM) had much less effect on the diffusion.

The rate of diffusion after the second bleaching, $D_{0,t=90-180}$, was slower with the higher $C_{12}E_6$/LAS concentrations. With 500

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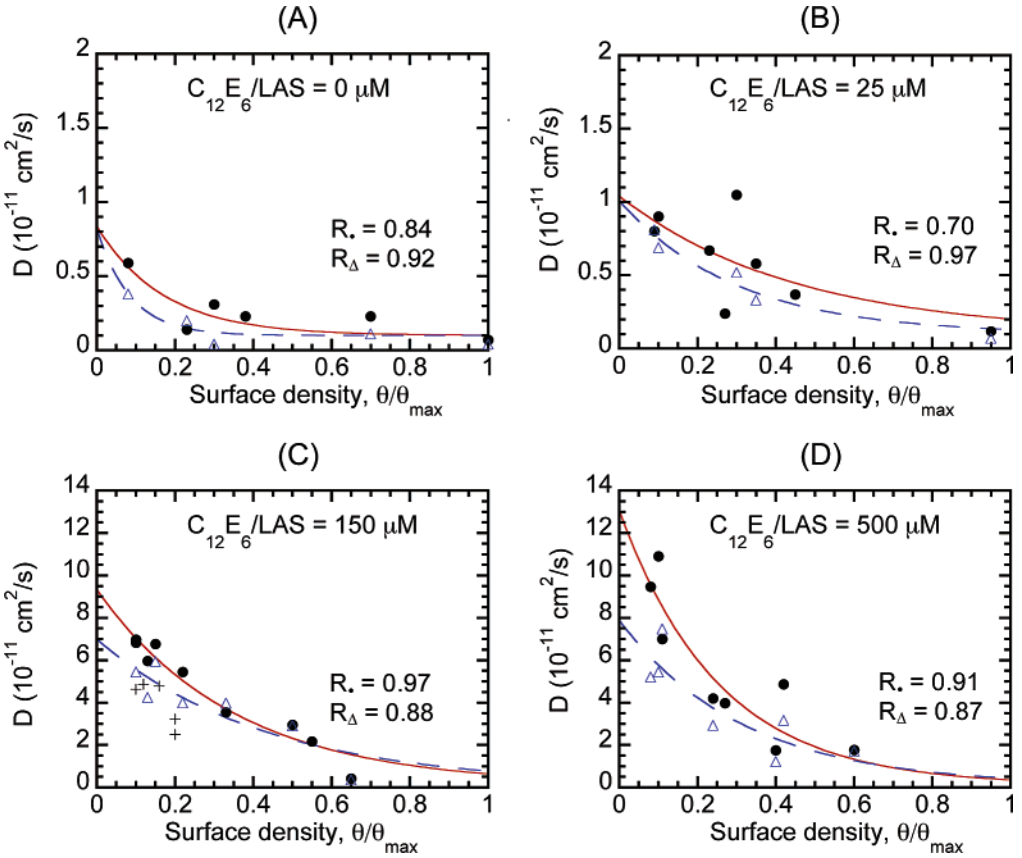

Figure 4. TLL diffusion from FRAP experiments on OTS surfaces plotted as a function of the relative surface density, θ/θ_{max}, when coadsorbed with different concentrations of $C_{12}E_6$/LAS; 0 μM (A), 25 μM (B), 150 μM (C), and 500 μM (D). The diffusion was measured in two time intervals after adsorption: 0−90 min ($D_{t=0-90}$ = ●) and 90−180 min ($D_{t=90-180}$ = △). Exponential functions were fitted for 0−90 min after adsorption (solid lines) and 90−180 min after adsorption (dashed lines) and are used to derive D_0. For $C_{12}E_6$/LAS = 150 μM (C), $D_{t=0-90}$ under flow of surfactants was also measured (+).

Table 1. Diffusion Coefficients and Mobile Fractions of Adsorbed TLL

protein	$C_{12}E_6$/LAS (1:2 mol %) (μM)	$D_{0,t=0-90}$ (cm^2/s)	$D_{0,t=90-180}$ (cm^2/s)	mobile fraction[a]	
TLL	0	0.8×10^{-11}	0.8×10^{-11}	0.38 ± 0.08	($n = 3$)
TLL	25	1.0×10^{-11}	1.0×10^{-11}	0.40 ± 0.04	($n = 4$)
TLL	150	9.3×10^{-11}	7.0×10^{-11}	0.70 ± 0.04	($n = 2$)
TLL	500	13.1×10^{-11}	7.9×10^{-11}	0.83 ± 0.10	($n = 4$)

[a] Mobile fraction was measured on surfaces with θ/θ_{max} between 0.1 and 0.15, 0−90 min after adsorption. n = the number of measurements performed to estimate the mobile fraction.

μM $C_{12}E_6$/LAS, D_0 decreased from 13.1×10^{-11} cm^2/s (averaged 0−90 min after adsorption) to 7.9×10^{-11} cm^2/s (averaged 90−180 min after adsorption) (see Table 1). $D_{0,t=0-90}$ was also estimated under flow conditions for $\theta/\theta_{max} < 0.20$ with $C_{12}E_6$/LAS = 150 μM (Figure 4C), which led to a decrease in the observed diffusion as compared to the no-flow situation.

3.5. Displacement of TLL. The displacement of TLL was measured both in the confocal microscope and from SPR experiments. In the CLSM, TLL was co-injected with $C_{12}E_6$/LAS to a θ/θ_{max} of TLL between 0.10 and 0.15, and the surface intensity was measured as a function of time and concentration of $C_{12}E_6$/LAS (Figure 5A). The intensity values in the graphs are normalized at every time point to the values found with 0 μM surfactants, so that effects from drift in instrument, autobleaching due to long-time exposure to the laser, etc. are eliminated. Compared to when no surfactants were present, the intensity decreased with time and increasing $C_{12}E_6$/LAS concentration. This suggested that a large fraction, from 10% with 25 μM $C_{12}E_6$/LAS to 45% with 500 μM $C_{12}E_6$/LAS, of the adsorbed TLL was displaced during FRAP experiments.

The ability of $C_{12}E_6$/LAS to displace TLL was also confirmed by SPR. In one set of SPR experiments, 0.9 μM unlabeled TLL was coadsorbed with different concentrations of $C_{12}E_6$/LAS in the buffer. Figure 5B shows the adsorbed amount of TLL on the surface plotted versus the $C_{12}E_6$/LAS concentration. As a reference, the $C_{12}E_6$/LAS adsorption with no TLL is plotted in the same graph. Surface excess (mg/m^2) is plotted before end-of-injection. By comparison of the surface excess data with and without enzyme, the net contribution of enzyme to the surface excess may be estimated. Note that as complete wetting is achieved (at 150 μM $C_{12}E_6$/LAS) all lipase is displaced from the surface (Figure 5B). With $C_{12}E_6$/LAS above 150 μM, the residual surface excess 6 min after end-of-injection was very low and similar to the value found with the surfactant alone (data not shown).

4. Discussion

The aim of this study was to investigate the generic effect of surfactants on TLL surface adsorption and mobility. As a model system for a fat/oil-like substrate surface, we have used self-assembled monolayers (SAMs) of long-chain hydrocarbons. The surfactant system $C_{12}E_6$/LAS (1:2 mol %) was used as a model system for the main fraction of surfactants in common detergent formulations. For this mixture of surfactants, the surface tension measurements indicated that micelles start to form in the concentration region of 100−200 μM (Figure 1A). The broad micellar formation region is probably due to the polydisperse nature of the anionic technical surfactant LAS. Corresponding results were found with SPR, where the surface was saturated

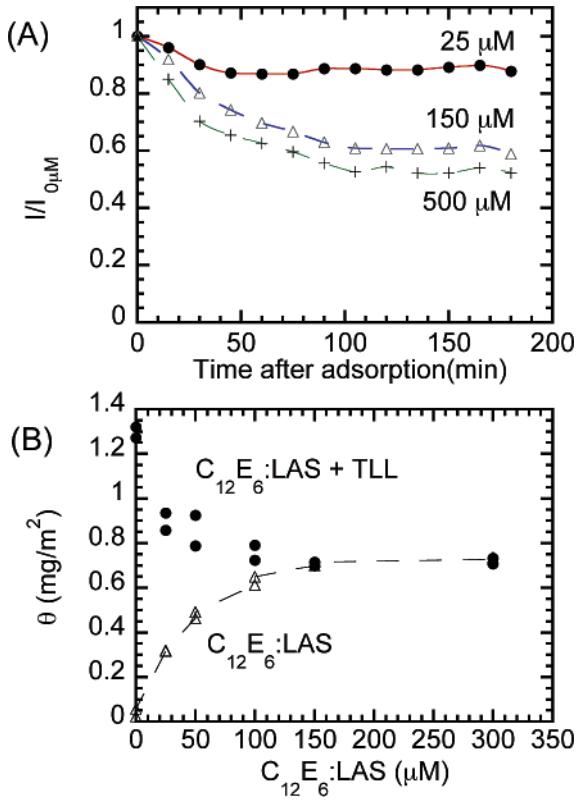

Figure 5. (A) Time evolution of the surface fluorescence intensity in the CLSM from TLL coadsorbed with different concentrations of $C_{12}E_6$/LAS. θ/θ_{max} is between 0.1 and 0.15. (B) Steady-state surface excess in SPR as a function of surfactant concentration upon injection of surfactants alone (△) or with 0.9 μM TLL + $C_{12}E_6$/LAS (●).

with surfactants at a bulk concentration of 150 μM (Figure 1B). Assuming that the critical concentrations for wetting and micelle formation are similar, higher surfactant concentrations would render additional micellar formation in solution.

To establish the maximum adsorbed amount (θ_{max}), adsorption of TLL was measured by ellipsometry on OTS surfaces and by SPR on HPA surfaces. The two methods were used to provide complementary information. The ellipsometer setup used requires rather large volumes of sample solution (min 3 mL) for each measurement; thus, it was not practical to establish an adsorption isotherm with this method. Our SPR on the other hand is limited to gold-based substrates, which has not been utilized in the CLSM measurements. SPR analyses resulted in lower absolute values of the adsorbed amount than ellipsometry (Figure 2A and B). The discrepancy could arise from different models used to calculate the adsorbed amount from the raw data in the ellipsometer [20,21] as compared to SPR.[24,25] Another reason might be different surface properties of the OTS and HPA surfaces (e.g., packing density of acyl chains and defects or impurities in the surface layer). The relative adsorbed amount at 0.1 μM as compared to 1 μM gave similar results with the two methods; around half the value found at 1 μM. We hence assume the results to be qualitatively comparable. According to the isotherm (Figure 2C), adsorption at a concentration of 1 μM should lead to the maximum surface density (θ_{max}) on a hydrophobic surface. θ_{max} on OTS surfaces was consequently set to a TLL density of 1.75 mg/m^2, as found by ellipsometry at 1 μM on this model surface.

To convert surface fluorescence intensity to surface density of lipase, TLL samples were adsorbed to the OTS surface in the confocal microscope. Samples at a total concentration of 1 μM with different fractions of the labeled TLL were used to study the relationship between surface intensity and bulk fraction of labeled TLL. The relation was found to be close to linear (Figure 3), with only small deviations at low and high fractions of labeled TLL. The fluorescence intensity from the adsorbed TLL on the OTS surface could then be converted to relative surface density, θ/θ_{max}, by normalizing to the maximum intensity value in Figure 3. The lower intensity found at 10% TLL might indicate a lower surface affinity by the labeled species, but it would be of no relevance for the CLSM measurements in this study since only the labeled population was used in the diffusion experiments. The lower intensity found compared to the linearity at 100% labeled TLL might be an indication of a small concentration quenching of Alexa-488 at high surface density. Quenching in this case would only affect the diffusion calculations at surface densities close to θ_{max} (i.e., quenched TLL might give rise to a minor increase in fluorescence when diffusing into the bleached area). However, as TLL at θ_{max} was found to be close to immobile, the possible quenching had no significant effect on the plots of D versus θ/θ_{max} in Figure 4.

A FRAP-based method, developed and presented earlier,[5] was used to measure TLL diffusion on OTS surfaces at different relative surface densities and in the presence of surfactants. Both amount and diffusion coefficient of the mobile fraction was obtained. The immobile fraction is thought to arise from lipase molecules adsorbed in an orientation that minimizes the free energy of adsorption. This could correspond to an orientation with the active site, the most hydrophobic part, facing the hydrophobic surface. The conformational change within TLL from a closed state to an open and more hydrophobic state is likely to alter the rate of desorption and the surface diffusion of this enzyme considerably[1]. There is also a possibility that some of the immobile fraction is related to partially unfolded or denatured lipases. However, TLL is a rigid molecule except for the flexibility of the lid domain, and the helix content has been shown to be unaffected after adsorption onto OTS surfaces.[26] Moreover, TLL adsorbed onto hydrophobic surfaces has been found to be enzymatically active;[27] therefore, it is unlikely that the molecule unfolds to any particular extent at the surface.

The diffusion rate of the TLL variant used in this study was similar to previous results using the method with TLL on hydrophobic surfaces.[5] The diffusion coefficients found earlier of TLL were $D_{t=0-90} = 0.8 \times 10^{-11}$ cm^2 and $D_{t=90-180} = 0.5 \times 10^{-11}$ cm^2/s with a mobile fraction of 0.45. In this study both $D_{t=0-90}$ and $D_{t=90-180} = 0.8 \times 10^{-11}$ cm^2/s with a mobile fraction of 0.38 (Figure 4 and Table 1). These diffusion rates are some orders of magnitude slower than reported for other proteins[28] and for protein diffusion in membranes,[29] suggesting that TLL adheres strongly to the surface. The lateral diffusion was found to be a decreasing function of surface density, as has been proposed by theoretical modeling[23] and Brownian dynamics studies.[30] This

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is consistent with sterical hindrance for 2D diffusion at high surface densities of TLL.

The mobility of TLL measured with FRAP was affected by the presence of high concentrations of $C_{12}E_6$/LAS whereas below CMC of the surfactant system only a small increase in TLL diffusion was seen (Figure 4). In the surfactant CMC region and above, there was more than a 10-fold increase in the diffusion coefficient estimated at infinite surface dilution (see Figure 4 and Table 1). A study of competitive adsorption of β-lactoglobulin and β-casein with the surfactants Tween 20, SDS, and CTAB at the air−water interface reported an increase in protein diffusion at the air−water interface with surfactant concentration,[36] but this is to our knowledge the first report on surfactant effect on protein diffusion on solid surfaces. Different studies have suggested that anionic and nonionic surfactants can affect lipases in bulk solution. NMR and microcalorimetry studies have revealed that only weak complex formation in bulk occurs between the *T. lanuginosus* lipase and the anionic surfactant SDS or the cationic TTAB.[31,32] However, molecular dynamics simulations have indicated that although the overall 3D structure of a lipase might be unaffected by charged surfactants, flexibility, and conformation movements as well as internal motion is affected.[33] Studies on the thermal stability of *T. lanuginosus* revealed that SDS and TTAB significantly destabilized the enzyme whereas nonionic detergents had no impact on stability.[34] In the same study, it was found that low concentrations of all studied surfactants enhanced the activity of TLL and that high concentrations led to an enzyme-inhibiting complex. Jutila et al.[35] have proposed that a small nonionic surfactant at low concentrations might intercalate into the active site region, competing with a monomeric substrate and thereby leading to reduced activity and at the same time a stabilization of the open conformation. It is unclear to what extent these observations affect lipase/surfactant/surface interactions at solid−liquid interfaces.

To study the mechanism behind the surfactant modulation of lipase diffusion at a solid surface, the ability of the surfactant mixture to displace the protein from the hydrophobic surface was tested in the CLSM (Figure 5A). Displacement of TLL by surfactants definitely occurred, since the TLL signal intensity decreased about 40% for surfactant concentrations at or above the CMC region. To account for effects of autobleaching, the signal was normalized with the signal found when no surfactants were present in the system. The results were supported by SPR competition experiments (Figure 5B). The adsorbed amount on the surface after coadsorption of TLL and $C_{12}E_6$:LAS was dominated by a signal that is identical to surfactants alone at concentrations at CMC and above, suggesting that TLL essentially is competed off the surface by the surfactants. Nonionic surfactants are known to desorb both lipases from silica surfaces[37] as well as other protein from air−water interfaces.[12,38] The TLL surface density for the FRAP experiments was always measured prior to the bleaching, and the θ/θ_{max} displayed in Figure 4 is a measure of the initial surface density. However, since the surfactants evidently displaced some of the protein, this means that the effective surface density may decrease during the experiment due to TLL displacement to the bulk phase. This effect may contribute to the apparent increase in the diffusion as compared to when no surfactants were present: lower effective density of protein at the interface results in a reduced sterical repulsion for the diffusing molecules.

We have earlier observed a time dependence on the diffusion coefficient of a different variant of TLL, most evidently at low surface densities and on hydrophobic surfaces.[5] The results suggested that the decrease in mobility with time was due to a rotation−diffusion process of the adsorbed TLL molecules: With time a larger fraction of the TLL molecules assume an orientation that minimizes the free energy of adsorption. The redistribution of the adsorbed TLL molecules with respect to surface orientation might well be coupled with a conformational change in structure of TLL (movement of the so-called lid), exposing the active site and a more hydrophobic region of the enzyme. This "docked configuration" of TLL is expected to have a very low mobility and thus contribute to the immobile fraction derived from our FRAP experiments. We also found a decrease in surface mobility of TLL as a function of time when coadsorbed with high $C_{12}E_6$/LAS concentrations (Table 1). We explain the decrease in diffusion rate with time in the presence of surfactants as a combined effect of the rotation−diffusion equilibration process and a surfactant-induced desorption of the most mobile TLL from the surface. Rebinding of displaced enzymes would contribute to an apparent increase in mobility of the lipase population at the solid−liquid interface. However, this phenomenon is counteracted by the scattering into bulk solution of a large fraction of the lipases which are competed off the surface (cf. Figure 5). In this way the most mobile fraction of adsorbed lipases is diluted from the surface, thereby contributing to the apparent decrease in surface diffusion with time. For no or low surfactant concentration there seem to be a decrease in surface mobility, yet the exponential fit of the data gives $D_{0,t=90-180} = D_{0,t=0-90}$ (Table 1). The reason for this might be the few data points used for the exponential fit. The mobile fraction was only measured during 0−90 min after adsorption and might have decreased with time as well. Taking this into account, the diffusion coefficients measured during the second time interval (90−180 min) would have been even lower if calculated with the mobile fraction measured during the same time interval.

To get additional insight into the surfactant-induced increase of TLL mobility, FRAP measurements under flow of the surfactant solution were performed. Diffusion was measured at low TLL surface densities ($\theta/\theta_{max} = 0.1-0.2$) with a surfactant concentration in the CMC region (150 μM) (see Figure 4C). The flow would dilute the displaced TLL more effectively into the bulk, reducing the probability of TLL to rebind to the surface. Since these measurements led to a lower apparent diffusion rate compared to the measurements performed under no-flow conditions, desorption−rebinding was likely to have a significant contribution to the recovery of the fluorescence signal. The diffusion coefficients of TLL estimated with surfactants should therefore not be taken as a measure of pure lateral diffusion on a surface but rather as a description of the total migration capacity of TLL under the present experimental conditions without external stirring. The FRAP method used in this study was based on measuring the magnitude of the maximum slope between the bleached and unbleached areas. If both unbleached and bleached TLL are equally displaced by the surfactants, it should have no impact on the data analysis procedure. We expect that the

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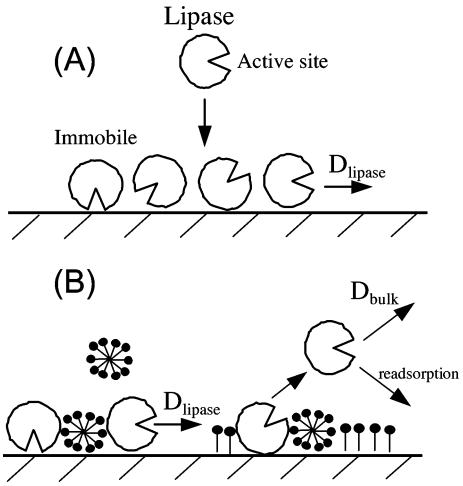

Figure 6. Model for lipase migration at a solid/liquid interface without surfactants (A) and in the presence of surfactants (B). The model is further explained in the text.

rebinding of a TLL molecule in our experimental setup is a local phenomenon occurring close to the desorption site. If the lipases were rebound at random distance from the desorption site, the fluorescence recovery in the bleached square would have occurred more equally throughout the whole bleached region. This was not observed in the collected images: the intensity increased only at the border of the bleached and unbleached areas, and the relaxation of the intensity profile could be effectively described by the derived lateral diffusion equation.

A proposed model for lipase surface mobility is given in Figure 6. Without surfactants, lipases migrate across the hydrophobic surface through lateral diffusion (Figure 6A**)**. The docked configuration, with the active site exposed to the surface, is assumed to represent an adsorbed state of very low mobility, which is practically immobile in the FRAP experiment. In the presence of surfactants, a fraction of the adsorbed lipases are more prone to be displaced from the surface by surfactants the more mobile they are (Figure 6B). The redistribution via desorption−rebinding, without diffusion into the bulk phase, contributes to an apparent higher diffusion coefficient as well as to a decrease in observed mobility with time since the most mobile species are diluted off the surface.

5. Conclusions

We have shown that surfactants perturb the binding of a TLL to hydrophobic surfaces. SPR and fluorescence CLSM analyses showed that enzyme was competed off the surface by $C_{12}E_6$/LAS starting at concentrations below the CMC. When lipase and surfactants were co-injected over the hydrophobic surface, SPR indicated that above the CMC the surface excess (at steady-state and after end-of-injection) essentially was due to surfactants at the interface. When lipase was adsorbed onto a hydrophobic surface and displacement by $C_{12}E_6$/LAS was monitored by fluorescence CLSM, desorption of enzyme depended both on time exposed to the surfactant solution and surfactant concentration. A maximum of 50% desorption of initially bound lipase was observed after 2 h at three times the CMC of the $C_{12}E_6$/LAS system.

The mobility at a hydrophobic surface of the fluorescently labeled lipase was quantified by FRAP as a function of the surfactant concentration and initial surface density of the enzyme. Upon coadsorption of lipase and $C_{12}E_6$/LAS, and subsequent exposure to the surfactants, the apparent surface diffusion at infinite surface dilution increased by an order of magnitude, and the mobile fraction doubled on passing the CMC of the surfactant system. In the presence of surfactants we suggest that migration has contributions of both rotation−diffusion of the enzyme on the surface and desorption−rebinding processes.

Acknowledgment. Novozymes A/S is acknowledged for financial support of the project.

LA0531244