Study of the interaction and activation of lipase from *Pseudomonas fluorescens* in surfactant monolayers and precipitates

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The activation of lipase from *Pseudomonas fluorescens* (PFL) upon its immobilization in surfactant coprecipitates (hexadecane-1,2-diol (HDD), cetyl alcohol (CetOH), *N*-cetylacetamide (CetAA), and cetylamine ($CetNH_2$)) organized in monolayers at the interface were studied by the Langmuir—Blodgett monolayer technique. Incorporation of the enzyme into surfactant monolayers at the surface pressure $\pi = 10$ mN m^{-1} results in an apparent increase in the area per molecule. In the series of noncharged surfactants CetOH—HDD—CetAA, this effect increases in proportion to the amount of the enzyme incorporated in the monolayer. The catalytic activity of the lipase—surfactant coprecipitates in an organic solvent as regards esterification increases in the same sequence, indicating similarity of the interaction of lipase with surfactant monolayers and coprecipitates. For $\pi = 10$ mN m^{-1}, the $CetNH_2$ monolayer with liquid-expanded state incorporates the largest amount of the enzyme (PFL : $CetNH_2$ = 1 : 290); the CetOH monolayer, which exists in the condensed state under the same conditions, incorporates the smallest amount (PFL : CetOH = 1 : 1700). The hydrolytic activity of PFL in mixed monolayers with surfactants increases 1.5—11-fold; the esterification activity in surfactant coprecipitates, 1.6—9-fold. The lipase activation effects are explained by facilitated transport of substrates into mixed monolayers and surfactant—enzyme precipitates in aqueous and organic media, respectively.

Key words: lipase, immobilization, monolayers, surfactants, Langmuir film balance.

The formation and use of organized monolayers attracts considerable attention in relation to fundamental research dealing with the simulation of biological membranes, study of lipid—protein interactions, and targeted changes in the properties of oriented molecules of synthetic and natural compounds. In enzymological studies, the method of monolayers is mainly used to study the kinetics of enzymatic reactions occurring at air—water interfaces and to study the structures of lipid—protein films.[1—3]

Lipases (EC 3.1.1.3) occupy a special place among hydrolytic enzymes, as they can be used both in the hydrolysis of lipids and esters and in enantioselective esterification and transesterification in organic solvents or in water-organic systems.[4] Lipids, natural lipase substrates, are highly associated in an aqueous medium and enzyme activation takes place in contact with the interface.[5] Adsorption of lipases on hydrophobic substrates is known to increase their catalytic activity.[6—8] Previously,[9,10] we have demonstrated an increase (up to 12.5-fold) in the catalytic activity of lipase with respect to esterification upon incorporation into precipitates of some surfactants, namely, hexadecane-1,2-diol (HDD), cetyl alcohol (CetOH), cetylamine ($CetNH_2$), and *N*-cetylacetamide (CetAA). However, these studies have not been concerned with the nature of lipase interaction with these surfactants, which is the goal of the present work.

To study the enzyme activation upon immobilization, we propose for the first time to use the method of monolayers. This method allows determination, with high accuracy, of the surface concentration and the two-dimensional arrangement of the surfactant monolayer and observation of the variation of the monolayer properties during incorporation of the lipase. The use of this method allowed us to study the effect of the nature of the surfactant and the monolayer properties on the catalytic activity of lipase precipitates at the molecular level. This is important for the understanding of the mechanism of lipase activation upon its immobilization and opens up the way for the development of highly efficient catalysts.

Experimental

A freeze-dried sample of purified lipase from *Pseudomonas fluorescens* (PFL) (Fluka, Switzerland) and a commercial sample of lipase from *Pseudomonas fluorescens* (enzyme content 0.25% w/w) provided by the RÖHM Pharma Polymers company (Germany) were used. The specific activities of these samples in

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hydrolysis of triacetin were 12 and 0.12 mmol AcOH h^{-1} (mg of the specimen)$^{-1}$, respectively. Cetylamine ($CetNH_2$), hexadecane-1,2-diol (HDD), cetyl alcohol (CetOH), *tert*-butyl methyl ether (Bu^tOMe), *p*-nitrophenyl propionate (NPP), 1-(*R,S*)-phenylethanol, vinyl acetate (Fluka, Switzerland), tris(hydroxymethyl)aminomethane (Reanal, Hungary), KH_2PO_4 (analytical grade), $CHCl_3$, and DMSO (Reakhim) were used as received. *N*-Cetylacetamide (CetAA) was prepared as described previously.[10]

The enzymatic activity of lipase in the hydrolysis of triacetin was determined by potentiometric titration with alkali of the acetic acid formed in the reaction using a Radiometer Copenhagen TTT 60 instrument (Denmark).[9,10]

The enzymatic activity of lipase in esterification was determined from the initial rate of acetylation of 1-(*R,S*)-phenylethanol with vinyl acetate in Bu^tOMe.[9,10]

The enzymatic activity of PFL in hydrolysis of NPP was calculated from the initial rate of accumulation of the *p*-nitrophenolate anion (ε = 12800 L mol^{-1} cm^{-1}) using the Fletcher method[11] by spectrophotometric monitoring of the absorption at 405 nm on a Gilford Instrument 2400-2 spectrophotometer (USA). The change in the optical density in a cell containing 25 μL of a 0.1 *M* solution of *p*-nitrophenyl propionate in DMSO, 50 μL of the lipase solution, and 0.925 mL of 0.05 *M* Tris—HCl buffer (pH 7.5) was used to calculate the enzymatic activity (nmol of *p*-nitrophenolate anion h^{-1} (mL of enzyme solution)$^{-1}$).

All experiments with monolayers were carried out on a Film Balance instrument (Lauda, Germany) operating according to the Langmuir film balance principle.[2]

The monolayers were formed by applying a solution of a surfactant in $CHCl_3$ onto the surface of a 0.01 *M* KH_2PO_4 solution, pH 7.5, or onto the surface of lipase solutions of various concentrations in this buffer at 20 °C. A 10 m*M* solution of a surfactant (12 μL) in $CHCl_3$ was applied onto a thoroughly cleaned buffer surface between the moving and measuring barriers using a microsyringe (Hamilton, USA). The monolayers were kept for ~10 min for complete evaporation of the solvent and for 1 h for lipase incorporation into surfactant monolayers. Then the monolayer film was compressed by moving the barrier at a constant rate (1 cm min^{-1}), and the isothermal dependences of the surface pressure (π) and the surface potential (U) on the area per surfactant molecule in the monolayer (A) were recorded. Each isotherm was recorded 2—4 times, the error of measurements being ±5%. The curves for time dependence of the monolayer area (A) were measured at a constant pressure π = 10 mN m^{-1} over periods of 15 min and 1 h. The area per molecule at a constant pressure (A_f) in the monolayer was determined by extrapolating the straight section of the time dependence of the monolayer area to zero time.

The change in the monolayer area at a pressure of 10 mN m^{-1} was calculated as

$$\Delta A_\pi\,(\%) = [(A_f^{10} - A_{15})/A_{15}] \cdot 100,$$

where A_f^{10} is the area per molecule in the monolayer found by extrapolating the straight section of the isotherm at π = 10 mN m^{-1} to zero in time, A_{15} is the area per molecule upon compression (after 15 min).

The relative stability of the mixed monolayer with respect to the monolayer of the pure surfactant was calculated as the ratio of the slopes of the straight sections of the corresponding time dependences of the area per molecule at π = 10 mN m^{-1}.

The change in the monolayer area (ΔA) caused by incorporation of the PFL molecule at different protein concentrations in the subphase was calculated from the relation

$$\Delta A\,(\%) = [(A_f - A_f^{Surf})/A_f^{Surf}] \cdot 100,$$

where A_f is the area per molecule after incorporation of lipase into the surfactant monolayer; A_f^{Surf} is the area per molecule for the pure surfactant.

After closing the moving barrier, the monolayer was withdrawn from the tank surface using a 100 μL microsyringe (Hamilton, USA). Removal of the monolayer from the surface of the aqueous phase resulted in a change in the area confined by the tank walls and the moving barrier, which maintained the surface pressure equal to 10 mN m^{-1}.

The wetting angle was determined by applying drops of water (2.5 μL) on the surface of pellets obtained by pressing the substances in a 5-mm deep mold 15 mm in diameter on a hydraulic press (Carl Zeiss, Jena (Germany)) at a pressure of 10 MPa for 10 min. The drop image was recorded using an OLYMPUS E-20 digital camera (Japan). On the photographs of the drops thus obtained, the average wetting angle for each compound was measured by a protractor.

Microscopic examination of the structure of the precipitates obtained was done using an optical microscope (Carl Zeiss, Jena (Germany)).

Results and Discussion

All the surfactants we studied are able to form stable monolayers at the water—air interface. The isotherms for the dependence of the surface pressure (π) on the area per surfactant molecule in a monolayer (A) are shown in Fig. 1. It can be seen that the isotherms for the monolayers of different surfactants follow qualitatively different patterns. These differences are related to the interaction of the hydrophilic part of the molecule with the aqueous phase because the hydrophobic moieties of the surfactant molecules are identical ($-C_{16}H_{33}$). The collapse pressure for the surfactants studied is ~48 mN m^{-1} for CetAA, CetOH, and HDD and 37 mN m^{-1} for $CetNH_2$, which suggests their relative stability at the water—air interface.[2] At the collapse pressures, *i.e.*, with the closest packing of the hydrophobic parts of the molecules, the areas (A) for the CetOH, HDD, CetAA, and $CetNH_2$ monolayers are equal to 0.19, 0.17, 0.16, and 0.14 nm^2, respectively. A different order is found for the liquid-expanded state of monolayers; for π = 5 mN m^{-1}, the area (A) decreases in the sequence $CetNH_2$, HDD, CetAA, and CetOH: 0.40, 0.34, 0.28, and 0.25 nm^2, respectively. The increase in the monolayer area for the surfactants implies a decrease in the degree of ordering of molecules in the monolayers due to different polarities of the hydrophilic heads of the surfactant molecules. Of the surfactants considered, only CetOH forms a condensed monolayer with a very low compressibility. This suggests the perpendicular orientation of the CetOH molecules relative to the interface at surface pressures above 10 mN m^{-1}. The average area of

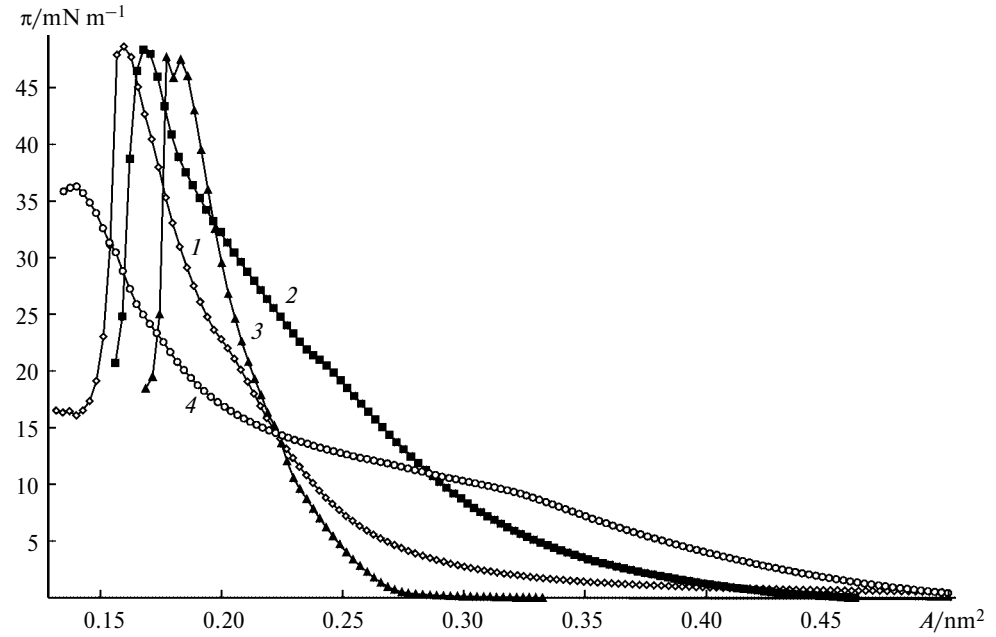

Fig. 1. Surface pressure (π) *vs.* area per molecule (*A*) isotherms for the monolayers of various surfactants: CetAA (*1*), HDD (*2*), CetOH (*3*), and $CetNH_2$ (*4*).

the CetOH molecule is 0.23 nm^2, *i.e.*, it is determined by the hydrocarbon fragment whose cross-section was reported[12] to be 0.22 nm^2.

To estimate the interaction of lipase with the surfactants, we studied the sorption of PFL molecules on the surfactant monolayers. The monolayers were formed directly on the protein solution rather than the protein was introduced in the subphase after the formation of a surfactant monolayer, as is usually done in the studies of lipid and surfactant monolayers with proteins.[1,12] This procedure allows one to observe the uniform distribution of the protein in the film, as was shown previously for cytochrome *C* by fluorescence microscopy.[13]

The effect of the protein concentration in the subphase on the change in the area of the surfactant monolayer after incorporation of the protein can be studied most conveniently by monitoring the changes in the area of a mixed monolayer at a constant pressure. At π = 10 mN m^{-1}, the surfactants we used are stable (see Fig. 1), their parameters being retained for 1 h at a specified constant pressure. The initial sharp decrease in the area (*A*) with time (less than 2 min) is due to the monolayer compression by the moving barrier until a constant pressure of 10 mN m^{-1} is reached. Note that the CetOH monolayer is the most stable; the next are CetAA, HDD, and $CetNH_2$, for which the monolayer areas change after compression to 10 mN m^{-1} by 1, 2.5, 6.2, and 17% (Table 1). The decrease in the area (*A*) for the monolayers of pure surfactants can be explained by mutual reorientation of molecules, resulting in equilibration and a closer packing of molecules in the monolayer. The CetOH monolayer exists, at the given pressure (π = 10 mN m^{-1}), in the condensed state, and exhibits the smallest change in the monolayer area. In the case of $CetNH_2$ whose monolayer undergoes transition from the liquid-expanded to the liquid-condensed state in the pressure range of 9—12 mN m^{-1}, an appreciable change in the monolayer area occurs at 10 mN m^{-1}.

Table 1. Decrease in the monolayer area (ΔA) over a period of 15 min at a pressure of 10 mN m^{-1}

Surfactant	ΔA_π (%)		ΔS^a
	I[b]	II[c]	
CetOH	0.9	1.3	1.74
CetAA	2.5	3.4	1.81
HDD	6.2	7.4	1.2
$CetNH_2$	17	13	0.9

[a] Relative stability of the mixed monolayer compared to the surfactant monolayer.
[b] For the individual surfactant.
[c] For the lipase—surfactant mixed monolayer; lipase concentration 100 μg L^{-1}.

Figure 2 shows the time dependences of the area per surfactant molecule at a constant pressure 10 mN m^{-1} and at different lipase concentrations within the bulk of the Langmuir tank. The apparent increase in the area per surfactant molecule in a mixed monolayer is related to incorporation of the enzyme into the monolayer and occurs for all four surfactants upon an increase in the lipase concentration. This indicates an enhanced incorporation

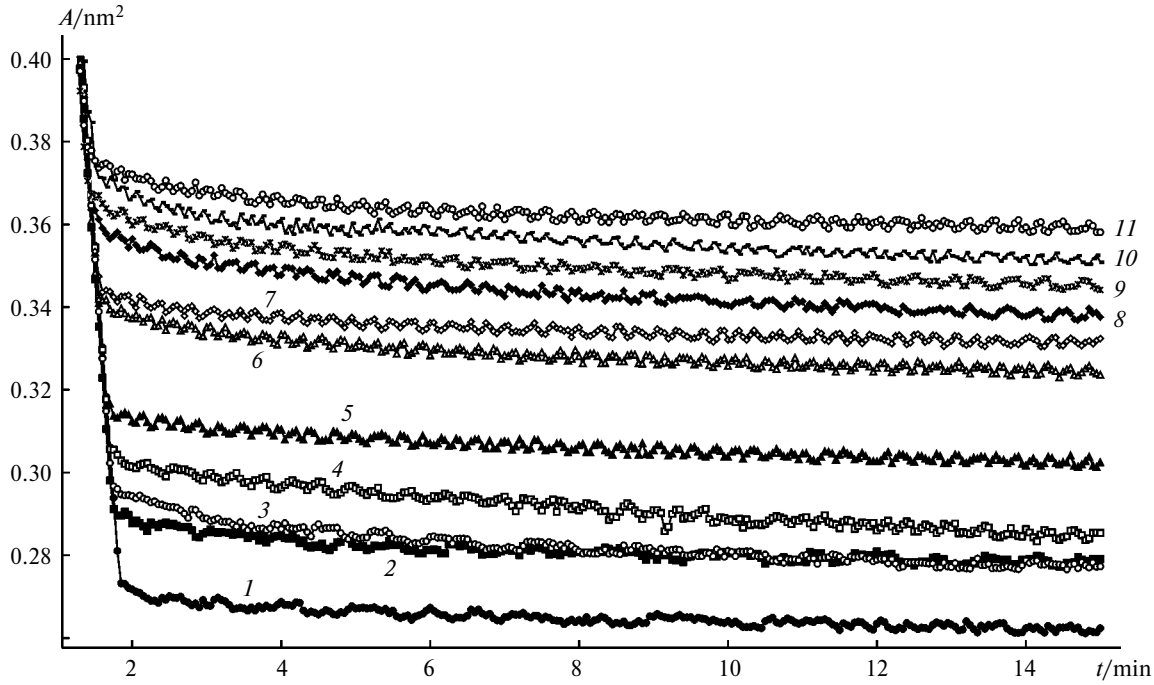

Fig. 2. Area per molecule (*A*) *vs.* time (*t*) for CetAA (*1*) at a surface pressure of 10 mN m^{-1} and different lipase concentrations in the subphase: 1 (*2*), 2 (*3*), 10 (*4*), 20 (*5*), 30 (*6*), 40 (*7*), 60 (*8*), 80 (*9*), 100 (*10*), and 200 μg L^{-1} (*11*).

of PFL into the surfactant monolayer following an increase in the concentration of this lipase in the subphase. The dependences of the area of the mixed monolayer (ΔA) on the lipase concentration in the subphase for all the surfactants used are shown in Fig. 3. When the protein concentration in the subphase is 3 nmol L^{-1} (100 μg L^{-1}), the mixed monolayer is saturated with lipase (see Fig. 3). The most pronounced change in the monolayer area upon incorporation of the lipase (35%) is observed for CetAA. This can be explained by relatively weak interaction of CetAA with water and, correspondingly, by high free energy of the surface of contact between them. The smallest change in the area (10%) after incorporation of the lipase into the CetOH monolayer can be due to the rigid condensed structure of the initial CetOH monolayer, which prevents the lipase molecule from passing from the subphase to the monolayer. The increase in the monolayer area after lipase incorporation by 28% for HDD and by 20% for $CetNH_2$ is due to building-in of the protein into the surfactant monolayers that are less dense than that of CetOH. An increase in the number of absorbed PFL molecules on the surface of a lipid monolayer with an increase in the protein concentration in the subphase has been observed previously.[14–16]

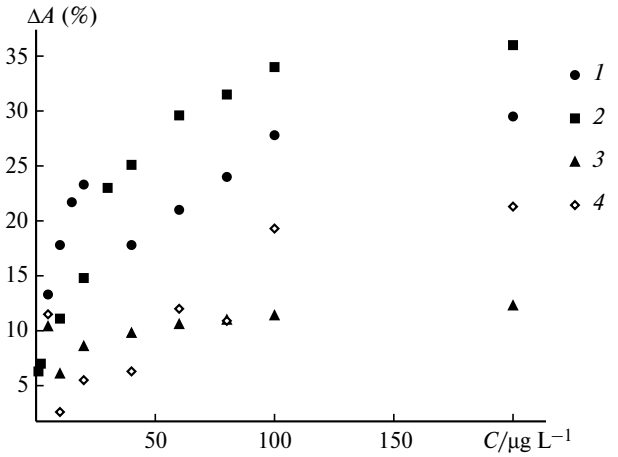

Fig. 3. Change in the area of the lipase—surfactant mixed monolayer (ΔA) *vs.* the concentration of lipase (*C*) for HDD (*1*), CetAA (*2*), CetOH (*3*), and $CetNH_2$ (*4*).

The change in the slope of the time dependences (see Fig. 2) prompts the conclusion that inclusion of lipase into the surfactant monolayers changes the stability of the mixed monolayer (see Table 1). Upon the saturation of monolayers with the protein, a certain stabilizing effect is observed only for $CetNH_2$, which may be due to the formation of $CetNH_2$—lipase associates caused by the electrostatic interaction between $CetNH_2$ acting as a base ($pK = 10.6$)[17] and PFL acting as a weak acid ($pI = 4.46$)[18] at neutral pH of the subphase. On sorption of lipase on the surfactant monolayers, the stability of the resulting mixed monolayers decreases. The observed increase in the slope of the time dependences can be due to either squeezing-out of some of the embedded protein molecules to the subphase or reorientation of surfactant molecules and the protein globules in the monolayer, which typically occurs with time at a constant pressure. The

Table 2. Activity[a] of lipase in lipase—surfactant mixed monolayers and in the bulk of the Langmuir tank after sorption of the enzyme on the monolayer

Surfactant	A_1[b]	A_2[c]	A'[d] (%)	A_3[e]	A_4[f]	A_4/A_3[g]
$CetNH_2$	94	62.4	34	31.6	47.0	1.5
CetAA	100	78.3	22	21.7	69.0	3.1
HDD	96	85.8	11	10.2	111.0	11.0
CetOH	100	93.6	6	6.4	62.4	9.8

[a] In nmol of *p*-nitrophenolate anion h^{-1} (mL of enzyme solution)$^{-1}$.
[b] The initial activity of lipase solution, *U*; lipase concentration 100 μg L^{-1}.
[c] Activity in the bulk of the Langmuir tank, *U*.
[d] The amount of the protein incorporated in the monolayer was calculated from the relation $[(A_1 - A_2)/A_1] \cdot 100$.
[e] The activity in monolayers calculated with the assumption of the absence of lipase activation, *U*; $A_3 = A_1 - A_2$.
[f] The observed activity in the mixed monolayer, *U*.
[g] The apparent activation of lipase in the monolayer.

influence of the lipase on the structure of surfactant monolayers will be considered below.

In order to confirm the inclusion of lipase into the surfactant monolayer and to estimate the amount of the included enzyme, we determined the lipase activity simultaneously in the mixed monolayers and in the subphase (Table 2). The known amount of lipase in the subphase can be used to estimate the amount of lipase adsorbed on the monolayer. The largest amount of the enzyme has been incorporated into the $CetNH_2$ monolayer; the next are CetAA, HDD, and CetOH, namely, 222, 120, 63, and 38 μg of lipase m^{-2}, respectively (or 34, 22, 11, and 6% of the initial lipase). Thus, one can conclude that the change in the area of surfactant monolayers upon lipase incorporation is proportional to the amount of lipase incorporated into the monolayer, except for the basic $CetNH_2$ (see Fig. 3, Table 2). The maximum inclusion of lipase into $CetNH_2$ coprecipitates (more than 99%) has been observed in our previous study[10] (Table 3). Taking into account the amounts of lipase and the surfactant in the monolayer and the fact that a pure lipase preparation was used in this study, we find that the monolayers contain one PFL molecule per 290 $CetNH_2$ molecules, 530 CetAA molecules, 1000 HDD molecules, and 1700 CetOH molecules. Activation of the enzyme on the monolayer surface is observed for each of the surfactants, as the overall activity of the protein in the subphase and in the monolayer is higher than the initial activity. The most pronounced (11-fold) activation of lipase takes place upon incorporation into the HDD monolayer; this is followed by CetOH, CetAA, and $CetNH_2$ (9.8-, 3.1-, and 1.5-fold, respectively). The activation of lipase at an interface is known to be related to conformational changes in its macromolecules, in particular, to displacement of the

Table 3. Enzymatic activity of the initial lipase and its coprecipitates with various surfactants[10]

Surfactant	Amount of the enzyme in the coprecipitate (%)	Activity, *U**	
		I**	II***
—	—	52000	1530
CetAA	65	24000	19000
$CetNH_2$	99	30000	13600
HDD	85	58800	9300
CetOH	80	18000	2500

* In μmol of the product h^{-1} (mg of the enzyme)$^{-1}$.
** For triacetin hydrolysis at 25 °C.
*** For acetylation of 1-(*R*,*S*)-phenylethanol at 37 °C.

polypeptide loop, which shields the active site of the enzyme.[19]

In the system we studied, the highest enzyme activity was found for mixed monolayers of lipase and the surfactants whose hydrophilic fragments contain a hydroxy group. Note that the least pronounced activation of the enzyme, found for $CetNH_2$, corresponds to the largest amount of PFL included into the monolayer of this amine and that a decrease in the protein inclusion into the surfactant monolayer entails an increase in the hydrolytic activity of PFL in the mixed monolayer (see Table 2). A similar situation is also observed upon variation of the enzyme load on insoluble substrates. With a decrease in the lipase load on solid substrates, the specific activity of the immobilized substance increases. This is related to the efficiency of the diffusion flux of the substrate in an enzymatic reaction, which is increased for low contents of the enzyme.[20]

Using the monolayer technique, one can gain an idea of the monolayer structure before and after the interaction of lipase with the surfactant. Lipase does not form a stable monolayer at the water—air interface (Fig. 4); therefore, one can consider the interaction of the enzyme with the surfactant by comparing the isotherms obtained for the pure surfactant and for the mixed surfactant—protein monolayer. For CetOH, HDD, and CetAA, the monolayers of pure surfactants are more stable than the mixed monolayers with lipase: the collapse pressure and the stability with time at a constant pressure is higher for pure surfactants (see Fig. 4, Table 1). When the protein is embedded in the CetOH monolayer, the monolayer structure does not change much. This is indicated, first, by the fact that the isotherms of the mixed and neat monolayers have the same shape and differ only in the apparent A_f, which has increased for the mixed monolayer upon binding of PFL. Second, in the region of collapse of the mixed monolayer, their isotherms coincide, which may be due to squeezing-out of the protein molecules to the subphase. In addition, CetOH showed the smallest change in the

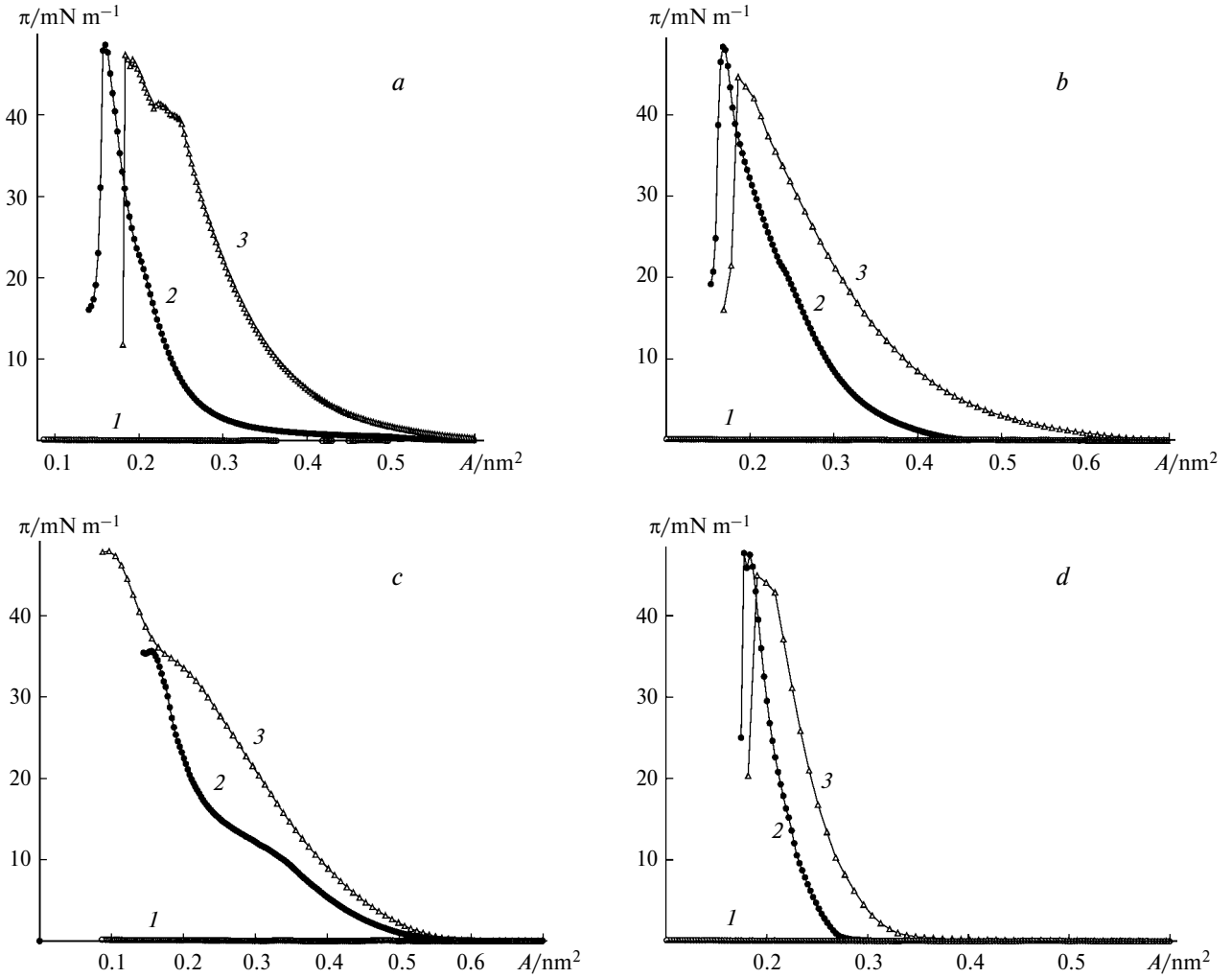

Fig. 4. Surface pressure (π) *vs.* area per molecule (*A*) isotherms for CetAA (*a*), HDD (*b*), $CetNH_2$ (*c*), and CetOH (*d*): lipase, 100 μg L^{-1} (*1*), surfactant (*2*), lipase—surfactant (*3*).

monolayer area after inclusion of the protein molecule, which is due to the rigid condensed structure of the CetOH monolayer poorly accessible for incorporation of the enzyme molecule. Thus, the CetOH monolayer tends to incorporate the protein molecules without structural changes in the surfactant monolayer.

The interaction of CetAA, HDD, and $CetNH_2$ with lipase (complex formation) is inferred from the fact that the π—*A* isotherms of the corresponding mixed monolayers differ appreciably from the isotherms of pure surfactants. The lipase—HDD and lipase—CetAA mixed monolayers are characterized by marked liquid-expanded states at relatively low pressures and show no inflection of the isotherm on passing from the liquid-expanded states to the condensed states, which is observed for pure HDD and CetAA monolayers. One can suggest that the interaction of lipase with the HDD and CetAA monolayers involves both the formation of associates and incorporation of proteins into surfactant monolayers as in the case of CetOH. The interaction with lipase is more pronounced for CetAA due to the change in the mixed monolayer structure at a pressure of 40 mN m^{-1}, which is due apparently to squeezing-out of protein globules to give the condensed structure of the mixed monolayer whose strength is virtually equal to the strength of the neat monolayer.

A complete change in the structure of the mixed monolayer compared to the monolayer of a pure surfactant is observed for $CetNH_2$. This is due to electrostatic interactions, which prevent close packing of molecules in the mixed monolayer, and to the fact that the $CetNH_2$ monolayer has adsorbed the largest amount of lipase. The lipase—$CetNH_2$ mixed monolayer is the most stable and is characterized by a collapse pressure of 48 mN m^{-1}, which markedly exceeds the collapse pressure for the monolayer of pure $CetNH_2$ (35 mN m^{-1}). We noted a stabilizing effect of PFL on the $CetNH_2$ monolayer related to the electrostatic attraction between oppositely charged molecules when discussing the change in the area (*A*) at a

constant pressure (see Table 1). It is noteworthy that at a pressure of ~10 mN m^{-1}, the monolayer of pure $CetNH_2$ passes from the liquid-condensed to liquid-expanded state and subsequently, at ~20 mN m^{-1}, the monolayer passes into a highly organized condensed state. These effects are not observed for the mixed monolayer. At a pressure of $\pi < 35$ mN m^{-1}, *i.e.*, before the collapse pressure of the monolayer of pure $CetNH_2$, the mixed monolayer has a liquid-condensed structure, which passes into the condensed state due to squeezing-out of some of the protein molecules into the subphase. Thus, inclusion of the protein into the $CetNH_2$ monolayer not only changes the structure of the mixed monolayer, but also increases its strength and stability. It is of interest to compare the results obtained with the results[21] of investigation of glucose oxidase—lipid complexes at a water—air interface. The addition of the cationic head group of the lipid to the negatively charged surface of glucose oxidase gave a complex comprising 150—250 lipid molecules per enzyme molecule, which contained 24% (w/w) of the protein, the yield of the complex being 84%, whereas the noncharged lipid formed a complex containing 3% protein in 30% yield.

Thus, a more pronounced liquid-expanded state of the monolayer at moderate pressures (<15 mN m^{-1}) and an increase in the monolayer area upon reorientation of surfactant and protein molecules and squeezing-out of some of the enzyme molecules at a pressure close to the monolayer collapse is a typical feature of lipase—surfactant mixed monolayers. The amount of absorbed lipase depends, first of all, on the structure of the surfactant (in our case, on the nature of the hydrophilic group) and on the protein concentration in the subphase.

The formation of lipase—surfactant associates can also be judged from the change in the electric properties of the monolayer. The orientation of the molecular electric dipoles in the monolayer can be monitored by following the changes in the surface potential (ΔU).[22] For the surfactants in question, the differences ΔU between the potential for the lipase—surfactant mixed monolayer and the potential for the monolayer of the pure surfactant are equal to 45, 60, 70, and 190 mV for CetOH, HDD, $CetNH_2$, and CetAA, respectively. As noted above, the hydrophobic parts of the surfactant molecule are the same; therefore, ΔU is determined by the nature of protein interaction with the hydrophilic head of the surfactant. The largest ΔU and, hence, the most pronounced dipole-dipole interaction is observed upon inclusion of lipase into the CetAA monolayer and the smallest value is found for CetOH. This is in line with the data on the increase in the area (ΔA) per molecule in the mixed monolayer relative to the monolayer of the pure surfactant (see Fig. 3).

It is of interest to compare the results obtained in this study using the monolayer technique with our data[9,10] on the catalytic activities of coprecipitates of the same surfactants with lipase. The activity of the coprecipitate particles was measured both in water and in an organic solvent. In both cases, the activity of the lipase preparation is limited by the rate of diffusion of the substrate to the enzyme active site. It is clear that the diffusion rate is directly related to the permeability of the coprecipitate particles for the solvent. In Bu^tOMe, the coprecipitates are fully wettable, whereas in water, wetting of these compounds increases in the sequence CetAA, $CetNH_2$, CetOH, and HDD (the wetting angles are 60, 100, 108, and 145°, respectively). In the aqueous medium, the coprecipitate having the highest wettability, *i.e.*, HDD, is the most active. The hydrolytic activity of the enzyme in the aqueous medium increases both in the HDD precipitate and in the monolayer collected from the surface. In both cases, the lipase—HDD associates are the most accessible for the substrates (triacetin and NPP). Apart from wetting effects, more specific interactions of the enzyme with the surfactant might also be involved; lipase stabilization under the action of *vic*-diols (for example, sorbitol) has been noted previously.[23] In the case of other surfactant—lipase coprecipitates, only near-surface layers of the coprecipitate are accessible for the substrate due to poorer wetting, and the reaction rate is limited to a larger degree by the specimen wettability. For the lipase coprecipitates considered, the hydrolytic activity in the bulk is lower than the activity of the native enzyme, although it is activated in appropriate surfactant monolayers, as was noted above. The limited wettability of coprecipitates in water is clearly illustrated by optical microscopy.

Figure 5 shows the photomicrographs of the freeze-dried lipase—CetAA coprecipitate in water and in Bu^tOMe. Both dry and wetted coprecipitates of the enzyme and the surfactant are amorphous porous agglomerates of very small particles (~4 μm). In an aqueous medium, as in the solid state, these particles are opaque, *i.e.*, they are not wetted. Conversely, in Bu^tOMe, they are transparent because they are soaked with the solvent (the refractive index of the swollen porous particles is close to the solvent refractive index). In the aqueous medium, the coprecipitate aggregates are situated at the water—air interface (the white line in Fig. 5, *b*) due to poor wetting, whereas in Bu^tOMe, they are uniformly distributed throughout the whole bulk; as a result, the substrate transport inside the particles is facilitated and the lipase molecules are more accessible for the substrate.

Note that the catalytic properties of the enzyme in the monolayer and in the coprecipitate wetted by an organic solvent bear some similarity. Freeze-dried coprecipitates of lipase and the surfactant are accessible for a solution of the substrate in Bu^tOMe due to good wettability. In a mixed monolayer, lipase is open for substrate molecules due to nonhindered diffusion of the substrate from the bulk to the two-dimensional monolayer structure. Therefore, in both the Bu^tOMe medium and the monolayer,

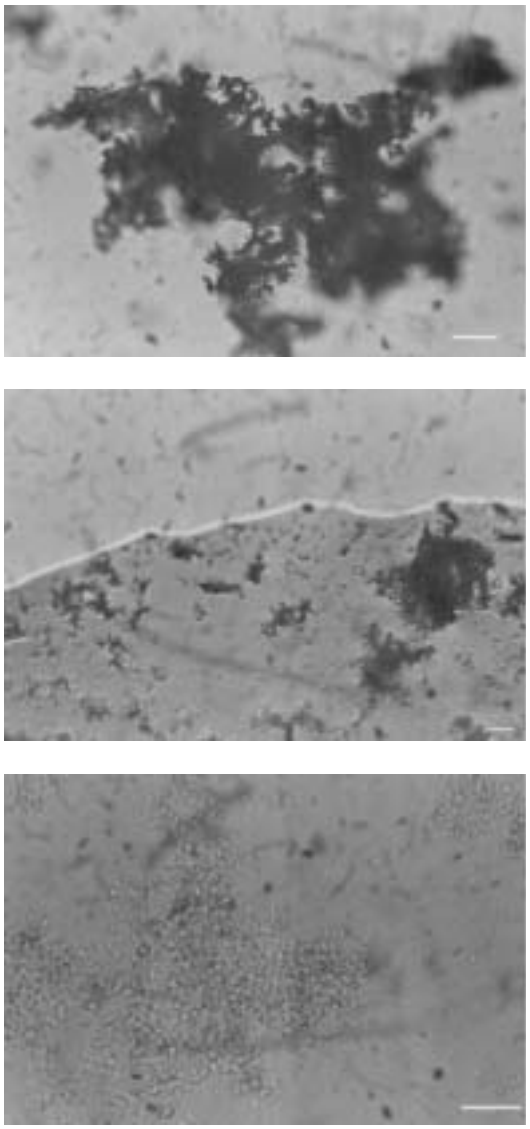

Fig. 5. Photographs of the lipase—CetAA coprecipitate in the freeze-dried form (*a*), in an aqueous medium (*b*), and in Bu^{t}OMe (*c*). Drawing scale 20 μm.

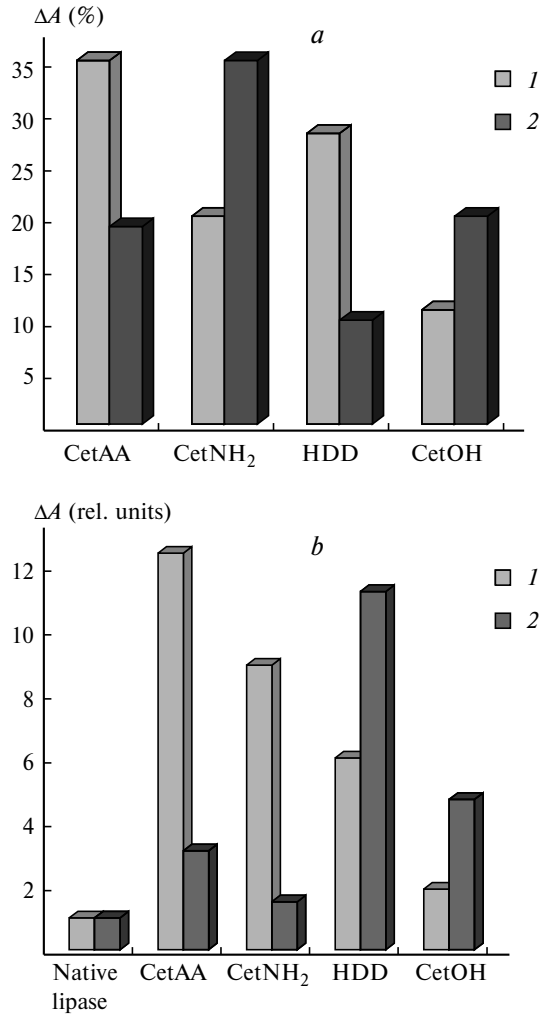

Fig. 6. Diagram of the increase in the catalytic activity (ΔA) of lipase in surfactant coprecipitates and monolayers (*a*): change in the area of the lipase—surfactant mixed monolayer (*1*) and amount of lipase incorporated in the monolayers (*2*). Amount of lipase incorporated into surfactant monolayers and change in their area (*b*): esterification activity of the precipitates (*1*) and hydrolytic activity in the monolayer (*2*).

lipase possesses a higher catalytic activity than in the initial state due to easier transport of substrates to the enzyme active site (Fig. 6, *a*).[10]

By comparing the parameters for lipase activation and inclusion into monolayers (see Fig. 6, *a* and *b*) with account of the differences in the structures of lipase—surfactant mixed monolayers (see Fig 4), the following conclusion can be drawn. A larger amount of lipase incorporated into the monolayer and a larger apparent area per molecule in mixed monolayers (see Fig. 6, *b*) entails a higher esterification activity of the corresponding precipitates in an organic solvent (see Fig. 6, *a*). This correlation indicates that incorporation of lipase into surfactant monolayers and precipitates occurs in similar ways, so the interaction of the enzyme with monolayers can mimic the processes of its immobilization upon coprecipitation with hydrophobic compounds. Cetylamine ($CetNH_2$) does not fit in this series, as the structure of its monolayer differs from those of other surfactants. The largest change in ΔA upon inclusion of PFL in $CetNH_2$ monolayers occurs at a surface pressure of 20 mN m^{-1} rather than at 10 mN m^{-1}, as accepted in this study; therefore, ΔA found for $CetNH_2$ cannot serve as a measure of the change in the activity of its precipitate by analogy with other surfactants.

Thus, in this work we confirmed at the molecular level the interaction of lipase from *Pseudomonas fluorescens* with a number of surfactants containing the hydrophobic $C_{16}H_{33}$ radical. The incorporation and activation of lipase in the monolayers and precipitates of the surfactants studied is a complex physicochemical phenomenon whose

parameters depend strongly on the properties of both the surfactants themselves and their monolayers. These may include the presence or absence of ionogenic functional groups, wettability of the surfactant by aqueous and organic solvents, specific features of the monolayer structure, and the capacity of monolayers for structural rearrangements in response to enzyme binding. Under conditions close to the saturation of surfactant monolayers by lipase, the resulting monolayer contains up to 20% (w/w) of the enzyme for noncharged surfactants and more than 30% (w/w) for $CetNH_2$ protonated in the aqueous medium. An important factor in displaying the potentially high lipase activity in mixed monolayers and in precipitates is accessibility of the enzyme for the substrate, which is determined by the efficiency of the diffusion flux of the substrate and related to the amount of the enzyme in the monolayers and to the permeability of the precipitate particles for solvents.

For the practical purposes of lipase immobilization in surfactant coprecipitates and their subsequent use in enzymatic esterification, one should apparently choose least water wettable surfactants (like CetAA) that simultaneously form monolayers, which possess a clear-cut liquid-expanded state and can be dissolved in hydrophilic organic solvents (acetone) needed for preparative coprecipitation with the enzyme.

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