Detergent-Induced Conformational Changes of *Humicola lanuginosa* Lipase Studied by Fluorescence Spectroscopy

Arimatti Jutila,* Keng Zhu,* Shamkant Anant Patkar,† Jesper Vind,† Allan Svendsen,† and Paavo K. J. Kinnunen*

*Helsinki Biophysics and Biomembrane Group, Department of Medical Chemistry, Institute of Biomedicine, University of Helsinki, Helsinki, Finland, and †Enzyme Design, Novo Nordisk, Bagsværd, Denmark

ABSTRACT Detergent (pentaoxyethylene octyl ether, C_8E_5)-induced conformational changes of *Humicola lanuginosa* lipase (HLL) were investigated by stationary and time-resolved fluorescence intensity and anisotropy measurements. Activation of HLL is characterized by opening of a surface loop (the "lid") residing directly over the enzyme active site. The interaction of HLL with C_8E_5 increases fluorescence intensities, prolongs fluorescence lifetimes, and decreases the values of steady-state anisotropy, residual anisotropy, and the short rotational correlation time. Based on these data, we propose the following model. Already below critical micellar concentration (CMC) the detergent can intercalate into the active site accommodating cleft, while the lid remains closed. Occupation of the cleft by C_8E_5 also blocks the entry of the monomeric substrate, and inhibition of catalytic activity at [C_8E_5] less than or equal to CMC is evident. At a threshold concentration close to CMC the cooperativity of the hydrophobicity-driven binding of C_8E_5 to the lipase increases because of an increase in the number of C_8E_5 molecules present in the premicellar nucleates on the hydrophobic surface of HLL. These aggregates contacting the lipase should have long enough residence times to allow the lid to open completely and expose the hydrophobic cleft. Concomitantly, the cleft becomes filled with C_8E_5 and the "open" conformation of HLL becomes stable.

INTRODUCTION

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3.) catalyze the hydrolytic cleavage of the ester bonds of long-chain triacylglycerols (Simons et al., 1997). The activity of these enzymes is greatly increased at the lipid-water interface, a phenomenon known as interfacial activation (Sarda and Desnuelle, 1958), which is also well documented for phospholipase A_2 (Pieterson et al., 1974). The models proposed to explain the interfacial activation of lipases can be grouped into two categories, substrate models and enzyme models (Simons et al., 1997; Volwerk and de Haas, 1982). In the former, factors such as an increase in substrate concentration at the interface (Brockman et al., 1973), better orientation of the scissile ester bond (Wells, 1974; Thurén et al., 1984), and reduction in the hydration shell around the ester bond (Brockerhoff, 1968) have been suggested to contribute. In the enzyme models conformational rearrangements are thought to lead to an optimized active site geometry and thus to enhanced catalytic activity of the protein. Obviously, the two models are not mutually exclusive.

There are abundant data to also support the enzyme model, as exemplified below for the lipase from *Humicola lanuginosa* (HLL). The crystal structure of HLL has been solved at 1.8-Å resolution (Derewenda et al., 1994a). It is roughly spherical in shape, with a size of 35 × 45 × 50 Å, and contains a central eight-stranded, predominately parallel β-sheet structure with five interconnecting α-helices. The active site comprises a Ser-His-Asp catalytic triad closely resembling that found in serine proteases (Brady et al., 1990). Recent studies of the S146A mutant of HLL indicate that the active site Ser also contributes to the stability of HLL (Peters et al., 1998). Part of the HLL structure is the "lid," an α-helical mobile surface loop consisting of amino acids 86–93 that covers the active site serine (Brady et al., 1990; Berg et al., 1998). According to x-ray crystallographic studies this lid is also highly mobile in the crystals (Derewenda et al., 1994b). One of the four Trp residues of HLL, Trp^{89}, is located in the lid. Holmquist et al. (1995) have demonstrated the importance of this residue for efficient hydrolysis by showing that site-directed mutagenesis at Trp^{89} decreases the catalytic rate of HLL. In their study on the interfacial activation of HLL, Berg et al. (1998) presented a strategy to obtain kinetic and equilibrium parameters and suggested that the activation is controlled through the cationic residues around the lid.

HLL has been suggested to adopt either a "closed" or an "open" conformation, depending on its environment (Aires-Barros and Cabral, 1994). Accordingly, in an aqueous medium HLL adopts the closed structure, and access to the catalytic triad is blocked by the lid. When HLL is complexed with substrate analogs or serine protease inhibitors, the lid is open, exposing the active site (Brzozowski et al., 1991; Derewenda et al., 1992; Holmquist et al., 1994). The interfacial activation of HLL would thus be due to a conformational change, opening of the lid (Martinelle et al., 1995), which generates a hydrophobic cleft containing the active site. The open conformation is stabilized by adsorption to a hydrophobic interface (Brzozowski et al., 1991). The ability of the lipase to efficiently form the acyl-enzyme

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Address reprint requests to Dr. Paavo K. J. Kinnunen, Department of Medical Chemistry, Institute of Biomedicine, University of Helsinki, P.O. Box 8 (Siltavuorenpenger 10 A), FIN-00014 Helsinki, Finland. Tel.: 358-9-1918237; Fax: 358-9-1918276; E-mail: paavo.kinnunen@helsinki.fi.

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intermediate at the interface is thus dependent on three separate steps: 1) the lipase has to bind to the lipid/water interface, 2) the active open conformation must be formed, and 3) the active site has to be saturated with substrate (Holmquist et al., 1994). The above scheme could be applicable to other lipolytic enzymes as well.

To this end, it has been found that many enzymes are characterized by conformational changes coupled to the binding and release of substrate. For triosephosphate isomerase, dihydrofolate reductase, and protein tyrosine phosphatases, for example, the conformational change is restricted to a localized movement of a hinged flexible loop. As these enzymes bind to their substrates, the loop folds over the active site-substrate complex to promote catalysis (Juszczak et al., 1997). These two conformations, closed and open, have been verified by crystallographic studies (Bolin et al., 1982; Lolis and Petsko, 1990; Bystroff and Kraut, 1991). In addition to these structural studies, the functional role of the coverage of the catalytic domains by the loop has also been investigated. The loop in lipoprotein lipase, hepatic lipase (Dugi et al., 1995), and pancreatic lipase (Carriere et al., 1997) play a crucial role in determining the substrate specificity of these lipases and can thus be considered a part of the active site.

X-ray crystallography studies indicate that the pancreatic lipase-procolipase complex adopts the open structure in the presence of mixed micelles of phosphatidylcholine and bile salt (van Tilbeurgh et al., 1993). Simons et al. (1997) have reported a conformational change in *S. aureus* lipase to be induced by Triton X-100. Hermoso et al. (1997) have shown that micelles of both nonionic and ionic detergents induce the closed → open conversion of the pancreatic lipase. The activity of HLL toward *p*-nitrophenyl butyrate has been shown to be enhanced by sodium dodecyl sulfate (Martinelle et al., 1995). In the present study detergent (pentaoxyethylene octyl ether, C_8E_5)-induced changes in the conformation of HLL were investigated by utilizing both steady-state and time-resolved fluorescence intensity and anisotropy measurements to monitor changes in the environment of the Trp residues of wild-type as well as mutant (W117F, W221H, W260H) HLL. In the latter the single Trp residue at position 89 in the lid should specifically indicate conformational as well as microenvironmental changes in this region of the protein.

EXPERIMENTAL PROCEDURES

Materials

C_8E_5, *p*-nitrophenyl butyrate, HEPES, and EDTA were from Sigma. Potassium iodide (KI) and sodium thiosulfate ($Na_2S_2O_3$) were from Merck (Darmstadt, Germany), and tryptophan was from Eastman Kodak (Rochester, NY). Diphenylhexatriene was purchased from EGA Chemie (Steinheim, Germany).

Site-directed mutagenesis and expression of the lipases

The gene from *Humicola lanuginosa* encoding the triacylglycerol lipase was obtained from Boel and Huge-Jensen (1988). The single Trp variant W89m (W117F, W221H, W260H) of HLL was constructed with the Chameleon double-stranded site-directed mutagenesis kit by the USE method (Svendsen et al., 1997), as instructed by the manufacturer (Stratagene, La Jolla, CA). Three primers 36–40 nucleotides in length, each containing one of the three mutations of interest, were made and subsequently annealed to the template pAHL (Svendsen et al., 1992), along with the selection oligos. The latter also included substitution of *Mlu*I for the *Sca*I site within the Amp gene, so as to abrogate the *Sca*I site in the lipase gene without amino acid changes. To verify the mutations the entire gene was sequenced using primers 19670 (CCCCATCCTTTAACTATAGCG) and 19671 (CTCCCTTCTCTGAACAATAAACCC) and a Bigdye Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) and an ABI373 sequencer. The sequences obtained were analyzed using Sequencer (Gene Codes Corp., Ann Arbor, MI). The plasmids were then transferred into *Aspergillus oryzea* and expressed as described by Christensen et al. (1988).

Purification of lipases

Filtered fermentation supernatants were adjusted to 0.8 M solid ammonium acetate and subjected to hydrophobic chromatography on Toyopearl Butyl 6000 C (Toso Haas, Montgomeryville, PA). Bound proteins were eluted with distilled water. Fractions containing lipase activity were pooled and adjusted to pH 9 by dilute NaOH. Ionic strength was adjusted to 2 mSi by diluting with distilled water, followed by anion exchange chromatography on Fast flow Q Sepharose (Pharmacia Biotech, Uppsala, Sweden). Bound proteins were eluted using a linear salt gradient up to 0.5 M NaCl at pH 9. The purity of the isolated HLL wt enzyme and the W89m variant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Protein concentrations of the pure samples were determined using the extinction coefficients calculated (Program Manual for the Wisconsin Package, Version 8; Genetics Computer Group, Madison, WI) for HLL wt and the mutant with correction for the lack of three Trps in the latter protein (Mach et al., 1992). Lipase activity was determined using tributyroylglycerol as a substrate (Svendsen et al., 1997). Absorption at 295 nm was measured with a Perkin-Elmer Lambda Bio 40 UV/VIS spectrometer, using a spectral bandwidth of 2 nm. Lipase solutions were made in 20 mM HEPES, 0.1 mM EDTA (pH 7.0), prepared in freshly deionized (Milli RO/Milli Q; Millipore, Bedford, MA) water.

Steady-state fluorescence spectroscopy

Steady-state fluorescence measurements were carried out with a Perkin-Elmer LS 50B luminescence spectrometer. In addition to Trp, HLL also contains Tyr. To selectively observe Trp, 295 nm was used as the excitation wavelength (Das and Mazumdar, 1995; Jennifer and Kathleen, 1997), while 345 and 355 nm were used as emission wavelengths for lipases and free Trp, respectively. Both excitation and emission bandwidths were 5 nm. To improve the signal-to-noise ratio, bandwidths of 10 nm were used, and a time average of 10 s was recorded in anisotropy measurements. All of the steady-state measurements were repeated three times, and standard deviations are presented as error estimations for anisotropy values. For fluorescence intensity values the errors were less than 2%. All instruments were equipped with magnetic stirrers and a circulating water bath to maintain a constant temperature (25°C, unless otherwise indicated). To avoid nonequilibrium effects the actual measurements were started at least 3 min after mixing of the components. Besides conventional emission spectra we also utilized synchronous fluorescence measurements, with simultaneous scanning of both emission and excitation wavelengths, while keeping their

difference ($\Delta\lambda = 60$ nm) constant. When $\Delta\lambda \geq 55$ nm, only the fluorescence of tryptophan is seen, whereas that of tyrosine is excluded. When indicated the detergent C_8E_5 was added in increasing concentrations, so as to observe changes in the lipase fluorescence induced by micelles. The critical micellar concentration (CMC) at ~7 mM was determined for this detergent by measuring the increase in the fluorescence emission of 100 μM diphenylhexatriene as a function of [C_8E_5]. In some experiments KI was used as a collisional quencher for Trp. A KI stock solution (1.0 M) was freshly prepared and contained 1 mM $Na_2S_2O_3 \cdot 5H_2O$ to inhibit production of I_3^- (Lehrer, 1971).

Determination of fluorescence lifetimes and time-resolved anisotropies

Time-resolved fluorescence measurements were performed with a commercially available system (PTI, London, Ontario, Canada). A train of 500-ps excitation pulses at 298 nm at a repetition rate of 10 Hz was produced by a dye laser pumped by a nitrogen laser. The beam from the dye laser was channeled to a frequency doubler. Emission decays for lipases and free Trp at 345 nm and 355 nm, respectively, were detected by a photomultiplier tube (Hamamatsu, Hamamatsu, Japan). The minimum lifetime accessible to the instrument is 200 ps. Each intensity decay curve represents an average of five subsequent measurements. As polarizers decrease the emission intensity, to obtain a good signal-to-noise ratio an average of 10 subsequent measurements was used to calculate anisotropy decays. Instrument response functions were measured separately, using aqueous glycogen solution. The decay curves were analyzed by the nonlinear least-squares method and fitted to a sum of exponentials. The presented error values for fluorescence lifetimes are calculated by the software from the instrument manufacturer. Anisotropy decays $r(t)$ are constructed from fitted curves and thus contain no experimental noise. Accordingly, typical fit criteria do not apply, and meaningful error values for rotational correlation times and residual anisotropy cannot be calculated.

Assay for catalytic activity

The kinetics of hydrolysis of *p*-nitrophenyl butyrate (PNPB) were monitored as the change in the absorbance at 405 nm due to *p*-nitrophenolate anion (Berg et al., 1998). Measurements were carried out with Perkin-Elmer Lambda Bio 40 UV/VIS spectrometer. The reaction was initiated by the addition of HLL to a solution containing PNPB and C_8E_5. The slope of the initial zero-order phase of the absorbance curve was taken as the relative rate of hydrolysis. The minor hydrolysis evident in the absence of the lipase was measured separately and subtracted as background.

RESULTS

Steady-state fluorescence spectroscopy.

To allow for an unambiguous interpretation of the data on the effects of C_8E_5, we first studied both wild-type and W89m HLL in solution, in the absence of the detergent (Fig. 1). For comparison the spectrum for free Trp is also shown. Emission of Trp is sensitive to the microenvironment of the fluorophore (van Tilbeurgh et al., 1993). Notably, the fluorescence quantum yield of W89m is high, and the emission of its single Trp is ~50% of that measured for the wild-type HLL with four Trps. This is likely to be explained by low quantum yields of the other three Trps, W117 and W221 in particular. More specifically, the latter two reside in locations with both anionic and cationic residues in their immediate vicinity, which could be sufficient to perturb their electronic configuration so as to cause efficient quenching of their fluorescence.

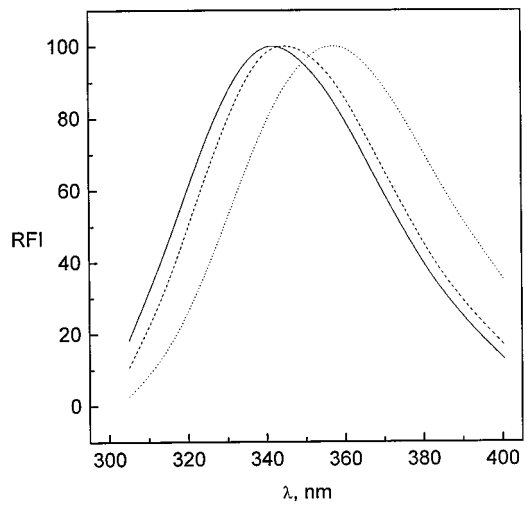

FIGURE 1 Fluorescence emission spectra of free tryptophan (·····) and wild-type (——) and W89m HLL (– – –). The spectra were normalized to the same maximum value for emission intensity. The peak intensity values corrected with respect to Trp concentrations were 100, 70, and 130 for free Trp, wt, and W89m. The excitation wavelength was 295 nm. The concentrations of Trp and the lipases were 1 μM in 20 mM Hepes, 0.1 mM EDTA (pH 7.0). The temperature was 25°C.

Trp residues in the proteins can be divided into three discrete spectral classes (Brustein et al., 1973), depending on whether they are buried in nonpolar regions of the protein ($\lambda_{max} \approx 330$ nm), completely exposed to water ($\lambda_{max} \approx 350$ nm), or located in an environment of intermediate polarity ($\lambda_{max} \approx 340$ nm). An increase in the I_{350}/I_{330} fluorescence intensity ratio thus suggests a more hydrophilic environment for Trp. The values of I_{350}/I_{330} for free Trp, wt HLL, and W89m were 2.00, 1.11, and 1.20, respectively. Judged from these values, the single Trp in the W89m is not in direct contact with the aqueous phase. These spectral differences between the two lipases can be seen in the steady-state emission spectra, where W89m has its emission maximum at a longer wavelength than the wild type (Fig. 1). Further evidence supporting the location of Trp^{89} in an environment of intermediate polarity was provided by synchronous fluorescence spectroscopy (Miller, 1979), which revealed the maximum emission intensity of the W89m to be at 340 nm (data not shown).

Iodide I^- is a commonly used collisional quencher of Trp fluorescence. Because of its negative charge and relatively large size, it cannot penetrate into the tightly packed interiors of proteins and can only quench the fluorescence of Trps located on the surface of the protein (Das and Mazumdar, 1995; Lakowicz, 1983). The relative fluorescence intensity of HLL is presented as a function of [KI] in Fig. 2.

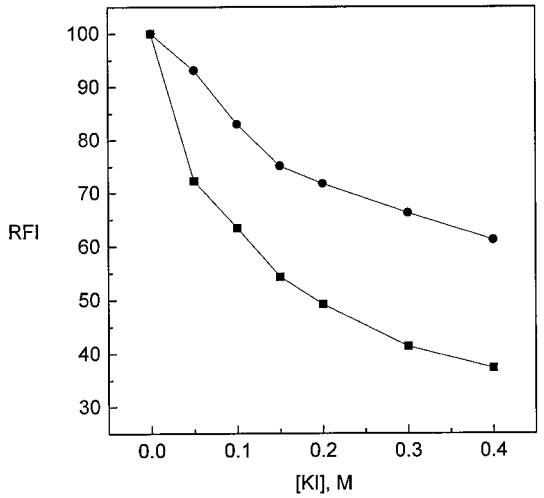

FIGURE 2 Quenching of tryptophan emission by KI in wt (●) and W89m (■). The relative fluorescence intensity values in the absence of KI are normalized to 100. The conditions were as described in Experimental Procedures.

Up to 0.2 M KI emission intensity diminished progressively with increasing [KI] for both wt (by 28%) and W89m (by 51%), revealing a accessibility of Trp^{89} in particular to I^-. The microenvironment of Trp can also be assessed by the temperature sensitivity of the fluorescence quantum yield, as in a polar environment (i.e., on the surface of a protein, contacting the aqueous phase or polar residues) its quantum yield decreases significantly with increasing temperature. The integrated area of the emission spectrum (comparable to fluorescence quantum yield) decreased by 47, 26, and 28% with an increase of the temperature from 20°C to 50°C for free Trp, wt, and W89m, respectively (data not shown). Neither 0.2 M KI nor increasing temperature had any effect on the absorbance of the lipases at 295 nm, thus confirming that the above differences are due to decreased quantum yield of Trp fluorescence (data not shown).

The sensitivity of Trp emission to its environment can be utilized to study conformational changes of the lipase and the interaction between HLL and C_8E_5. At $[C_8E_5]$ less than the CMC, emission intensities for the two lipases as well as free Trp remain virtually unaltered (Fig. 3). However, when $[C_8E_5]$ is increased beyond the CMC, up to 16 mM, there is a 17, 13, and 44% increase in relative fluorescence intensity (RFI) for free Trp, the wild type, and the mutant, respectively (Fig. 3 *B*). However, there was no shift in the maximum emission wavelengths, 342 and 345 nm, for the wild type and the mutant, respectively. Notably, the increase (ΔI) due to 16 mM C_8E_5 in the emission intensities was 24% and 66% for the wt and the W89m, respectively (Fig. 3 *A*). When we take into account the Trp content of the lipases, these data indicate that for the wt lipase the increment in Trp emission at $[C_8E_5]$ greater than the CMC results also solely from Trp^{89}.

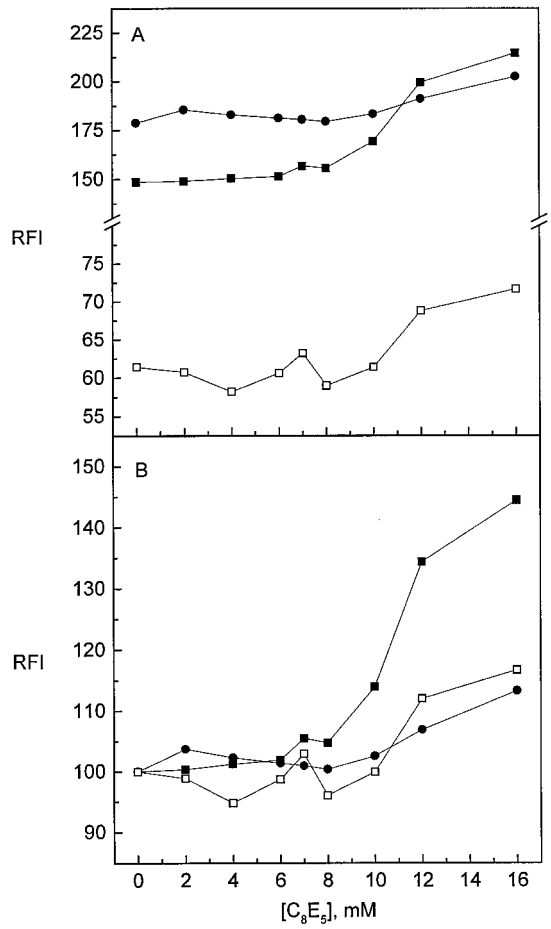

FIGURE 3 (*A*) Relative fluorescence intensity of free tryptophan (□) and wild-type (●) and mutant (■) HLL as a function of $[C_8E_5]$. (*B*) Emission intensity values in the absence of C_8E_5 are normalized to 100. The conditions used were identical to those described in the legend for Fig. 1.

Steady-state fluorescence anisotropy

The anisotropy of intrinsic protein fluorescence reflects both segmental motions of the fluorophore as well as the movement of the whole protein (Lakowicz, 1983). Below the CMC of the detergent the measured anisotropies remained almost constant, 0.005 for free Trp and 0.13 for both lipases (Fig. 4*)*. However, at higher $[C_8E_5]$, the anisotropy of free Trp increases almost linearly, reaching 0.010 at $[C_8E_5]$ = 16 mM. The opposite is observed for Trp of the lipases, and above CMC their fluorescence anisotropy decreases (Fig. 4). At $[C_8E_5]$ = 16 mM the wild type and the mutant yield values of 0.111 and 0.090, respectively.

Time-resolved fluorescence spectroscopy

To get more insight into the detergent-induced changes in the conformation of HLL and the concomitant changes in the microenvironment of the Trp residues, fluorescence

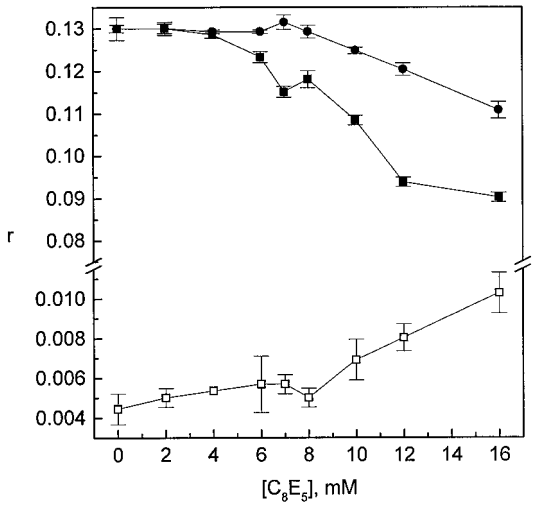

FIGURE 4 Steady-state fluorescence anisotropy of free tryptophan (□) and wild-type (●) and mutant (■) HLL as a function of $[C_8E_5]$, measured at 25°C in 20 mM Hepes, 0.1 mM EDTA (pH 7.0). The concentration of Trp and lipases was 1 μM.

lifetimes of free Trp, the wild type, and the mutant were measured (Chen et al., 1991). Reduced dielectricity due to, for instance, diminished exposure to water is normally associated with prolonged fluorescence lifetimes of Trp (Kungl et al., 1998). This was also evident in the measurements for free Trp at different $[C_8E_5]$, both below and above CMC (Fig. 5). In keeping with two solvent equilibrated states for Trp (Rayner and Szabo, 1978), the emission decay is described by a two-exponential function, the components of which have lifetimes in the range of 0.4–1.6 ns (τ_1, Fig. 5 *A*) and 2.7–3.1 ns (τ_2, Fig. 5 *B*). As expected, both lifetimes increase as the environment of Trp becomes more hydrophobic upon association with C_8E_5 micelles when $[C_8E_5]$ exceeds the CMC. Interaction with the detergent is likely to favor the state with the shorter lifetime τ_1 as the fractional intensity of τ_2 decreases with increasing $[C_8E_5]$ (Fig. 5 *C*).

At all studied $[C_8E_5]$ levels, fluorescence decays of the wild type also comply with two-exponential kinetics. The longer lifetime τ_2 increases gradually from 4.9 ns in the absence of detergent to 5.7 ns at $[C_8E_5] = 16$ mM (Fig. 5 *B*), whereas the shorter lifetime τ_1 apparently varies randomly between 0.93 and 1.7 ns (Fig. 5 *A*). The increase in τ_2 coincides with the decrease in the fractional contribution of this component (Fig. 5 *C*).

Compared to the average values measured for the four Trps of the wild type, the fluorescence decays for W89m are slower in all conditions studied. Interestingly, at $[C_8E_5] < 6$ mM, the decay of W89m fluorescence is one-exponential, with only the longer lifetime component ($\approx$ 5.3 ns) being evident. At higher $[C_8E_5]$, however, the decays become two-exponential, and both components have a tendency to increase with the detergent concentration (Fig. 5). Similarly

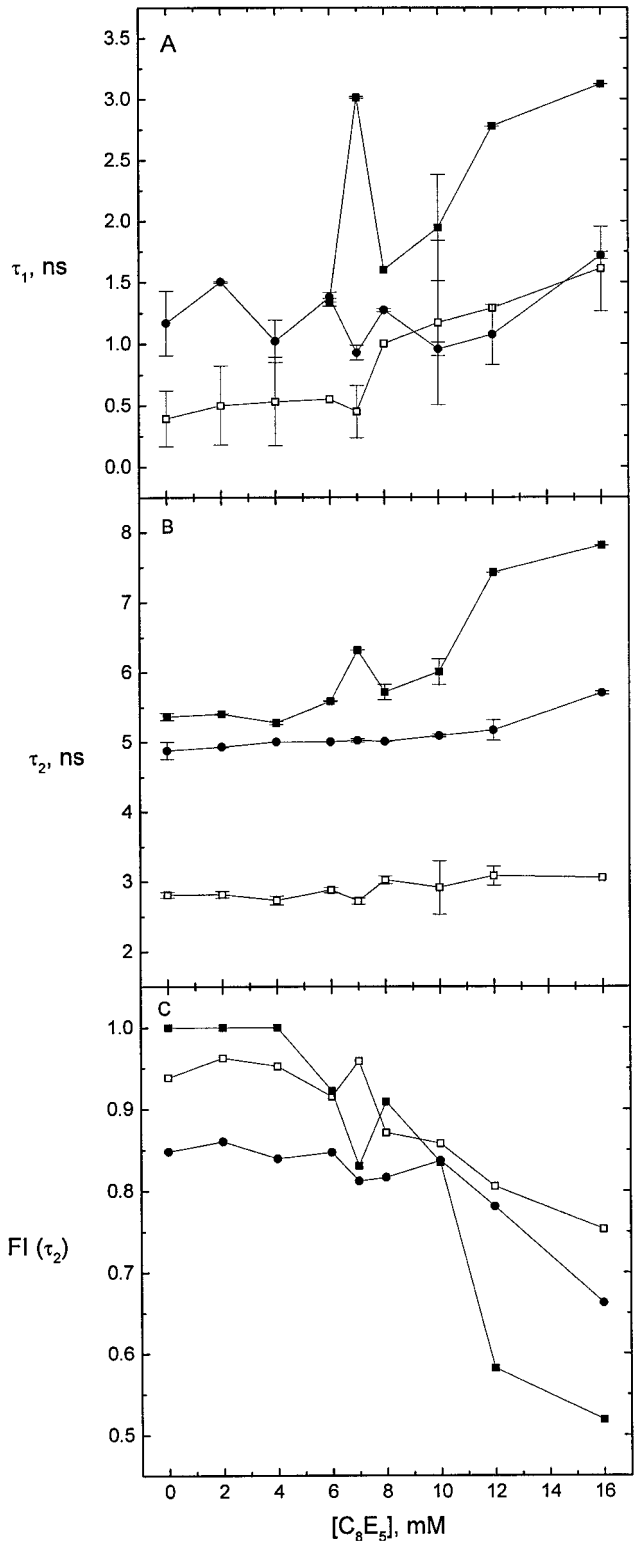

FIGURE 5 Fluorescence lifetimes τ_1 (*A*) and τ_2 (*B*) and the fractional intensity of the component with a lifetime τ_2 (*C*) of free tryptophan (□) and wild-type (●) and mutant (■) HLL at different $[C_8E_5]$. Conditions were as stated in the legend for Fig. 1.

to the wild type, the fractional intensity of τ_2 decreases by almost 50% when [C_8E_5] is increased from 0 to 16 mM (Fig. 5 *C*). Interestingly, anomalous behavior is detected at the CMC, where both τ_1 and τ_2 are exceptionally long.

Time-resolved anisotropy

Anisotropy decay of intrinsic fluorescence is an informative tool for studies on macromolecular interactions as well as on protein conformational changes and local motions (Lakowicz, 1983). Accordingly, if protein binds to another particle its overall motion slows down and the longer rotational correlation time ϕ_2 (typically of the order of 10–50 ns) is prolonged. Two-exponential anisotropy decay indicates a contribution from segmental motions, with variation in the shorter correlation time ϕ_1 (typically subnanosecond), indicating changes in protein conformation. Alterations in segmental motion should also be evident as changes in the values for the residual anisotropy r_∞.

Because of the complexity of the fluorescence of the wild type HLL (due to its four Trps), time-resolved anisotropy studies were carried out only with the mutant (Fig. 6). At all studied [C_8E_5] levels, two-exponential anisotropy decays were evident. It is commonly assumed that the fluorescence of proteins is depolarized 1) by rapid local motions of the Trp side chains and 2) by the overall rotation of the entire macromolecule (Steiner, 1991). In the present case the shorter correlation time ϕ_1 can thus be attributed to the movements of Trp^{89} in the lid, and the longer correlation time ϕ_2 to the rotations of the lipase either as such or as a complex with C_8E_5. Increasing detergent concentration up to 8 mM shortens ϕ_1 significantly, from 0.24 to 0.06 ns, suggesting detergent-induced conformational changes in W89m already well below the CMC (Fig. 6 *A*). There is a minimum for ϕ_1 of ~0.06 ns at [C_8E_5] = 8 mM, whereafter it increases slightly, to ~0.12 ns. The longer correlation time ϕ_2 has an average value of ~19 ns, with no clear dependency on [C_8E_5] (Fig. 6 *B*). Interestingly, when [C_8E_5] exceeds the CMC, the value of r_∞ decreases from 0.20 to 0.025, indicating that the amplitude of the segmental motions of W89m increases significantly (Fig. 6 *C*). At [C_8E_5] ≈ 16 mM interfering effects due to light scattering may have to be considered.

Effect of C_8E_5 on the catalytic activity

As interactions between HLL and C_8E_5 were revealed by the above evidence from fluorescence spectroscopy data, it was of interest to study the effect of the detergent on the catalytic activity of the lipase. The relative activities of wt and W89m as a function of [C_8E_5] are illustrated in Fig. 7. For W89m, increasing detergent concentration decreases the rate of hydrolysis below CMC, and at [C_8E_5] = 6 mM the lipase is nearly inactive. Above the CMC the nature of

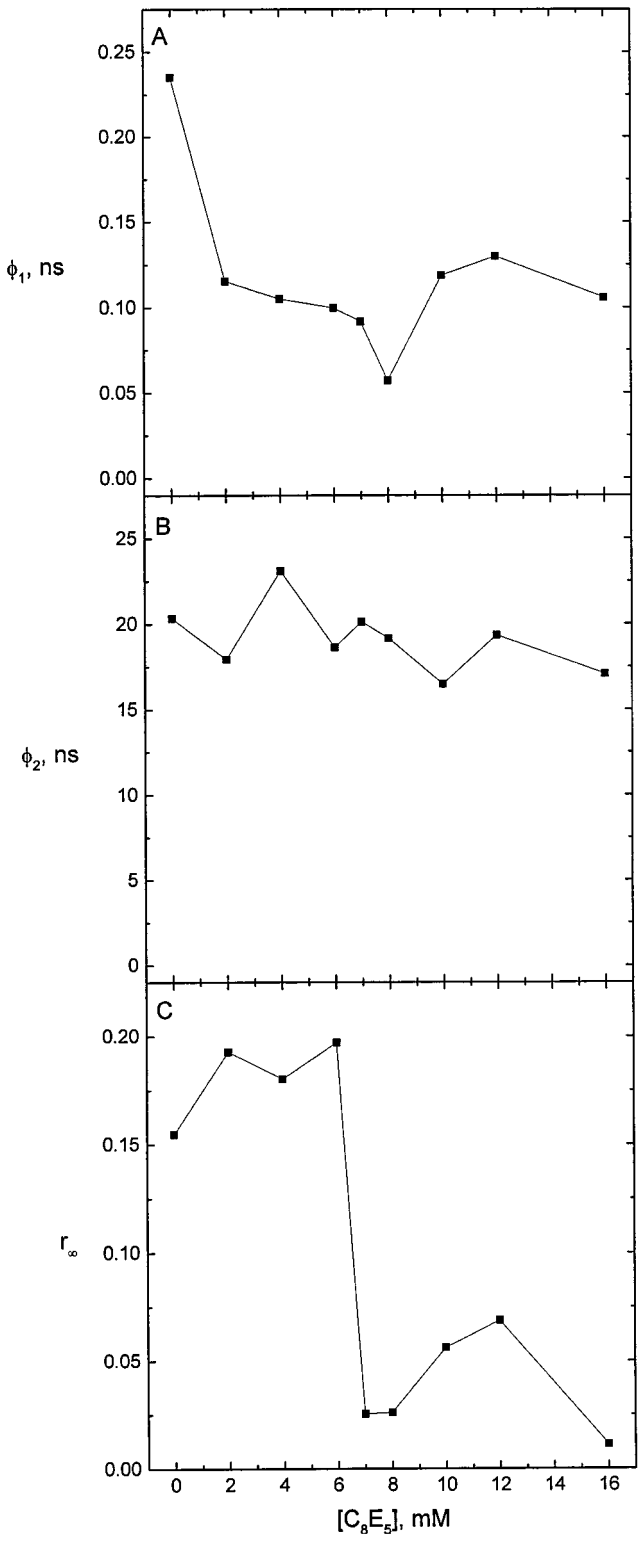

FIGURE 6 Rotational correlation times ϕ_1 (*A*) and ϕ_2 (*B*) and the residual anisotropy r_∞ (*C*) of W89m HLL at different [C_8E_5], measured as described in Experimental Procedures. The temperature was 25°C.

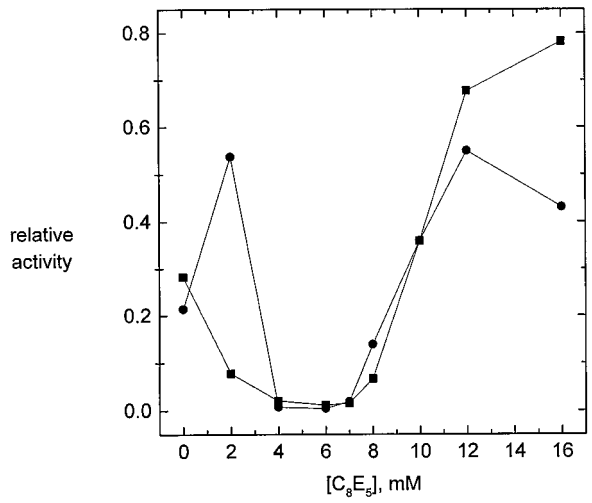

FIGURE 7 Relative activity of wt (●) and W89m (■) HLL toward PNPB at different C_8E_5 concentrations, as indicated by the change in absorbance at 405 nm. The concentrations of HLL and PNPB were 0.33 μM and 1 mM in 20 mM HEPES, 0.1 mM EDTA (pH 7.0). The temperature was 25°C.

this interaction changes and the catalytic activity is enhanced by the detergent. At $[C_8E_5] \geq 10$ mM the activity of W89m exceeds that measured in the absence of the detergent. The behavior of wt is similar to that of W89m, with two exceptions, as follows. A low concentration (2 mM) of C_8E_5 increases the activity, and at the other end, increasing the concentration from 12 to 16 mM slows down the hydrolysis. The reason for this consistently observed behavior of wt remains unknown at present. However, wt is also almost inactive just below the CMC, at $[C_8E_5]$ from 4 to 7 mM.

DISCUSSION

The aim of the present investigation was to study conformational changes in HLL induced upon micelle formation by the neutral detergent C_8E_5. Particular emphasis was placed on detergent-induced changes in the microenvironment of Trp^{89} in the lid of HLL. The wild-type HLL has four Trp residues, at positions 89, 117, 221, and 260. Accordingly, to resolve the contribution from Trp^{89} to the spectral properties of the lipase we were particularly interested in the W89m mutant of HLL, where the other three Trp residues are substituted (W117F, W221H, W260H) by site-directed mutagenesis.

Four different approaches were undertaken to address the microenvironment of Trp^{89} in the mutant HLL in the absence of the detergent, as follows. The value of $I_{350/330}$ was higher for the mutant than for the wild type (1.20 versus 1.11), indicating that on average the other three Trps reside in a more hydrophobic environment than Trp^{89}. I^- quenches Trp^{89} fluorescence quite efficiently. Accessibility of Trp^{89} to this quencher thus reveals that iodine can enter into the activity site accommodating hydrophobic cavity covered by the lid. A significant decrease in the fluorescence quantum yield with an increase in temperature from 20°C to 50°C suggests that W89 resides in a relatively polar environment. This is further supported by the synchronous emission spectrum of the mutant, which reveals an intensity maximum of Trp^{89} at 340 nm.

The association of free Trp with C_8E_5 micelles increases its quantum yield (Fig. 3), in keeping with an increase in the hydrophobicity of its average environment. Increased quantum yields were also measured for both lipases upon their interaction with C_8E_5 micelles. However, the relative increase in RFI as $[C_8E_5]$ exceeds the CMC is more than three times higher for the mutant than for the wild type. Comparison of the magnitudes of the changes in fluorescence further suggests that in contrast to W89 the microenvironments of the three other Trps (W117, W221, and W260) of wt HLL do not undergo major changes. Accordingly, we can conclude that the detergent-induced conformational changes of the lipases affect W89 in the lid, and that this residue becomes accommodated in a more hydrophobic environment. A likely possibility is that above the CMC Trp^{89} is in contact with the octyl chains. Accordingly, the opening of the lid above the CMC would be accompanied by a hydrophobicity-driven interaction between C_8E_5 and the hydrophobic active site-containing cleft of HLL. However, although the overall change in the hydrophobicity is clear when judged by the increase in quantum yield, no shift was observed in the maximum emission wavelength. This could be due to two simultaneous processes making opposite contributions to the quantum yield and λ_{max}.

The results from steady-state anisotropy measurements are in agreement with the above. Above the CMC the movement of free Trp is hindered by interactions with micelles; the micelle-bound fluorophores move more slowly than those in solution. As expected, at all $[C_8E_5]$ levels, HLL has considerably higher anisotropy values than free Trp; the movements of the lipases are torpid compared to free Trp, and the surrounding polypeptide structures further restrict the local mobility of the Trp residues. Interaction with micelles *decreases* emission anisotropy for both the wild type and the mutant, this effect being more pronounced for the latter. This aligns with C_8E_5 above its CMC to cause conformational changes in the lipases, and these changes are sensed by Trp^{89} in the lid. The most appealing conclusion is that the closed $\leftrightarrow$ open equilibrium is shifted toward open at $[C_8E_5] >$ CMC, and consequently Trp^{89} in the lid can move more freely, which is evident as decreased steady-state anisotropy.

Except for W89m at detergent concentrations below CMC, two-exponential Trp fluorescence intensity decays were measured in the present study. A possible explanation for this is that the excited-state Trp^{89} cannot adopt the state yielding τ_1, until there is a C_8E_5-induced conformational

change in W89m. In the wild type both components are observed, even in the absence of C_8E_5, perhaps because the conformational dynamics of the other three Trp residues are less restricted. For free Trp as well as the wild-type and W89m lipase, the fractional intensity of τ_1 increases with increasing [C_8E_5], suggesting that this change indicates a more hydrophobic microenvironment. Furthermore, both lifetimes τ_1 and τ_2 are prolonged in the presence of increasing detergent concentrations, which can be attributed to a more hydrophobic microenvironment of the fluorophore (Kungl et al., 1998). An exception to the above, however, is the shorter lifetime τ_1 of the wild-type HLL that did not show any correlation to [C_8E_5]. A probable explanation is the complex photophysics resulting from the four Trps of the wt lipase residing in four different environments.

Somewhat puzzling behavior was observed for W89m at the CMC, where both τ_1 and τ_2 were exceptionally long. One possible explanation is that the nucleation of micelle formation is not a simple transition from a solution of detergent monomers below CMC to a system above CMC, with micelles in equilibrium with monomers. Instead, there may be short-lived detergent/lipase complexes already below the CMC. One possibility for the complex geometry is lipase dimers linked by micelle-like aggregates of C_8E_5. We did not pursue this finding in more detail at this stage.

Interaction between C_8E_5 and HLL well below CMC is indicated by the anisotropy decay measurements for W89m, with the short correlation time ϕ_1 decreasing drastically already at the lowest [C_8E_5] studied. It is possible that C_8E_5 monomers have access to Trp^{89} in the active site-accommodating cavity, even if the lid is closed, similarly to I^-. The shortest value for ϕ_1 was measured at [C_8E_5] = 8mM. A likely possibility is that at this concentration the closed-open equilibrium of the lid is shifted toward open, resulting in more rotational freedom for Trp^{89} and thus shorter ϕ_1. However, at [C_8E_5] $>$ 8 mM, ϕ_1 increases slightly, indicating changes in the nature of the C_8E_5-lipase interaction.

Perhaps somewhat unexpectedly, the longer rotational correlation time ϕ_2 is not affected by C_8E_5, its value varying between 16 and 24 ns. The average diameter of C_8E_5 micelles and HLL should be on the same order of magnitude (Timmins et al., 1994), and the binding of HLL to the micelles should thus increase ϕ_2. All other data reported here clearly demonstrate C_8E_5 and HLL to interact above the CMC. One possible explanation for this apparent contradiction is that instead of HLL associating with C_8E_5 micelles, the lipase and the detergent form a complex where the hydrophobic active site-containing cavity of the lipase is filled by the detergent molecules. The hydrodynamic radius of this complex would be close to that of HLL, and, accordingly, this complex formation would not influence ϕ_2. At the CMC the value of residual anisotropy r_∞ drops from $\sim$0.18 to 0.03, indicating that the amplitude of the motion of Trp^{89} increases. This finding could be attributed to the opening of the lid and the concomitant formation of the C_8E_5-HLL complex.

CONCLUSIONS

The above results lead us to propose the following model for the interaction between HLL and C_8E_5. Already below the CMC C_8E_5 molecules can intercalate into the active site-accommodating cavity and suppress the activity of HLL, while the lid remains closed. This inhibition of catalytic activity could result from conformational changes in HLL, induced by C_8E_5. Another and perhaps more likely mechanism is that the intercalation of the detergent into the active site containing cleft causes a steric block to the access of the substrate into the cleft. At a threshold concentration of the detergent and close to its CMC (yet below the CMC), the cooperativity of the hydrophobicity-driven binding of C_8E_5 to the lipase increases. The reason for the increase in the cooperativity is an increase in the number of detergent molecules participating in the nucleation of aggregates on the hydrophobic surface of the lipase. These aggregates should have a long enough lifetime to allow the lid to open completely, so as to expose the hydrophobic active site-accommodating cleft. Concomitantly, the cleft becomes filled with C_8E_5 and the open conformation of the lipase is stabilized. The hydrodynamic radius of this detergent-lipase complex would not be significantly different from that of the lipase. In this complex Trp^{89} resides in a hydrophobic environment and the amplitude of its segmental motions increases. Above the CMC the catalytic activity of HLL is enhanced by C_8E_5 because of detergent-induced opening of the lid. As the aggregation number of the detergent increases above the CMC, binding to the hydrophobic cleft and blocking of the active site become less favorable.

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