Spectroscopic and Kinetic Studies of Lipases Solubilized in Reverse Micelles

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Received December 2, 1992; Revised Manuscript Received February 1, 1993

ABSTRACT: The conformation and activity of three different lipases have been studied in reverse micelles formed by sodium bis(2-ethylhexyl) sulfosuccinate (AOT) in isooctane. In the case of human pancreatic lipase, the conformation of the polypeptide chain—as judged from far-UV circular dichroism measurements—is only slightly altered after the enzyme is transferred from a bulk aqueous solution into the microenvironment of reverse micelles. Significant spectral changes in the near-UV circular dichroism and fluorescence spectrum indicate, however, that the solvation of aromatic amino acid side chains is considerably different in reverse micelles. Conversely, the circular dichroism spectra of the lipases from *Candida rugosa* and *Pseudomonas* sp. are considerably different in reverse micelles, compared with the spectra in aqueous solution, indicating that both enzymes loose the native structure at the water/AOT/oil interface. Bound substrate and/or product can prevent this denaturation. While *Pseudomonas* sp. and human pancreatic lipase are inhibited by tetrahydrolipstatin (THL), the lipase from *Candida rugosa* is not. These data, together with additional activity and inhibition data, indicate that the micellar microenvironment accentuates the difference between the different enzymes in terms of the relation structure/activity.

Lipases are enzymes that typically act at lipid/water interfaces, the lipase being water-soluble and most of the characteristic (and physiological relevant) substrates being water-insoluble (Sémériva & Desnuelle, 1979; Verger, 1980; Brockman, 1984). Although lipases are widely distributed in animals, plants, and microorganisms, studies on the mechanism of activation at the water/lipid interface have mainly been limited to porcine pancreatic lipase (Verger, 1984) and more recently to porcine pancreatic carboxylester lipase [e.g., see Muderhwa and Brockman (1990)].

Recently, the interest in lipases has been increasing, owing to the observation that under certain conditions dispersed lipase powders are able to catalyze a variety of enantioselective transformations of water-insoluble compounds in almost anhydrous water-immiscible organic solvents [e.g., see Zaks and Klibanov (1985) and Chen and Sih (1989)]. Although a number of such studies have been carried out, most investigations have focused on technical applications with rather crude lipase preparations.

Basic research concerning the mode of action and the conformation of lipases in predominantly organic milieu or at the water/oil interface has not yet received due attention. The reason for this lies in experimental difficulties: lipases are insoluble in apolar solvents, and spectrophotometric methods, such as circular dichroism or fluorescence, cannot be applied. One possibility to overcome this problem is offered by the finding that lipases (and all other kind of proteins) can be solubilized in an organic solvent by the help of reverse micelles (Luisi & Magid, 1986; Martinek et al., 1986; Waks, 1986; Laane et al., 1987; Walde & Luisi, 1989; Holmberg & Oesterberg, 1990; Walde, 1990). We therefore thought that reverse micelles could be useful systems to investigate the conformational behavior of lipases at water/lipid interfaces, which would eventually contribute to a better understanding of the mode of action and interfacial activation of this class of enzymes.

In this paper, we report on spectroscopic and kinetic properties of three lipases in reverse micelles formed by sodium bis(2-ethylhexyl) sulfosuccinate (AOT)[1] in isooctane. In addition, comparative spectroscopic measurements have been made in aqueous solution. The main aim of this work is to investigate the effect of the micellar environment upon the structure of three related enzymes, and in particular to clarify whether, in comparison to water, structural and/or activity properties are accentuated in reverse micelles, or rather canceled. We will report in the case of the three enzymes separately CD studies in water and reverse micelles, as well as activity data.

MATERIALS AND METHODS

Reagents. Oleic acid (99%) and AOT were from Sigma and used as obtained (Walde & Luisi, 1989). Isooctane (for UV spectroscopy) and tridecanoylglycerol (>99%) were from Fluka, and *p*-nitrophenyl palmitate was from Serva. THL was a gift from Prof. A. Fischli (Hoffmann-La Roche, Basel). Lipase from *Candida cylindracea*, recently renamed as *Candida rugosa* was purchased from Boehringer. The lyophilisate was solubilized in 75 mM Tris/HCl buffer, pH 8.1, and filtered through Centricon-30 (from Amicon, 30 000 molecular weight cutoff) to remove the inherent salts in the preparation and to adjust the pH. The molecular weight as estimated by analytical SDS–PAGE was 60 000. The protein concentration was determined spectrophotometrically by using $E_{279}{}^{1\%} = 7.94$ (Tomizuka et al., 1966). *Pseudomonas* sp. lipase was purchased from Boehringer and treated with the same procedure as described for *C. rugosa* lipase, with the exception that 50 mM Tris/HCl, pH 7.0, was used as the buffer. The molecular weight as determined with SDS–PAGE was 33 000. For the determination of the protein concentration, $E_{280}{}^{1\%} = 11.05$ was used (Sugiura & Oikawa, 1977). Colipase-free human pancreatic lipase was a gift from Dr. F. Winkler (Hoffmann-La Roche), the molecular weight of the lipase was 52 000 as estimated from SDS–PAGE, and $E_{280}{}^{1\%}$ was taken as 14.4 (7 Trp, 15 Tyr), based on $E_{280}{}^{1\%} = 13.3$ for

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[1] Abbreviations: AOT, sodium bis(2-ethylhexyl) sulfosuccinate; CD, circular dichroism; FTIR, Fourier transform infrared; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; THL, tetrahydrolipstatin; $w_0 = [H_2O]/[AOT]$, molar concentrations.

0006-2960/93/0432-4029$04.00/0

porcine pancreatic lipase (6 Trp, 16 Tyr; Donnér et al., 1976). Two porcine pancreatic lipase samples were obtained from Merck and Sigma. Both samples were rather crude as judged by analytical SDS–PAGE. One porcine pancreatic lipase preparation ("lipase-B, FE-98") was obtained from Prof. R. Verger (CNRS, Marseille, France). This sample contained, apart from the lipase (M_r 52 000), also colipase (M_r 11 000). Electrophoretically pure porcine pancreatic colipase was obtained from Boehringer, $E_{280}{}^{1\%} = 4.2$ (Donnér et al., 1976).

Electrophoresis. Analytical SDS–PAGE was carried out on a PhastSystem from Pharmacia (gel 10–15) with the following molecular weight standard kit from Pharmacia: phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), and α-lactalbumin (14 000). The proteins were stained with Coomassie Blue.

Preparation of Reverse Micelles. The enzymes were solubilized in AOT reverse micelles by injecting small amounts of concentrated buffered enzyme solutions as described before [e.g., see Walde and Luisi (1989)]. If necessary, the final solution was centrifuged to obtain optically clear solutions.

Spectroscopy. UV absorption spectra were recorded on a Uvikon 810 spectrophotometer by using quartz cells with a path length of 1 cm. Fluorescence spectra were measured on a SPEX Fluorolog F112XE with 1-cm quartz cells. FTIR spectra were measured on a Nicolet 5SXC FT-IR spectrometer by using CaF_2 cells of path length 0.01 cm. CD spectra were measured on a JASCO J-600 spectropolarimeter by using quartz cells of 1-cm path length for the near-UV range (350–250 nm) and 0.02-cm path length for the far-UV range (250–190 nm); the spectra were accumulated 16 times. Mean residue ellipticities, $[\theta]$, were calculated from the measured ellipticity by taking into account the overall concentration, the molecular weight of the enzyme (see above), and the number of amino acids per enzyme molecule: 465 for *C. rugosa* lipase, 324 for *Pseudomonas* sp. lipase (Sugiura & Oikawa, 1977), and 449 for human pancreatic lipase (Winkler et al., 1990). Estimations of the secondary structure content were carried out either by using Provencher's CONTIN program for the data between 240 and 190 nm (Provencher & Gloeckner, 1981) or by the approximative method of Chen et al. (1972), using the mean residue ellipticity at 222 nm.

Lipase Assays. Lipase activities in reverse micelles were measured throughout this work in four different ways: (A) according to Han et al. (1987) using olive oil as substrate (20%, v/v); (B) according to Walde and Luisi (1989) using tridecanoylglycerol as substrate (47 mM) and an assay based on FTIR spectroscopy; (C) according to Walde (1990) using cosolubilized phenol red as acid–base indicator and again 47 mM tridecanoylglycerol; and (D) according to Fletcher et al. (1985) using *p*-nitrophenyl palmitate (1 mM) and measuring the release of *p*-nitrophenol at 333 nm. Details of the assay are given in the corresponding original papers.

It is worthwhile to mention here the importance of the order of adding lipase and substrate. In the case of pancreatic lipase, the best experimental procedure with respect to highest enzyme activity is attained by first mixing lipase and colipase in aqueous solution and then solubilizing this mixture in reverse micelles containing substrate. Another successful possibility is to solubilize first substrate and colipase and then add lipase. If lipase is first solubilized in the absence of colipase, the enzyme is rapidly inactivated, and inactivated lipase in AOT reverse micelles could not be reactivated by adding colipase.

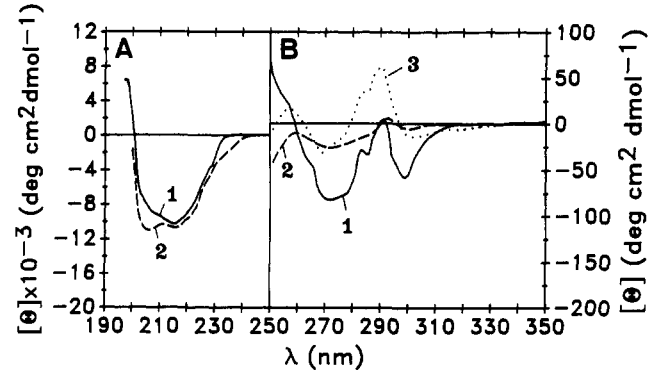

FIGURE 1: Far-UV (A) and near-UV (B) circular dichroism spectra of human pancreatic lipase in aqueous (solid line 1) and in AOT reverse micellar solutions (dashed and dotted lines 2 and 3). Mean residue ellipticities are plotted as a function of wavelength. (1) In 50 mM Tris/HCl, pH 7.5; (2) in 50 mM AOT/isooctane reverse micelles, $w_0 = 10$ (prepared with 50 mM Tris/HCl, pH 7.5); (3) like (2), but in the presence of 10 μM tetrahydrolipstatin (THL). [Lipase] = 6.9 μM.

RESULTS AND DISCUSSION

Human Pancreatic Lipase. We will begin our study with human pancreatic lipase, as this is one of the few lipases whose X-ray structure is known. Apart from human pancreatic lipase (Winkler et al., 1990), crystal structures with details on the active-site residues are only known for two other lipases, namely, *Rhizomucor miehei* lipase (Brady et al., 1990; Brzozowski et al., 1991; Derewenda et al., 1992) and a lipase from *Geotrichum candidum* (Schrag et al., 1991). All these lipases are serine hydrolases with a catalytic serine that is activated by the same (or a similar) charge-relay system found in serine proteases, such as α-chymotrypsin or subtilisin. One common feature of the three lipases is that the active serine is completely buried by a surface loop which must be moved to give access to a substrate molecule (Winkler et al., 1990; Van Tilbeurgh et al., 1992; Brady et al., 1990; Brzozowski et al., 1991; Schrag et al., 1991). The crystal structures of these three lipases are therefore structures of catalytically inactive forms of the lipases, at least with respect to the folding of the region around the active site of the enzyme.

To the best of our knowledge, the solution conformation of human pancreatic lipase has not yet been studied in detail. Figure 1 shows the CD spectrum of pure (colipase-free) human pancreatic lipase in aqueous solution at pH 7.5 (50 mM Tris/HCl) and in 50 mM AOT/isooctane reverse micelles at $w_0 = 10$, prepared with the same Tris buffer as the aqueous component. w_0 represents the ratio of the molar concentrations of added water to surfactant ($w_0 = [H_2O]/[AOT]$).

In aqueous buffer solution, the CD spectrum of human pancreatic lipase (curve 1 in Figure 1) is comparable to the CD spectrum of porcine pancreatic lipase (Donnér et al., 1976), although the intensities of the two characteristic negative bands around 270 and 295 nm are higher in the case of the human enzyme. The α-helix content in the lipase as determined with Provencher's CONTIN program (Provencher & Gloeckner, 1981) from the far-UV CD spectrum of a 6.8 μM aqueous lipase solution is 20 ± 2%. The corresponding value for the amount of β-sheet structure is 62 ± 3%. [With the simpler method of Chen et al. (1972) which considers mean residue ellipticities at 222 nm only, an α-helix content of 16% is obtained.] While the amount of α-helix as calculated from the CD spectrum is in good agreement with the α-helix content in the crystal (Winkler et al., 1990), the β-sheet content seems to be overestimated by about 20%.

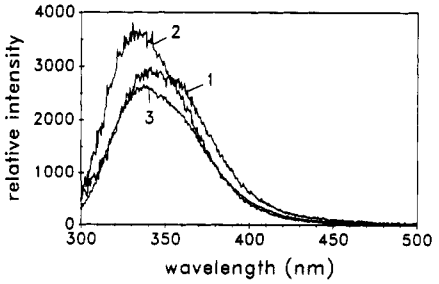

FIGURE 2: Fluorescence emission spectra of human pancreatic lipase after excitation at 280 nm in aqueous solutions (1) and in AOT reverse micellar solution (2, 3). For the meaning of spectra 1–3, see Figure 1.

The CD spectrum of human pancreatic lipase solubilized in AOT reverse micelles (curve 2 in Figure 1) indicates that the conformation of the polypeptide chain is slightly altered in reverse micelles with respect to an aqueous environment. Although the spectral changes in the far-UV region are relatively small, larger changes occur in the near-UV region, which is dominated by the signals arising from the aromatic amino acid side chains Trp and Tyr.

The changes observed in the CD spectrum of the lipase in going from water to reverse micelles are paralleled by changes in the intrinsic fluorescence properties of the lipase. The corresponding fluorescence emission spectra of human pancreatic lipase after excitation at 280 nm are shown in Figure 2. The emission maximum is shifted from 348 nm (in water, curve 1) to 334 nm (in AOT reverse micelles, curve 2), accompanied by changes in the relative fluorescence intensity (Figure 2). A blue shift in the emission spectrum has often been observed for proteins in reverse micelles [e.g., see Grandi et al. (1981), Nicot et al. (1985), and Marzola and Gratton (1991)]. This blue shift indicates a decreased polarity of the protein microenvironment inside reverse micelles.

CD as well as fluorescence measurements indicate that the microenvironment of at least 1 of the 7 Trp or 15 Tyr residues is changed upon solubilizing the lipase in AOT reverse micelles. However, the main chain conformation, as judged from CD, does not change drastically. It was therefore interesting to carry out activity measurements in reverse micelles. This has been accomplished by using two recently developed spectrophotometric techniques which both allow one to follow continuously the time course of triglyceride hydrolysis (Walde & Luisi, 1989; Walde, 1990). The results shows that pure (colipase-free) human pancreatic lipase is rapidly inactivated in AOT reverse micelles, in particular if solubilized in the absence of substrate. Although a small hydrolytic activity can be detected within 1 or 2 min after solubilization, complete inactivation of the enzyme occurs under the conditions used within 30 min after solubilization (data not shown).

Human pancreatic lipase could be solubilized in a catalytically active form in AOT reverse micelles if the enzyme was first premixed with human (or porcine) pancreatic colipase (Figure 3). Colipase therefore prevents the lipase from being inactivated in AOT reverse micelles. For our AOT system, colipase seems to be an essential cofactor of pancreatic lipase, and most probably, colipase cannot be replaced by other unspecific proteins, such as bovine serum albumin (data not shown). This is further supported by the observation that porcine pancreatic lipase samples containing only porcine colipase as an impurity ("lipase-B, FE-98"; see Materials and Methods) are also active in reverse micelles (data not shown).

In order to test whether in reverse micelles the binding of inhibitors acts as the binding of substrates, we have carried

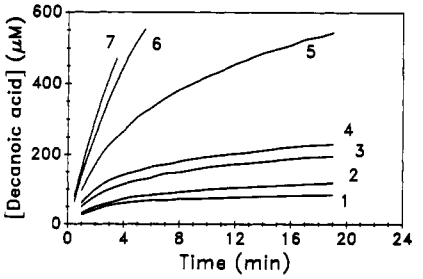

FIGURE 3: Effect of porcine pancreatic colipase on the activity of human pancreatic lipase at 25 °C in 50 mM AOT/isooctane reverse micelles, w_0 = 11 (prepared with 50 mM Tris/HCl, pH 9). Initial substrate concentration: 47 mM tridecanoylglycerol, $[\text{lipase}]_{ov}$ = 34 nM. Variation of the molar ratio (R_{CL}) of colipase to lipase. (1) No lipase, no colipase; (2) no colipase; (3) R_{CL} = 0.5; (4) R_{CL} = 0.9; (5) R_{CL} = 2.3; (6) R_{CL} = 3.7; (7) R_{CL} = 4.6. Determined with the phenol red method, $[\text{phenol red}]_{ov}$ = 5 μM.

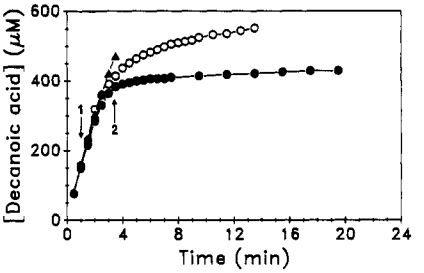

FIGURE 4: Effect of tetrahydrolipstatin (THL) on the activity of human pancreatic lipase (34 nM overall) in the presence of porcine pancreatic colipase (155 nM overall) at 25 °C in 50 mM AOT/isooctane reverse micelles at w_0 = 11 (prepared with 50 mM Tris/HCl, pH 9). Initial substrate concentration: 47 mM tridecanoylglycerol. Determined with the phenol red method, $[\text{phenol red}]_{ov}$ = 5 μM. (▲) No THL; (○) $[\text{THL}]_{ov}$ = 40 nM, added after 1 min 30 s (1); (●) $[\text{THL}]_{ov}$ = 2.3 μM, added after 3 min 30 s (2).

out inhibition studies of human pancreatic lipase. Tetrahydrolipstatin (THL) is a selective, covalent inhibitor of pancreatic lipase (Hadváry et al., 1988; Gargouri et al., 1991); THL binds as an ester to Ser-152 at the active site of the lipase (Hadváry et al., 1991; Lüthi-Peng et al., 1992). Since THL is almost insoluble in water but highly soluble in an apolar organic solvent such as isooctane, it was interesting—and also easy—to check whether THL is able to inhibit human pancreatic lipase also in AOT reverse micelles. Figure 4 shows the effect of adding THL during the initial stage of the reaction in the presence of colipase: pancreatic lipase is inhibited by THL, the time for complete inhibition being shorter the higher the THL concentration. As judged by FTIR measurements (see Materials and Methods), no reactivation of the lipase could be observed even after a long-time storage for several days (data not shown). Therefore, the lipase–THL complex seems to be rather stable in reverse micelles. Inhibition of human pancreatic lipase could also be achieved in the absence of colipase (data not shown). These studies were performed immediately after solubilization of the enzyme. They indicate that lipase–THL complex formation does not require the presence of colipase.

The effect of THL on the CD spectrum of human pancreatic lipase in AOT reverse micelles is shown in Figure 1 (curve 3). The spectrum of this lipase–THL complex is rather similar to the spectrum of THL-inhibited lipase in aqueous solution in the presence of bile salts and dimethyl sulfoxide (Lüthi-Peng & Winkler, 1992). As shown in Figure 2, THL has also an effect on the fluorescence spectrum of pancreatic lipase in reverse micelles, the fluorescence intensity being decreased in the presence of THL.

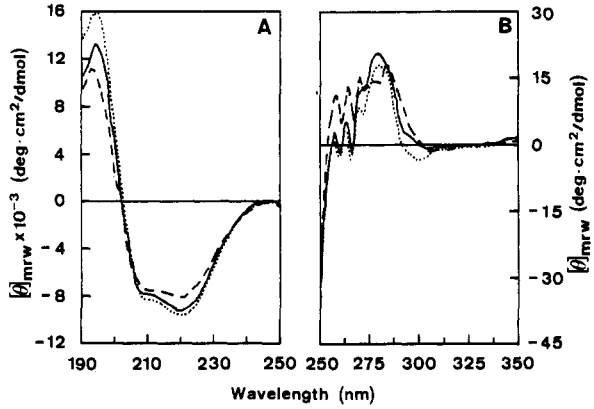

FIGURE 5: Effect of pH on the far-UV (A) and near-UV (B) circular dichroism spectra of *C. rugosa* lipase in aqueous solutions. Mean residue ellipticities are plotted as a function of wavelength. (···) In 50 mM KCl/HCl, pH 2.3; (—) in 75 mM Tris/HCl, pH 8.1; (- - -) in 50 mM sodium borate/HCl, pH 9.3.

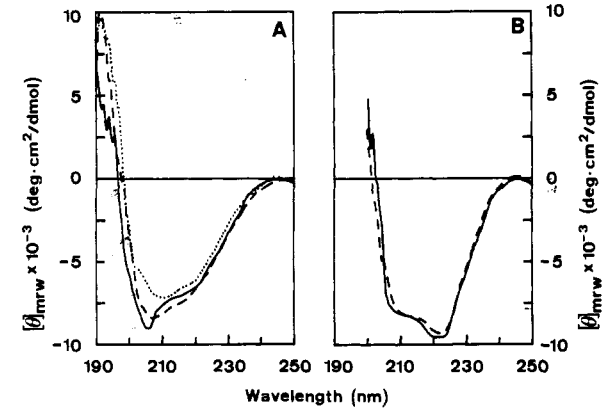

FIGURE 6: Effect of water content (A) and oleic acid (B) on the far-UV circular dichroism spectrum of *C. rugosa* lipase solubilized in 50 mM AOT/isooctane reverse micelles. w_0 is varied by adding 75 mM Tris/HCl, pH 8.1. (A) No oleic acid: w_0 = 10 (···); w_0 = 20 (- - -); w_0 = 30 (—). (B) 10 mM oleic acid: w_0 = 8 (- - -); w_0 = 20 (—).

Candida rugosa Lipase. As already mentioned, no X-ray structural data are known for this enzyme. Furthermore, no detailed spectroscopic studies of *C. rugosa* lipase are reported in the literature.

We have first measured the pH dependence of the CD spectrum of this lipase between pH 2.3 and 9.3 in aqueous solution (Figure 5). At pH 2.3 and 8.1, the spectra are almost superimposable with a very weak positive band in the near-UV range, around 280 nm, and two minima in the far-UV range, at 208 and 222 nm. At pH 9.3, the intensity of the bands in the far-UV range of the spectrum is slightly decreased, indicating most probably a lower content in secondary structure. The α-helix content as estimated with the method of Chen et al. (1972) is around 23–24% at pH 2.3 or 8.1 and about 18% at pH 9.3. These approximate values for the α-helix content are comparable to the values for the pancreatic lipase.

Upon solubilization of the lipase in AOT reverse micelles at different w_0 values, a change in the CD spectrum is observed with respect to water (Figure 6). The negative maximum at 210 nm in aqueous solution is shifted to 205 nm, indicating most probably a decrease in the α-helix content, in particular at higher w_0 values. A similar effect (lower secondary structure content at larger w_0) has been observed for other enzymes in reverse micelles (Steinmann et al., 1986). In the presence of oleic acid, however, the CD spectrum of the lipase in reverse micelles (at w_0 = 8 or 20) is rather similar to the corresponding (low-pH) spectrum in water (Figure 5), indicating an increase in conformational stability of the solubilized lipase in the presence of ligands. Higher lipase stability in reverse micelles in the presence of substrate or product has recently also been observed by others (Chang & Rhee, 1990; Tsai & Chiang, 1991).

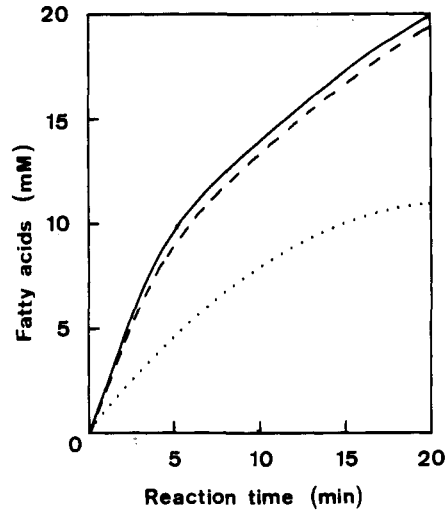

FIGURE 7: Effect of tetrahydrolipstatin (THL) on the activity of *Pseudomonas sp.* lipase at 30 °C in 50 mM AOT/isooctane reverse micelles, w_0 = 6 (prepared with 50 mM Tris/HCl, pH 7). Initial substrate concentration: 20% (v/v) olive oil, $[\text{lipase}]_{ov}$ = 340 nM. (—) No THL; (- - -) 113 nM THL; (···) 1 μM THL.

Since not much is known about the mechanism of action and the active site of *C. rugosa* lipase, we have also studied the effect of THL (10 μM) on the hydrolysis of olive oil (20% v/v) in reverse micelles in the presence of 75 nM lipase (data not shown). While under these conditions the colipase-dependent human pancreatic lipase is completely inhibited by THL (Figure 4), *C. rugosa* lipase is not. Within the initial time of the reaction (at least up to 25 min), there is no significant difference between the reaction without THL and the reaction with THL. This observation could be confirmed by using *p*-nitrophenyl palmitate as substrate (1 mM) and 63 nM lipase. The chemical nature of the active sites in these two lipases is therefore most likely quite different.

Pseudomonas sp. Lipase. The near-UV CD spectrum of *Pseudomonas sp.* lipase in aqueous solution at pH 7.0 (50 mM Tris/HCl) shows a rather low positive band similar to the case of *C. rugosa* lipase, the mean residue ellipticity being less than 10 deg cm^2 dmol^{-1} at 280 nm (data not shown). In the far-UV region, the shape of the CD spectrum is also very similar to the spectrum of the *Candida* enzyme, with minima around 220 nm (–7500 deg cm^2 dmol^{-1}) and 210 nm (–7000 deg cm^2 dmol^{-1}). Again, solubilization of the lipase in reverse micelles leads to changes in the CD spectrum, in analogy to the changes observed in the case of the *Candida* lipase; the intensity of the negative band at 220 nm is lowered by about 10% at w_0 = 10 (50 mM AOT/isooctane).

With respect to THL, the *Pseudomonas sp.* lipase behaves differently from the *C. rugosa* lipase (Figure 7). With an overall concentration of 340 nM lipase, 1 μM THL clearly acts as lipase inhibitor. Under the conditions of Figure 7, the olive oil hydrolysis in reverse micelles comes almost to a stop within less than 20 min. This inhibition study indicates that the active sites of the *Pseudomonas sp.* lipase have some common feature with (human) pancreatic lipase.

CONCLUSIONS

The aim of this comparative study of three different lipases is 2-fold: on the one hand, comparison for each enzyme of the

behavior in water and reverse micelles should shed light on the effect of the water pool microenvironment on the conformation; on the other hand, comparison of the three different enzymes with one another in the same environment should indicate whether the micellar environment is able to emphasize the difference in behavior among related enzymes. This second point is a particularly interesting one. In the case of α-chymotrypsin vis à vis trypsin, for example, it has been shown that certain properties of the two proteinases, which in water are very similar, became different in reverse micelles (Walde et al., 1988; Peng & Luisi, 1990). Concerning more generally the effect of the micellar environment on the enzymatic properties, much data have been accumulated in the literature (Luisi & Magid, 1986; Martinek et al., 1986; Waks, 1986). More recently, the case of lipases in reverse micelles has been the focus of much interest [e.g., see Han et al. (1987), Holmberg and Oesterberg (1990), Chang and Rhee (1990), and Walde and Luisi (1989)]. With this background, let us briefly analyze the meaning of the data of the present paper.

The most important finding in the case of pancreatic lipase is the rapid inactivation of the enzyme in AOT reverse micelles in the absence of colipase. The observation that (pure) pancreatic lipase is inactivated in reverse micelles is in agreement with earlier reports by Malakhova et al. (1983) and Kuboi et al. (1990) using porcine pancreatic lipase and tributyrin or triolein, although these authors did not directly address the question of the importance of colipase.

The mechanism of lipase inactivation is not known. One may propose that AOT molecules bind at the active site of the enzyme, thus hindering substrate molecules to reach the reactive Ser-152. This possibility appears to be in agreement with CD and fluorescence measurements in view of the fact that the inhibitor THL, known to bind covalently to Ser-152 (Hadváry et al., 1991; Lüthi-Peng et al., 1992), also leads to significant changes in the near-UV CD spectrum (Figure 1B). However, one does not see how according to this mechanism the presence of colipase would then prevent enzyme inactivation. In fact, on the basis of new X-ray evidence (Van Tilbeurgh et al., 1992), colipase is physically distant from the substrate binding site/active site of the lipase.

The reaction with THL permits a more general observation: that reverse micelles which can host water-soluble as well as water-insoluble compounds are rather convenient systems to study enzyme inhibition by water-insoluble inhibitors, such as THL. Reverse micelles could therefore be used as alternatives to the conventional aqueous emulsion media (Hadváry et al., 1988; Gargouri et al., 1991).

The situation in the case of *Candida rugosa* lipase is quite different, since this enzyme does not require a cofactor-protein for activity. The conformation of this lipase is altered on going from water into reverse micelles in the absence of substrate (or products). However, the presence of oleic acid stabilizes the native conformation of the enzyme (Figure 6). It seems that the overall polypeptide conformation of the *C. rugosa* lipase in water corresponds to that of the lipase during catalysis in reverse micelles. Since THL is not an inhibitor of *Candida rugosa* lipase, the active site of this enzyme must be considerably different from the catalytic site of pancreatic lipase. This finding can be understood on the basis of the fact that the *Candida* lipase belongs to the cholinesterase family of lipases (Shimada et al., 1990) while the colipase-dependent pancreatic lipase is in the lipase gene family (Hide et al., 1992).

Pseudomonas sp. Lipase. With respect to the behavior against THL, *Pseudomonas* sp. lipase seems to have common features with pancreatic lipase; this bacterial lipase is most probably a serine hydrolase.

ACKNOWLEDGMENT

We thank Dr. F. Winkler (Hoffmann-La Roche, Basel, Switzerland) for providing us with human pancreatic lipase and colipase and for the many discussions and information on the crystal structure of pancreatic lipase. We also thank Prof. A. Fischli (Hoffmann-La Roche) for THL samples and for his interest in the work and Prof. R. Verger (CNRS, Marseille, France) for the porcine pancreatic lipase samples and for his suggestions.

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