Cutinase from *Fusarium solani pisi* Hydrolyzing Triglyceride Analogues. Effect of Acyl Chain Length and Position in the Substrate Molecule on Activity and Enantioselectivity†

Maurice L. M. Mannesse,‡ Ruud C. Cox,‡ Bart C. Koops,‡ Hubertus M. Verheij,*,‡ Gerard H. de Haas,‡ Maarten R. Egmond,§ Harry T. W. M. van der Hijden,§ and Jacob de Vlieg§

Department of Enzymology and Protein Engineering, CBLE, Utrecht University, the Netherlands, and Unilever Research Laboratory, Vlaardingen, the Netherlands

Received November 23, 1994; Revised Manuscript Received February 22, 1995⊗

ABSTRACT: Triglyceride analogues were synthesized in which one of the primary acyl ester functions has been replaced by an alkyl group and the secondary acyl ester bond has been replaced by an acyl amino bond. The chain length at either position was varied, and both (*R*)- and (*S*)-enantiomers of each compound were synthesized. These pseudo triglycerides contain only one hydrolyzable ester bond, and they are ideally suited to studying the influence of the chain length at the 1-, 2-, and 3-position on lipase activity and on stereopreference. These substrates were used to characterize cutinase from *Fusarium solani pisi*. Our results show that the activity of cutinase is very sensitive to the length and distribution of the acyl chains and that the highest activities are found when the chains at positions 1 and 3 contain three or four carbon atoms. The enzyme preferentially hydrolyzes the (*R*)-enantiomers, but this preference is strongly dependent on the acyl chain length distribution, with (*R*) over (*S*) activity ratios varying from about 30 to 1. This enantioselectivity was found in three different assay systems: a mixed micellar, a reverse micellar, and a monolayer study. Our data suggest that at least two alkyl chains of the pseudo triglycerides must be fixed during hydrolysis. Therefore, these substrates were used to characterize mutants of cutinase with mutations in putative lipid binding domains. Two mutants (A85F and A85W) have increased activities. The results obtained with these mutants suggest an interaction of the acyl chain of the scissile ester bond with a surface loop, comprising residues 80−90, in the enzyme−substrate complex.

Lipases catalyze the hydrolysis of water-insoluble substrates, like triacylglycerols. They display a depressed activity on monomeric substrates and are fully active when a lipid−water interface is present. This event is known as interfacial activation (Sarda & Desnuelle, 1958). Until recently little was known about the molecular basis of interfacial activation. It was not until 1990 that high-resolution crystal structures of *Rhizomucor miehei* lipase, human pancreatic lipase, and *Geotrichum candidum* lipase were published (Brady *et al.*, 1990; Winkler *et al.*, 1990; Schrag *et al.*, 1991). In these X-ray structures the catalytic center is buried beneath one or more surface loops and is inaccessible to solvent and substrate molecules. Recently the comparison of the X-ray structures of *Rhizomucor miehei* lipase, irreversibly modified with hexyl phosphonate ethyl ester and diethyl *p*-nitrophenyl phosphate became available (Brzozowski *et al.*, 1991; Derewenda *et al.*, 1992). This revealed a displacement of the surface loop (or "lid") covering the active site upon inhibitor binding. It is thought that this movement is one of the main events in the interfacial activation process. The hexyl chain of the phosphonate inhibitor binds in a groove formed by the movement of this lid. Recently, the structure of *Candida rugosa* lipase, complexed with several covalent transition state analogue inhibitors, each containing one alkyl chain, was published (Grochulski *et al.*, 1994).

Cutinase from *Fusarium solani pisi* is a lipolytic enzyme that catalyzes the hydrolysis of the water-insoluble biopolyester cutin which covers the surface of plants. In addition, cutinase is able to hydrolyze a wide variety of esters ranging from soluble *p*-nitrophenyl esters to insoluble long-chain triglycerides (Kolattukudy *et al.*, 1984; Lauwereys *et al.*, 1990). Because cutinase hydrolyzes soluble esters and emulsified triacylglycerols as efficiently as esterases and lipases do, the enzyme forms a bridge between esterases and lipases. The X-ray structure of cutinase revealed an α/β-hydrolase fold with an active site serine accessible to solvent (Martinez *et al.*, 1992). This serine is a member of the Ser-Asp-His catalytic triad and is located in a crevice between two hydrophobic loops (80−90 and 182−189). It is thought that these loops are involved in both interfacial and substrate binding (Martinez *et al.* 1992, 1994). The knowledge of the substrate binding site of cutinase is mainly based on a crystallographic study of covalently inhibited cutinase with diethyl phosphate (Martinez *et al.*, 1994). This study gave accurate information about the atoms involved in the oxyanion hole formation during catalysis. However, the structure of the complex provides no insight into the interaction between cutinase and a triglyceride molecule. Until now no experimental structural data were known from

† This research was carried out with financial aid from the BRIDGE program of the European Community under Contract No. N-BIOT-0194 NL.

* Author to whom correspondence should be addressed: University of Utrecht, P.O. Box 80.083, 3508 TB Utrecht, The Netherlands. Telephone: +31-30-533526. Telefax: +31-30-522478. E-mail: m.mannesse@chem.ruu.nl.

‡ Utrecht University.

§ Unilever Research Laboratory.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1995.

0006-2960/95/0434-6400$09.00/0

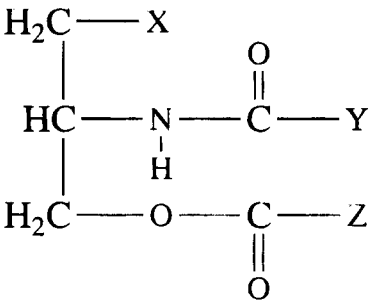

FIGURE 1: General structure of the synthetic triglyceride analogues. A pseudo-triglyceride is depicted with two acyl ester chains replaced with an alkyl chain and an acyl amino chain, respectively. The general formula of the substrates used in this paper is (*R*)- or (*S*)-*X*/*Y*/*Z*, with *X*, *Y*, and *Z* representing the number of carbon atoms on the alkyl, amide, and ester positions, respectively.

the literature about the interaction of lipases with a triglyceride analogue. For a better understanding of enzyme–substrate interaction it is important to determine the molecular background of the stereopreference and the influence of acyl chain length on activity. The determination of substrate selectivity and stereopreference of lipases with triglycerides as substrate is problematic, because the hydrolysis does not stop at the level of diglyceride. Moreover, acyl migration may occur at the level of both di- and monoglycerides. To avoid these problems, we decided to synthesize a number of triglyceride analogues containing only one hydrolyzable ester bond. The secondary ester group is replaced by an amide bond, while the third ester is replaced by an alkyl chain. Moreover, the starting materials to synthesize these compounds in both enantiomeric forms are in most cases commercially available. With the variation of the chain length on the three positions it is possible to determine the influence of the alkyl chain length on the hydrolysis of the ester bond. Both (*R*)- and (*S*)-enantiomers were prepared to study the influence of these chain lengths on the stereopreference as well. To study the binding of substrate in the active site of cutinase and particularly the involvement of a surface loop comprising residues 80–90, we also studied cutinase mutants.

MATERIALS AND METHODS

Chemicals. (*S*)- and (*R*)-norleucine, α-bromohexadecanoic acid, and $NaBH_4$ were purchased from Janssen Chimica. α-Bromododecanoic acid was purchased from Fluka. Thionyl chloride, triethylamine, decanoyl chloride, and hexadecanoyl chloride were purchased from Merck. Chloroacetyl-, acetyl-, propionyl-, butanoyl-, pentanoyl, hexanoyl, and octanoyl chloride were purchased from Janssen Chimica. Bis(2-ethylhexyl) sodium sulfosuccinate (AOT)[1] was purchased from Sigma. All other chemicals were of analytical grade.

Thin-layer chromatography was done on TLC plates from Merck. Optical rotations ($[\alpha]^{20}_D$) were determined with a Perkin Elmer polarimeter, Model 241 MC. ^{1}H-NMR measurements were carried out on a 360-MHz Bruker machine. Melting points (mp) were determined on a Leitz melting point microscope and are uncorrected.

Synthesis of (R)-3/3/3. The general structure of the synthetic triglyceride analogues is depicted in Figure 1.

For the synthesis of (*R*)-3/3/3, (*R*)-norleucine was esterified with methanol and thionyl chloride as described by Guttmann and Boissonnas (1958). Yield: 96%. $R_f = 0.86$ in $CHCl_3$/MeOH/H_2O (65/35/8, by volume); mp 140.5–141.5 °C; $[\alpha]^{20}_D = -20.3°$ ($c = 6$ in $CHCl_3$). The amino group was subsequently acylated with *n*-butanoyl chloride in dry $CHCl_3$ and triethylamine. Subsequently the methyl ester was reduced with $NaBH_4$ in tetrahydrofuran. After evaporation of tetrahydrofuran, the crude reaction product was acidified with 2.5% HCl and extracted with $CHCl_3$. The yield of the nonpurified alcohol product was 67%. Finally the hydroxyl group was acylated with *n*-butanoyl chloride in dry $CHCl_3$ and triethylamine. After acidification, the crude reaction product was extracted with $CHCl_3$. Pure (*R*)-3/3/3, a colorless solid, was obtained after column chromatography on silica gel using ether/hexane (4/6, v/v) as eluent. Yield: 60%. $R_f = 0.19$ in ether/hexane (1/1, v/v); $[\alpha]^{20}_D = +35.5°$ ($c = 3.5$ in $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ(ppm) 0.90–1.00 (9H, CH_3), 1.25–1.70 (10H, alkyl and β-CH_2), 2.10–2.20 (2H, αCH_2CON), 2.25–2.40 (2H, αCHCOO), 4.00–4.10 (1H, NCH), 4.15–4.25 (2H, OCH_2).

Synthesis of (S)-3/3/3. This enantiomer was synthesized from (*S*)-norleucine as described for the (*R*)-3/3/3 compound. $[\alpha]^{20}_D = -34.7°$ ($c = 5$ in $CHCl_3$). Chromatographic and NMR characteristics were identical to those of the (*R*)-enantiomer. Physical parameters are given in Table 1.

Synthesis of (R)- and (S)-3/Y/Z. All compounds derived from norleucine were prepared as described for (*R*)- and (*S*)-3/3/3, except for the choice of acyl chloride for the acylation reactions on positions *Y* and *Z*. The (*R*)- and (*S*)-3/*Y*/*Z* triglyceride analogues were all purified by column chromatography on silica gel with ether/hexane as eluent, except for (*R*)- and (*S*)-3/9/9; the latter two were crystallized from diethyl ether at −20 °C. Physical parameters are given in Table 1.

Preparation of Enantiomerically Pure (R)- and (S)-α-Aminododecanoic Acid. *rac*-α-Bromododecanoic acid was aminated with a molar excess of NH_3 (Marvel, 1955). After filtration and washing with methanol, pure *rac*-α-amino acid was obtained. Yield: 82%. *rac*-α-Aminododecanoic acid was *N*-chloroacylated with chloroacetyl chloride in dimeth-

Table 1: Physical Parameters of the Triglyceride Analogues[a]

	(*R*)-enantiomer			(*S*)-enantiomer		
substrate	mp (°C)	$[\alpha]^{20}_D$ (°)	R_f	mp (°C)	$[\alpha]^{20}_D$ (°)	R_f
3/3/3	36.5–37.0	+35.5	0.15	36.0–37.0	−34.7	0.16
3/3/9	40.0–41.0	+28.2	0.26	40.0–41.0	−28.7	0.23
3/9/3	30.5–32.0	+28.3	0.26	31.0–32.0	−27.9	0.29
9/3/3	47.0–48.0	+19.8	0.21	48.0–50.0	−22.6	0.24
9/9/9	78.5–79.0	+15.7	0.44	79.0–80.0	−16.9	0.44
9/9/3	48.0–48.5	+18.2	0.38	48.0–49.0	−19.8	0.38
9/3/9	83.5–84.0	+18.7	0.29	82.0–83.5	−19.9	0.30
3/9/9	44.0–45.5	+23.6	0.38	44.0–45.0	−20.6	0.40
3/3/15	69.0–70.0	+20.7	0.29	71.5–72.0	−20.6	0.29
3/15/3	52.0–52.5	+21.5	0.29	53.5–54.0	−22.2	0.33
13/3/3	66.5–67.0[b]	nd[b]	0.21[b]	70.0–71.0	−20.1	0.26
3/3/1	51.0–52.0	+34.6	0.10			
3/3/2	51.0–52.0	+39.3	0.12			
3/3/4	48.5–49.5	+34.2	0.16			
3/3/5	55.5–56.0	+30.4	0.16			
3/3/7	44.0	+28.0	0.22			
3/5/3	32.0–33.0	+29.9	0.22			
3/7/3	22.5–24.0	+26.7	0.28			

[a] R_f values were determined with thin-layer chromatography on silica gel with ether/hexane (1/1, v/v) as eluent. $[\alpha]^{20}_D$ values were determined with $CHCl_3$ as solvent. n.d. = not determined. [b] These physical parameters were obtained with the racemic 13/3/3, and therefore the optical rotation of 13/3/3 was not determined.

[1] Abbreviations: AOT, bis(2-ethylhexyl) sodium sulfosuccinate; DMF, dimethylformamide; DOC, sodium desoxycholate.

ylformamide (DMF). DMF was evaporated under reduced pressure at 35 °C, and (*rac*-chloroacetamino)dodecanoic acid was subsequently precipitated with ice−water. After crystallization from hexane with a small amount of $CHCl_3$, pure (*rac*-chloroacetamino)dodecanoic acid was obtained with a yield of 65%. mp 92.5−94 °C. Enantiomeric resolution of *rac*-α-chloroacetamide was performed as described by Mori and Funaki (1985). Pure (*S*)-α-aminododecanoic acid was obtained. Yield: 97%. $[\alpha]^{20}_D = +5.2°$ (c = 10, in 2 M NaOH). Pure (*R*)-(α-chloroacetamino)dodecanoic acid was obtained after crystallization from hexane. Yield: 61%. mp, 79.5−82.5 °C. $[\alpha]^{20}_D = -29.6°$ (c = 2, in $CHCl_3$). (*R*)-α-Aminododecanoic acid was prepared by acid hydrolysis of (*R*)-(α-chloroacetamino)dodecanoic acid as described by Mori and Funaki (1985). Pure (*R*)-α-aminododecanoic acid was obtained. The yield was 42% with respect to *rac*-α-aminododecanoic acid. $[\alpha]^{20}_D = -5.8°$ (c = 9.7 in 2 M NaOH).

Synthesis of (R)- and (S)-9/Y/Z. All these preparations were performed as described for the syntheses where the starting material was norleucine. In this case the starting material was α-aminododecanoic acid. (*R*)- and (*S*)-9/9/9 were purified by crystallization from acetone at 4 °C. (*R*)- and (*S*)-9/9/3 were purified by column chromatography on silica gel with $CHCl_3$/MeOH (1/1, v/v) as eluent. Physical parameters are given in Table 1. ^{1}H-NMR of (*R*)-9/3/3 ($CDCl_3$) δ(ppm) 0.85−1.00 (9H, CH_3), 1.10−1.75 (22H, alkyl and β-CH_2), 2.10−2.20 (2H, αCH_2CON), 2.25−2.35 (2H, αCHCOO), 4.05−4.10 (1H, NCH), 4.15−4.25 (2H, OCH_2). The NMR characteristics of (*S*)-9/3/3 were identical.

Synthesis of (S)- and rac-13/3/3. *rac*-α-Bromohexadecanoic acid was aminated with 25% NH_3 in tetrahydrofuran (Marvel, 1955). With a yield of 91%, pure *rac*-α-aminohexadecanoic acid was obtained; mp, 220−225 °C. The amino group was subsequently chloroacetylated with chloroacetyl chloride in DMF. Pure (*rac*-α-chloroacetamino)-hexadecanoic acid was obtained. Yield: 64%. mp 99−101.5 °C. The (*R*)- and (*S*)-enantiomers were enzymatically separated with amino acylase as described by Mori and Funaki (1985). Pure (*S*)-aminohexadecanoic acid was obtained with a yield of 43%; mp, 219−222 °C. Due to incomplete hydrolysis of (*S*)-chloroacetamide, pure (*R*)-hexadecanoic acid could not be obtained. Hence, only pure (*S*)- and *rac*-13/3/3 could be prepared. ^{1}H-NMR of *rac*-13/3/3 ($CDCl_3$) δ(ppm) 0.90−1.00 (9H, CH_3), 1.10−1.80 (30H, alkyl and β-CH_2), 2.10−2.20 (2H, αCH_2CON), 2.25−2.40 (2H, αCHCOO), 4.00−4.10 (1H, NCH), 4.10−4.30 (2H, OCH_2). The NMR characteristics of (*S*)-13/3/3 were identical.

Enzymes. Details of the produced wild-type cutinase and variants will be published elsewhere. All enzyme constructs were produced in a yeast expression system and purified from the fermentation liquor. The N-terminal amino acid sequence of all enzyme variants is identical to the enzyme produced by the original *Fusarium* species (Kolattukudy *et al.*, 1984). The identity of the purified enzyme samples was checked using electrospray mass spectrometry and determination of the isoelectric point. The specific activities of the samples were routinely measured using an olive oil emulsion system (Sigma). Relative to the wild-type enzyme, activities ranged from 133% for A85F to 85% for S54W, with 105% for A85W and 101% for [A85F+G82A].

Amino acylase (10 000 units/g) from *Aspergillus* was purchased from Tokyo Kasei Co. Protein concentrations were determined spectrophotometrically using calculated $E^{1\%}$ values at 280 nm (Mach *et al.*, 1992) of 7.13 for cutinase wild type, A85F, and [A85F+G82A] and 9.68 for cutinase S54W and A85W.

pH-stat Measurements. The synthetic substrates were solubilized with Triton X-100/DOC (9/1, mol/mol), in a 5 mM Tris-HCl and 50 mM NaCl buffer of pH 8. Released fatty acid was measured using a Radiometer titration set (a PHM-84 pH meter, a TTT 80 titrator, an ABU-80 autoburette, a TTT 60 titration assembly, and a Rec-80 servograph). Assays were carried out under a constant nitrogen flow with approximately 5 mM sodium hydroxide at 30 °C. In so-called three-dimensional micellar experiments the amount of micelles was varied with a fixed mole fraction of substrate. In two-dimensional micellar experiments the amount of micelles was kept constant, whereas the mole fraction of substrate was varied between 0 and 1 mol %. From the latter measurements interfacial specificity constants (k_{cat}/K^*_m) were obtained.

Monolayer Measurements. The monolayer experiments were performed on a KSV 5000 barostat (KSV Instruments, Helsinki), controlled by a Sperry PC/Microl T. A zero-order trough with two compartments (Verger & de Haas, 1973), a reaction compartment (190 mL, 97 cm^2) and a 330-mL reservoir compartment, connected by a small surface channel was used to measure surface pressure versus mean molecular area isotherms and to perform enzyme kinetics. The surface pressure was determined by a Wilhelmy plate attached to an electromicrobalance connected in turn to a microcomputer controlling the movement of the barrier.

Surface pressure versus mean molecular area isotherms were measured for the enantiomeric substrate pairs (*R*)- and (*S*)-9/3/9 and (*R*)- and (*S*)-3/9/9 (Figure 3). Stock solutions of individual substrates were prepared in distilled $CHCl_3$ just before use. Fifty microliters of substrate solution with an accurately known concentration was spread on the aqueous buffer. The monolayer was allowed to stabilize for 5 min before it was compressed with a continuous increase in surface pressure per minute [linear compression of 1.0 mN/(m min)].

The enzymatic determinations were performed on the zero-order trough as described above. The reaction compartment was magnetically stirred. A substrate film was spread from a $CHCl_3$ solution. The film was compressed to a desired surface pressure with a maximal barrier speed of 10 mm/min. The monolayer was allowed to stabilize for several minutes before enzyme was injected under the film of the reaction compartment. Constant surface pressure was maintained by a computer-controlled barrier movement. Activity appeared to be proportional to the amount of cutinase added. Kinetic data were analyzed according to a previous model (Verger *et al.*, 1973; Ransac *et al.*, 1990) and fitted by nonlinear regression analysis (Nelder *et al.*, 1965).

Activity Measurements in a Reverse Micellar System. Reverse micellar solutions were made by adding appropriate amounts of an aqueous cutinase solution (in 50 mM Tris, pH 8.5) to a 2-mL isooctane solution of 100 mM AOT and 100 mM substrate (w_0 = [H_2O]/[AOT] ratio = 10). Cutinase appeared to be unstable in this system with a 50% activity loss in 15 min. To improve the stability, 100 mM hexanol was added (Sebastião *et al.*, 1993). With this w_0 value and this hexanol concentration, the hydrolytic reaction rate was about 20-fold higher than the synthetic reaction rate (data not shown). The activity was determined with

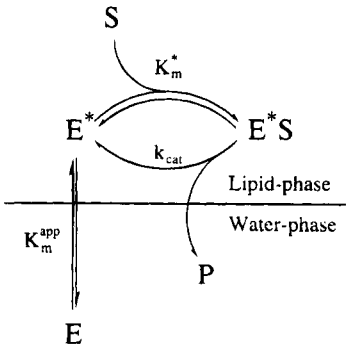

FIGURE 2: Model for the action of a lipolytic enzyme at an interface. The following symbols are used: E, enzyme in the water phase; E*, enzyme adsorbed to the interface; S, substrate; E*S, interfacial enzyme−substrate complex; K_m^{app}, apparent three-dimensional affinity constant; K_m^*, interfacial (two-dimensional) affinity constant; k_{cat}, catalytic rate constant; P, products of hydrolysis [model adopted from Verger and de Haas (1976)].

a spectrophotometric assay as described by Lowry and Tinsley (1976). Every 5 min 400 μL of the mixture was added to 850 μL of toluene. Two hundred and fifty microliters of cupric acetate/pyridine was added, and the mixture was subsequently vortexed for 30 s. After centrifugation, the absorbance of the toluene phase was measured at 715 nm. The following enantiomeric pairs were assayed: 9/3/3 and 3/9/3.

RESULTS

The kinetic behavior of cutinase from *Fusarium solani pisi* was studied with a number of triglyceride analogues of the general structure depicted in Figure 1. The influence of the chain length of the ester, amide, and alkyl positions on cutinase activity and stereoselectivity was investigated. Since these compounds are insoluble in water and since they do not spontaneously form micelles, we screened for detergents that readily form mixed micelles with these substrates. The solubility varied with the detergent used and with the hydrophobicity of the substrates. A low solubility was observed with anionic detergents like deoxycholate (DOC) or taurodeoxycholate especially, and the solubility decreased with increasing chain length (data not shown). In Triton X-100 the solubility was higher (at least 1 mol % for the most hydrophobic compound), but the enzymatic activity with Triton X-100 micelles was low. Satisfactory activities and solubilities were observed with mixed micelles composed of Triton X-100 containing 10% DOC.

Two steps are important for the activity of a lipolytic enzyme at an interface (see Figure 2). The first step involves the binding of the enzyme to the micellar interface. All enzyme can be bound to the interface by increasing the amount of micelles. In this case the three-dimensional substrate concentration (≡micellar surface) is varied. The second step is binding of a substrate monomer to the active site of the enzyme. This step can be studied by increasing the interfacial concentration (≡mole fraction) of substrate while keeping the amount of micelles constant. Thus by varying the two-dimensional substrate concentration, the equilibrium E ⇌ E*S can be shifted toward the E*S form. In these two-dimensional micellar measurements, the interfacial substrate concentration should be kept low for two reasons: First, the solubility of the substrate is limited. Second, the effect of the substrate molecules on the quality of the interface (Verger & de Haas, 1976) must be negligible, and therefore, the interfacial concentration must be kept low.

Table 2: Kinetic Properties of Cutinase with Triglyceride Analogues of the Form (*R*)/(*S*)-*X*/*Y*/*Z*[a]

	(*R*)-enantiomer	(*S*)-enantiomer	
substrate	k_{cat}/K_m^* (M^{-1} s^{-1})	k_{cat}/K_m^* (M^{-1} s^{-1})	(*R*)/(*S*) activity ratio
3/3/3	37 000	2400	15
3/9/3	27 000	1200	23
3/15/3	14 000	500	28
3/3/9	11 000	900	12
3/9/9	9000	300	30
9/9/3	3100	1000	3.1
3/3/15	2300	200	12
9/3/3	2000	2000	1.0
9/3/9	1000	800	1.3
13/3/3	800[b]	1000	
9/9/9	600	120	5.0

[a] *X*, *Y*, and *Z* represent the number of carbon atoms of the alkyl, amide, and ester positions, respectively. Experimental details are described in the experimental session. [b] This value represents the specificity constant for racemic 13/3/3 and not that for (*R*)-13/3/3.

In initial experiments three-dimensional binding curves were determined. The activity of cutinase was measured with varying concentrations of Triton X-100/DOC micelles containing fixed concentrations of 1 mol % of the (*R*)-enantiomers of the substrates 3/3/1, 3/3/3, and 3/3/15 and both enantiomers of the substrates 9/3/3 and 3/9/3. Saturation curves were obtained for these substrates, and it appeared that at a concentration of 150 mM Triton X-100/17 mM DOC virtually all cutinase is bound to the micelles. On the basis of these results we assume that saturation is reached for the other substrates also under these conditions. Therefore, all two-dimensional micellar measurements were carried out with 150 mM Triton X-100 and 17 mM DOC in the presence of 0.1−1 mol % substrate. For all substrates, a linear dependence between the interfacial concentration and cutinase activity was found. This finding indicates that K_m^* is significantly larger than the interfacial substrate concentration, and hence k_{cat} and K_m^* values are highly correlated and cannot be determined separately. However, from the slopes of the lines the interfacial specificity constants, k_{cat}/K_m^*, can be determined.

In Table 2, the interfacial specificity constants are given for a number of synthetic triglyceride analogues. From these data it is clear that cutinase hydrolyzes the (*R*)-enantiomers faster than the corresponding (*S*)-enantiomers. The magnitude of this stereopreference is strongly dependent on the alkyl chain length distribution and ranges from 1 to 30. In general a high ratio is found for substrates with a short chain on the alkyl position and a long chain on the amide position, whereas a low ratio is found when a long chain is present on the alkyl position and a short chain is present at the amide position. The results, summarized in Table 2, also show the influence of the chain length distribution on cutinase activity. Both for the series of the (*R*)-enantiomers and for the series of the (*S*)-enantiomers, it is obvious that cutinase activity decreases with an increasing number of carbon atoms on any of the three positions of the triglyceride analogues.

It is well known that the physicochemical properties of the substrate may strongly influence the kinetic behavior of lipases. Such effects cannot explain the results obtained with both enantiomers of the same substrate. Furthermore, the physicochemical differences between, for example, the three compounds 3/3/9, 3/9/3, and 9/3/3 are probably not sufficient to explain the different hydrolysis rates of these compounds.

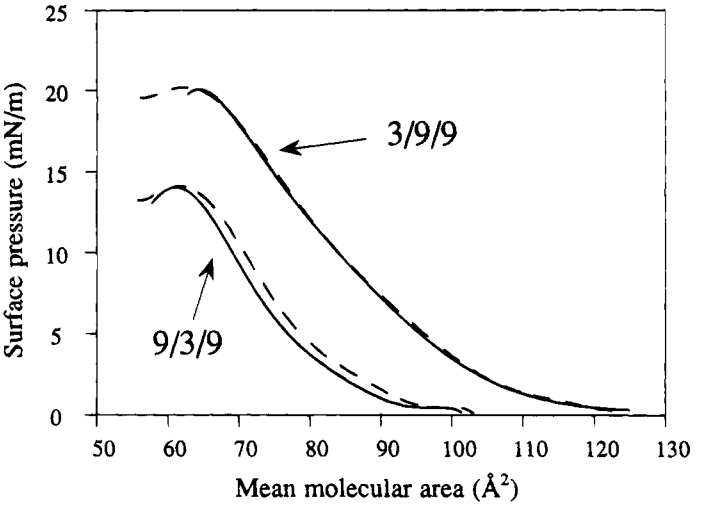

FIGURE 3: Surface pressure versus mean molecular area in monomolecular films of the enantiomeric pairs 3/9/9 and 9/3/9: (*R*)-enantiomers, (—); (S)-enantiomers, (— —). For further details, see Materials and Methods.

Table 3: Dependence of the Activity and the Enantioselectivity of Cutinase on the Enzyme Assay Used

	enzyme assay		
	pH-stat	reversed micelles	monolayer
substrate	k_{cat}/K_m* (M^{-1} s^{-1})	activity[b] (μmol μg^{-1} min^{-1})	activity × 10^{-12} [c] (molecules cm^{-2} min^{-1} μg^{-1})
(*R*)-3/9/9	9000	n.d.	7.5
(*S*)-3/9/9	300	n.d.	0.58
(*R*)-3/9/3	27 000	843	n.d.
(*R*)-3/9/3	1200	71	n.d.
(*R*)-9/3/9	1000	n.d.	2.9
(*S*)-9/3/9	800	n.d.	0.83
(*R*)-9/3/3	2000	84	n.d.
(*S*)-9/3/3	2000	77	n.d.

[a] The kinetic data obtained with the enantiomeric pairs 3/9/9, 9/3/9, 9/3/3, and 3/9/3 in a reversed micellar assay and in monolayer measurements are given. For reasons of comparison, the results of the bulk experiments are presented as well. n.d. = not determined. [b] Hydrolysis rate during the first 5 min of the reaction. [c] The enzyme activities shown here were determined at a surface pressure of 10 mN/m. In all cases the activities are given in molecules cm^{-2} min^{-1} (μg of added enzyme)$^{-1}$.

To test this assumption, we analyzed four pairs of enantiomers, exhibiting quite different (*R*) over (*S*) activity ratios. Unfortunately the enantiomeric pairs 3/9/9 and 9/3/9 are not soluble in the reverse micellar system AOT/isooctane, and the enantiomeric pairs 9/3/3 and 3/9/3 do not form stable monomolecular films at the air—water interface. This means that a complete comparison between the three assay systems with identical enantiomeric pairs is impossible. Therefore, the enantioselectivity of cutinase on the pairs 3/9/9 and 9/3/9 was determined in a monomolecular film assay. The enantiomeric pairs 3/9/3 and 9/3/3 were tested in a reverse micellar system.

In Figure 3 the surface pressure/mean molecular area isotherms of the enantiomeric pairs 3/9/9 and 9/3/9 are represented. The isotherms of the (*R*)- and (*S*)-isomers of both enantiomeric pairs coincide and indicate a high chemical purity of the triglyceride analogues. The 3/9/9 compounds form stable monolayers at the air—water interface up to 20 mN/m, and the 9/3/9 compounds do so up to 15 mN/m.

The results of the kinetic measurements in the two different assay systems are summarized in Table 3, and for reasons of comparison the data obtained with the pH-stat measurements are included as well. From these data it is clear that, although there are quantitative differences, qualitatively the stereopreference of cutinase for the four enantiomer pairs is independent of the assay system.

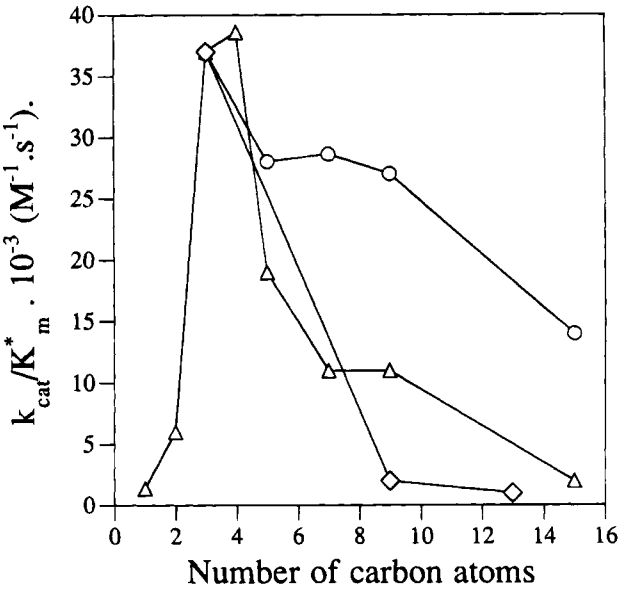

FIGURE 4: Influence of the alkyl chain length on cutinase activity. Three substrate series were studied. In the first series the number of carbon atoms of the ester position was varied: (*R*)-3/3/*Z*, with *Z* varying from 1 to 15 carbon atoms (△). In the second series the number of carbon atoms on the amide position was varied: (*R*)-3/*Y*/3, with *Y* varying from 3 to 15 carbon atoms (○). In the third series the number of carbon atoms on the alkyl position was varied: (*R*)-*X*/3/3, with *X* varying from 3 to 13 carbon atoms (◇). The reaction conditions are described under Materials and Methods.

To study in more detail the influence of the chain length on the three positions separately, three substrate series were prepared wherein one of the chains was varied in length while the other two were kept constant at three carbon atoms. The results of the two-dimensional micellar measurements of cutinase with these substrate series are given in Figure 4. Independent of the position of the alkyl chain, the activity of cutinase was low with substrates containing a large number of carbon atoms. While the activity rapidly dropped with increasing length (in general, larger than three or four carbon atoms) of the chains at the alkyl and the ester position, the chain length on the amide position only moderately influenced activity.

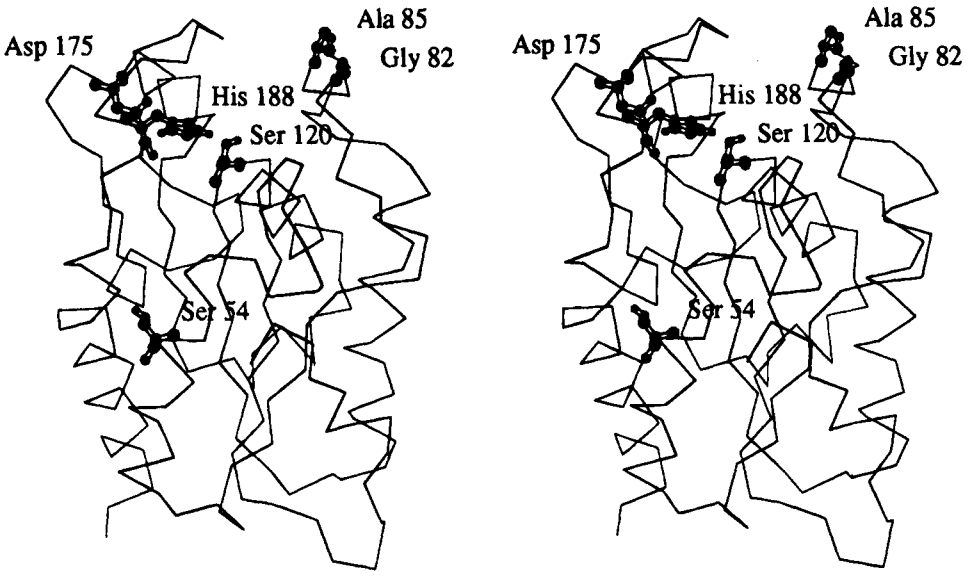

FIGURE 5: Stereoview of the X-ray structure of cutinase using the Molscript program (Kraulis, 1991). The positions of the mutations of the cutinase mutants studied in this paper are highlighted in ball and stick: Ala 85, Gly 82, and Ser 54. The active center residues Ser 120, Asp 175, and His 188 are depicted as well [X-ray structure adopted from Martinez *et al.* (1992)].

The results described above suggest that in cutinase different binding pockets may exist that can accommodate a short chain at the external (ester or alkyl) positions and a somewhat longer chain at the internal, amide position. The X-ray structure of native cutinase has been determined (Martinez *et al.*, 1992) and is depicted in Figure 5.

On the basis of the structure of cutinase, covalently inhibited with a diethyl phosphate, it has been suggested (Martinez *et al.*, 1994) that the surface loop (80−90) may play a role in substrate binding. To test this hypothesis, we assayed the mutants A85F and A85W. In addition, the double mutant [A85F+G82A] was tested because the glycine to alanine mutation could influence the flexibility of the loop. In Figure 5 serine 54 is depicted, too. This residue is of interest because of its position at one end of a hydrophobic channel. To test the involvement of this hydrophobic channel in substrate binding, we tested an S54W mutant as well. Since the introduction of large hydrophobic residues at the surface of the enzyme might improve the interaction with interfaces (the equilibrium E ⟺ E* in Figure 2), we first analyzed the kinetic behavior of the mutants in a three-dimensional assay. As shown in Figure 6, the A85W mutant has an affinity for micelles similar to that of the wild-type cutinase, but it is clear that the mutant has considerably higher activity. Similar results were obtained with mutants A85F and [A85F+G82A] (data not shown).

The results of the two-dimensional micellar measurements of the cutinase mutants are shown in Figure 7. It is evident (Figure 7a) that both wild-type cutinase and all loop mutants show narrow and high activity optima for the activity dependence on the chain length on the ester position. For the cutinase mutants A85W, A85F, and [A85F+G82A] the data suggest that optimal activity has been shifted toward slightly longer acyl ester chains. This conclusion is supported by the data presented in Figure 7d where the activities relative to native cutinase are plotted. From these curves it is clear that the mutations G82A and S54W do not influence enzymatic properties. In Figure 7, panels b and c, the influence of the chain length on the alkyl and amide positions is depicted. At first sight these curves suggest differences between native cutinase and some mutants, but inspection

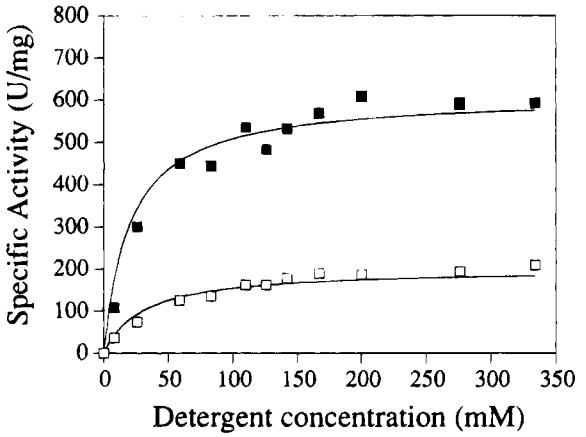

FIGURE 6: Activity dependence on the micelle concentration. In these experiments the amount of micelles was varied while the interfacial substrate concentration was kept constant. Saturation curves are given for cutinase wild type (□) and cutinase A85W (■) with 1 mol % (*R*)-3/3/3 as substrate. The detergent concentration depicted on the horizontal axis represents the total concentration of Triton X-100 and DOC (9/1, mol/mol). Specific activities on the vertical axis are given in units per milligram of enzyme, where 1 unit = 1 μmol min^{-1}.

of panels e and f, where the relative activities are plotted, shows that these chain length variations influence native and mutant cutinase in the same way. Again the mutations G82A and S54W do not influence the enzymatic activity.

DISCUSSION

It is known that the physical state of lipid substrates has a profound influence on the activity and substrate selectivity of lipases. To obtain comparable interfacial kinetic parameters (k_{cat}/K_m*), it is of great importance to present different substrates to the enzyme in a similar way. Furthermore, it is crucial that all enzyme molecules should be present at the interface to participate in catalysis. All triglyceride analogues are soluble in 150 mM Triton X-100 and 17 mM DOC to at least 1 mol %. In this micellar system essentially all cutinase is bound to the interface, and because of the low interfacial substrate concentrations used in the assays (0 ≤ 1 mol %), the quality of the interface is very likely to be preserved in

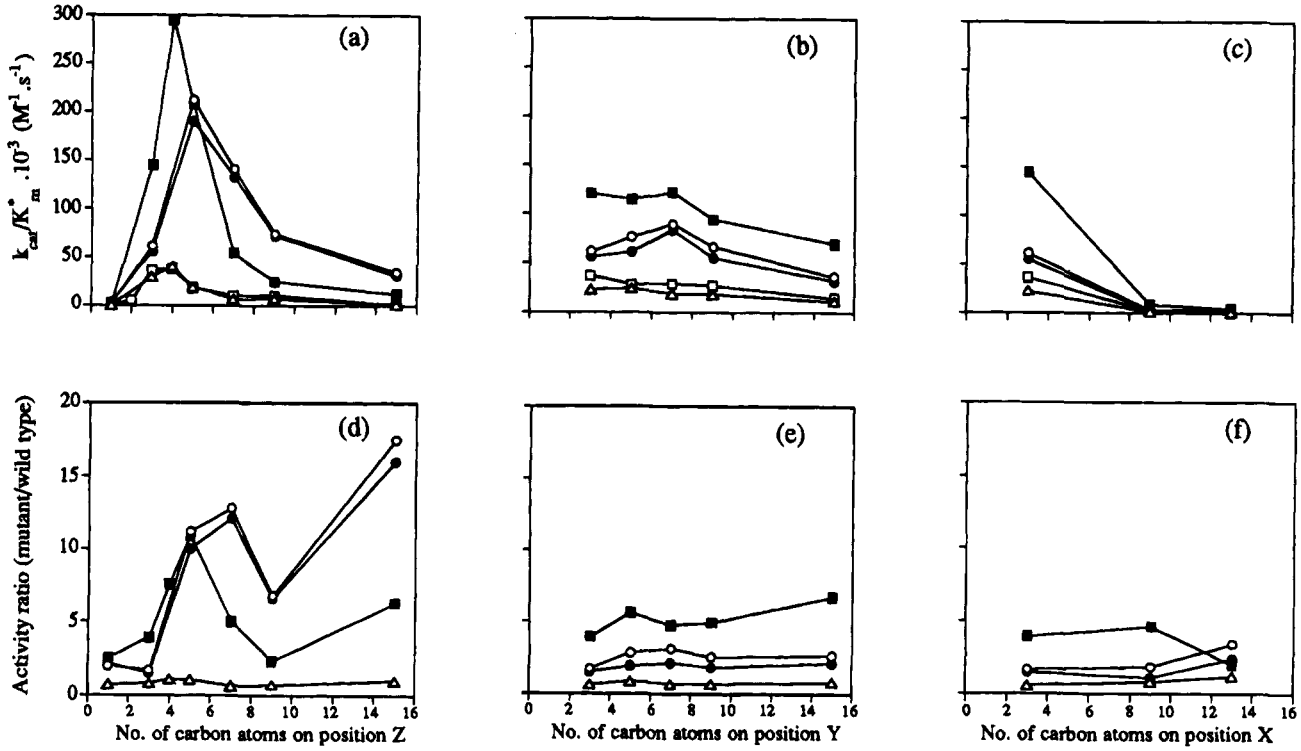

FIGURE 7: Activity ratio of cutinase wild type and mutants and activity dependence on the number of carbon atoms on the alkyl (*X*), amide (*Y*), and ester (Z) positions of the triglyceride analogues. In the three substrate series the number of carbon atoms on the nonvariable chain was kept constant at three carbon atoms. From two-dimensional micellar experiments, where the detergent concentration was kept constant at 150 mM Triton X-100 and 17 mM DOC and the interfacial substrate concentration was varied between 0 and 1 mol %, specificity constants (k_{cat}/K_m*) were obtained. In panels a, b, and c the activity dependence on the number of carbon atoms on the ester (a), amide (b), and alkyl positions (c) are given. In panels d, e, and f, the dependence of the activity ratios of the mutants and the native enzyme is given. For all panels the following symbols are used: (□) wild type, (■) A85W, (○) [A85F+G82A], (●) A85F, and (△) S54W.

all experiments. Moreover, the enzyme adsorption−desorption equilibrium (see the equilibrium E ⇌ E* in Figure 2) appears to be independent both of the kind and interfacial concentration of the triglyceride analogue used and of the character of the mutations in the enzyme studied in this paper.

From the two-dimensional micellar experiments (see Materials and Methods) it appears that the enantioselectivity of cutinase depends on the chain-length distribution of the synthetic substrate (Table 2). It is known that the stereoselectivity of *Candida rugosa* lipase in esterification reactions is governed by the alkyl chain length on the chiral carbon atom (Parida *et al.*, 1993). These authors suggest that at least two hydrophobic binding pockets must be present, one of them accommodating a short alkyl chain, while a longer alkyl chain can be accommodated in the other pocket. Our results obtained with cutinase also stress the significance of the size of the substituents on the chiral carbon atom. Particularly, the number of carbon atoms on the alkyl and amide positions of our synthetic substrates seems to affect the enantioselectivity quite dramatically (Table 2). Due to these changes in enantioselectivity when the chain-length distribution is altered, it is very likely that also cutinase contains more than one hydrophobic binding pocket to accommodate acyl moieties of lipid substrates. It should be taken into account that the enantioselectivity of enzymes in general can very well be solvent dependent (Wescott *et al.*, 1994). The hydrophobic micellar core can be considered as a hydrophobic solvent, and therefore micelles might be able to influence the enantioselectivity of the enzyme. Nevertheless, cutinase shows similar stereo selectivities in three different enzyme assays. Both the reversed micellar assay and the monolayer experiments resulted in a considerable stereopreference of cutinase for the (*R*)-enantiomer of the 3/9/9 enantiomeric pair and almost no stereopreference with the enantiomeric pair 9/3/9. These results are in good agreement with the data obtained with the pH-stat measurements (Table 3). We therefore conclude that the physical parameters of the micellar system do not dominate the enantioselectivity of cutinase. Earlier studies of lipase enantioselectivity (Rogalska *et al.*, 1993) have indicated that cutinase action on triacylglycerols is highly stereoselective; i.e., the *sn*-3 acyl chain is preferentially hydrolyzed. This observed enantioselectivity on natural substrates is completely in line with our findings on pseudoglycerides where the (*R*)-enantiomers are the preferred substrates in general (Figure 8).

Our results with cutinase wild type and three substrate series where in each case one of the alkyl chains is varied in length while keeping the other two constant at three carbon atoms show that the activity is only moderately influenced by an increasing chain length on the amide position (Figure 4). In contrast, the activity drops rapidly with an increasing number of carbon atoms on the ester and alkyl positions. It is therefore likely that cutinase contains two small hydrophobic binding pockets or cavities that can accommodate a limited number of carbon atoms. More insight into substrate binding was obtained with cutinase mutants. Three mutants contained mutations in loop (80−90), a putative substrate binding site. The locations of the mutations are depicted in Figure 5. The best way to interpret the results is to look at Figure 7, panels d, e, and f, where the activity ratios of cutinase mutants and native cutinase are given. From this representation of the results it is clear that the activity ratios for all the mutants containing a mutation on position 85 are

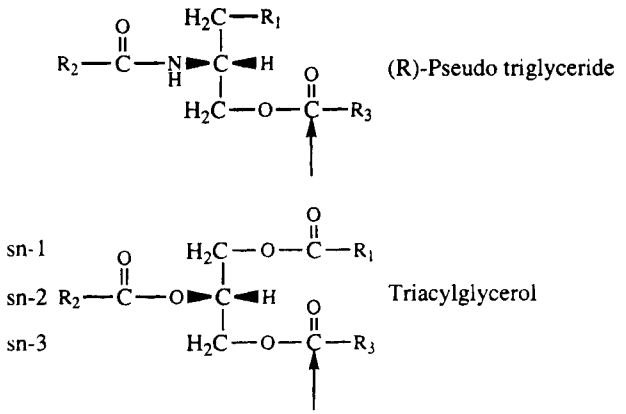

FIGURE 8: Comparison of the hydrolysis by cutinase of a natural triacylglycerol at the *sn*-3 position (Rogalska *et al.*, 1993) with the hydrolysis of an (*R*)-pseudo-triglyceride. The nucleophilic attack of the active site serine is directed at the carbonyl groups indicated by arrows.

independent of the number of carbon atoms on both the amide and the alkyl position of the synthetic substrates (Figure 7e,f). Because the mutation on position 85 only influences the dependence of the activity ratios on the number of carbon atoms on the ester position (Figure 7a,d), we suggest an interaction between loop (80−90) of cutinase and the acyl ester chain of the triglyceride analogue (position *Z* in Figure 1). Cutinases A85F and [A85F+G82A] show similar substrate selectivities (Figure 7), indicating that the G82A mutation has no influence on the enzymatic properties. Since we expect a decrease in the flexibility of loop 80−90 by mutation G82A, it is likely that the flexibility of this loop is not crucial for enzymatic activity, or that flexibility is not drastically affected by this mutation. Cutinase S54W shows a substrate selectivity similar to that of the native enzyme. We were therefore not able to prove the involvement of the hydrophobic groove in substrate interaction.

Summarizing our observations, it is clear that upon interaction of cutinase with a triglyceride analogue, depicted in Figure 1, the acyl ester chain points toward the hydrophobic loop (80−90), whereas the alkyl chain may be fixed in another small hydrophobic pocket or cavity. The acyl amide chain may either interact with a less well defined binding pocket or point away from the surface of the enzyme. This could explain the changes in enantioselectivity when the chain-length distribution of the substrate is altered. The information is not sufficient to give an accurate description of the substrate binding pocket. More cutinase mutants with mutations in putative substrate binding sites may lead to a more detailed knowledge of the binding of a triglyceride-like molecule to cutinase. Probably the most important outcome of our studies concerns the triglyceride analogues. With these compounds it appears to be possible to determine the stereopreference and the influence of the acyl chain length on the activity of lipases in general. Of course, there are structural differences between our triglyceride analogues and true triglycerides by the removal of one ester bond and the replacement of an ester bond by an amide bond. Nevertheless, the stereopreference of cutinase determined with our synthetic triglyceride analogues matches the preference determined by Rogalska *et al.* (1993) using true triglycerides. From our studies it appears that, with site-directed mutagenesis in putative lipid binding sites and testing of the mutant enzymes with our synthetic triglyceride analogues, it is possible to determine a specific interaction of one of the acyl moieties with the part in the protein of interest.

ACKNOWLEDGMENT

We would like to thank Chrislaine Martinez for reading and evaluating the manuscript and Ruud Dijkman for giving valuable suggestions concerning the synthesis of the triglyceride analogues.

REFERENCES

Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J. P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L., & Menge, U. (1990) *Nature 343*, 767−770.

Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S. A., & Thim, L. (1991) *Nature 351*, 491−494.

Dennis, E. A. (1973) *Arch. Biochem. Biophys. 158*, 485−493.

Derewenda, Z. S., Derewenda, U., & Dodson, G. G. (1992) *J. Mol. Biol. 227*, 818−839.

Grochulski, P., Bouthillier, F., Kazlauskas, R. J., Serreqi, A. N., Schrag, J. D., Ziomek, E., & Cygler, M. (1994) *Biochemistry 33*, 3494−3500.

Guttmann, S., & Boissonnas, R. A. (1958) *Helv. Chim. Acta 41*, 1852−1855.

Joerger, R. D., & Haas, M. J. (1994) *Lipids 29*, 377−384.

Kolattukudy, P. E. (1984) in *Lipases* (Borgstrom, B., & Brockman, H., Eds.) pp 470−504, Elsevier, Amsterdam.

Kraulis, P. (1991) *J. Appl. Crystallogr. 24*, 946−950.

Lauwereys, M., Geus, P. de, De Meutter, J., Stanssens, P., & Matthyssens, G. (1990) in *Lipases: structure, mechanism and genetic engineering* (Schmid, R. D., Alberghina, L., & Verger, R., Eds.) GBF Monographs, Vol. 16, pp 243−251, VCH, Weinheim, Germany.

Lowry, R., & Tinsley, I. J. (1976) *J. Am. Oil Chem. Soc. 53*, 470−472.

Mach, H., Middaugh, C. R., & Lewis, R. V. (1992) *Anal. Biochem. 200*, 74−80.

Martinez, C., Geus, P. de, Lauwereys, M., Matthyssens, G., & Cambillau, C. (1992) *Nature 356*, 615−618.

Martinez, C., Nicolas, A., Tilbeurgh, H. van, Egloff, M.-P., Cudrey, C., Verger, R., & Cambillau, C. (1994) *Biochemistry 33*, 83−89.

Marvel, C. S. (1955) *Organic Syntheses, Collect. Vol. No. III*, 495.

Mori, K., & Funaki, Y. (1985) *Tetrahedron 41*, 2369−2377.

Nelder, J. A., & Mead, R. (1965) *Comput. J. 7*, 308−313.

Parida, S., & Dordik, J. S. (1993) *J. Org. Chem. 58*, 3228−3244.

Ransac, S., Rogalska, E., Gargouri, Y., Deveer, A. M. T. J., Paltauf, F., Haas, G. H. de, & Verger, R. (1990) *J. Biol. Chem. 265*, 20271−20276.

Rogalska, E., Cudrey, C., Ferrato, F., & Verger, R. (1993) *Chirality 5*, 24−30.

Sarda, L., & Desnuelle, P. (1958) *Biochim. Biophys. Acta 30*, 513−521.

Schrag, J. D., Li, Y., Wu, S., & Cygler, M. (1991) *Nature 351*, 761−764.

Sebastião, M. J., Cabral, J. M. S., & Aires-Barros, M. R. (1993) *Biotechnol. Bioeng. 42*, 326−332.

Verger, R., & Haas, G. H. de (1973) *Chem. Phys. Lipids 10*, 127−136.

Verger, R., & Haas, G. H. de (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77−117.

Wescott, C. R., & Klibanov, A. M. (1994) *Biochim. Biophys. Acta 1206*, 1−9.

Winkler, F. K., D'Arcy, A., & Hunziker, W. (1990) *Nature 343*, 771−774.

BI942714Q