The role of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase

Mats Martinelle, Mats Holmquist, Ib Groth Clausen1, Shamkant Patkar1, Allan Svendsen2 and Karl Hult2

Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden and 2Novo-Nordisk A/S, DK-2880 Bagsvaerd, Denmark

1To whom correspondence should be addressed

The importance of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase for the hydrolytic activity at the water/lipid interface was investigated by site-directed mutagenesis. It was found that the effect on the hydrolytic activity upon the replacement of Trp89 with Phe, Leu, Gly or Glu was substrate dependent. The Trp89 mutants displayed an altered chain length specificity towards triglycerides, with a higher relative activity towards triacylglycerol than with the data.

Introduction

Triacylglycerol lipases (EC 3.1.1.3) are enzymes that catalyse the hydrolysis of neutral lipids in biological systems. By definition, lipases are enzymes which, in contrast to esterases, become activated when adsorbed to a water/lipid interface and display low activity with their substrate in a monomeric state (Verger and de Haas, 1976). To date the three-dimensional structures of several lipases have been reported (Brady et al., 1990; Winkler et al., 1990; Schrag et al., 1991; Grochulski et al., 1993; Noble et al., 1993; Derewenda et al., 1994). They all have a common αβ-hydrolase fold (Ollis et al., 1992), a catalytic triad (Ser–His–Asp/Glu) similar to that found in serine proteases (Kraut, 1977) and a lid covering the active site making it inaccessible to the substrate. However, lipolytic enzymes (lipases) with solvent-accessible active sites have also been found (Martinez et al., 1992; Hjorth et al., 1993; Uppenberg et al., 1994). It was suggested early that interfacial activation of lipases is due to a conformational change in the protein leading to increased activity (Desnuelle et al., 1960). X-ray crystallography of lipases in complex with inhibitors or co-crystallized with micelles revealed that the structural basis for interfacial activation of the lipases from Rhizomucor miehei (RML) (Brzozowski et al., 1991), *Humicola lanuginosa* (HLL) (Lawson et al., 1994), human pancreas (HPL) (van Tilburgh et al., 1993) and Candida rugosa (CRL) (Grochulski et al., 1994a). Interfacial activation of these lipases involves a conformational change of the enzyme where the lid covering the active site is displaced, creating an open accessible active site and a hydrophobic lipid-binding surface. As the catalytically active open lipase is adsorbed to the water/lipid interface, the amphiphilic lid interacts with both the substrate and the surface of the main body of the enzyme. Electrostatic interactions mediate the contact between the lid and the cortex of the protein in activated lipases (Derewenda et al., 1992; van Tilburgh et al., 1993; Grochulski et al., 1994b). Disruption of the electrostatic interactions displayed by a single residue in the lid decreases the catalytic activity of RML (Arg86) and HLL (Glu87) (Holmquist et al., 1993).

The ‘classical’ pancreatic lipase family and three out of four members of homologous lipases from filamentous fungi, *R. miehei* (RML), *H. lanuginosa* (HLL) and *Penicillium camembertii* (PCL), have a conserved tryptophan in the central part of the lid (Derewenda et al., 1994; Thirstrup et al., 1994). The *Rhizopus delemar* lipase (RD) has an alanine in the corresponding position (Derewenda et al., 1994). In the crystal structures of the activated conformation of HLL and RML the side chain of this tryptophan is in close contact with the acyl moiety of a transition state analogue bound to the active sites (Derewenda et al., 1992; Lawson et al., 1994). It has been discussed whether the conformation of Trp89 in the crystal structures of RML and HLL in complex with the small diethyl p-nitrophenyl phosphate inhibitor is influenced by intermolecular contacts (Derewenda et al., 1992; Lawson et al., 1994). However, Lawson et al. (1994) claim that in the open structure of HLL complexed with the large n-dodecyl chlorophosphonate ethyl ester the lid region could move freely without intermolecular contacts. Site-directed mutagenesis of Trp89 in the lid of *H. lanuginosa* lipase showed that this residue is important for efficient hydrolysis of triglycerin (Holmquist et al., 1994).

In this work, we investigated whether mutations of residues Glu87 (one of the residues stabilizing the open active conformation) and Trp89 (interacting with the substrate) in the lid of *H. lanuginosa* lipase alter the substrate specificity in the hydrolysis of emulsified substrates. Site-directed mutagenesis of Glu87 and Trp89 residues was carried out and the specific hydrolytic activities of wild-type and mutated enzymes were determined towards a variety of different ester substrates. The acyl chain length specificity of the lipase variants towards triglycerides was determined. The importance of Glu87 and
Table I. Hydrolytic activity ($v_0$) ($\mu$mol/min/mg) of wild-type and mutant *H. lanuginosa* lipase with triglycerides as a substrate at pH 7.5 and 25°C

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Acyl moiety</th>
<th>Leaving group</th>
<th>Diacylglycerol</th>
<th>Vinyl</th>
<th>Ethyl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>8800</td>
<td>23 000</td>
<td>990</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu87Ala</td>
<td>3300</td>
<td>8000</td>
<td>390</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Phe</td>
<td>2900</td>
<td>17 000</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Leu</td>
<td>2100</td>
<td>19 000</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Gly</td>
<td>1700</td>
<td>14 000</td>
<td>170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Glu</td>
<td>340</td>
<td>6200</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Butyrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>5400</td>
<td>10 000</td>
<td>210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu87Ala</td>
<td>1800</td>
<td>4000</td>
<td>78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Phe</td>
<td>750</td>
<td>5000</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Leu</td>
<td>410</td>
<td>5500</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Gly</td>
<td>290</td>
<td>4200</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp89Glu</td>
<td>63</td>
<td>1800</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The relative error in the $v_0$ values was estimated to be $\pm 10\%$, based on conducting each experiment in duplicate or triplicate.

Trp89 during the formation and cleavage of the acyl enzyme intermediate was examined using vinyl and ethyl esters as substrates. The activities and enantioselectivities of the lipase variants towards chiral substrates with the asymmetric centre in the acyl moiety were determined.

**Materials and methods**

**Chemicals**

Triglycerides, 3-(N-morpholino)propanesulphonic acid (MOPS) and gum arabic were purchased from Sigma (St Louis, MO). Ethyl and vinyl esters were bought from Aldrich-Chemie (Steinheim, Germany) and Tokyo Kasei (Tokyo, Japan), respectively. Calcium chloride was obtained from Merck (Darmstadt, Germany). All commercial chemicals used were of analytical grade.

(rac)-1-Hexyl 2-methyldecanoate and (rac)-1-heptyl 2-ethyldecanoate. These compounds were prepared as described previously (Berglund et al., 1993).

**Cloning and expression of the *H. lanuginosa* lipase**

The gene from *H. lanuginosa* encoding the triglyceride lipase was cloned, sequenced and expressed as described by Boel and Hauge-Jensen (1988), and the lipase produced was isolated from the culture medium.

**Site-directed mutagenesis**

The general method used for the mutation of the lipase gene has been described by Nelson and Long (1989). A detailed description of the procedure used has been described earlier (Holmquist et al., 1994). The correct mutation was verified by DNA sequencing.

**Lipase purification**

The recombinantly produced wild-type and mutated lipases were purified by a procedure containing three chromatographic steps with a diethylaminoethyl (DEAE) column (Pharmacia, Uppsala, Sweden), a Toyopearl Butyl-650C column (TosoHaas, Montgomeryville, PA) and a Q-Sepharose high-performance column (Pharmacia). A detailed description of the purification procedure has been published earlier (Holmquist et al., 1994).

No contaminants could be detected in the purified lipases after SDS–PAGE analysis and Coomassie Blue staining.

**Protein quantitation**

Protein concentrations were determined spectrophotometrically at 280 nm (wild-type and Glu87Ala *H. lanuginosa* lipase, $\varepsilon = 4.3 \times 10^4$ M$^{-1}$ cm$^{-1}$; Trp89X, $\varepsilon = 3.6 \times 10^4$ M$^{-1}$ cm$^{-1}$).

**Enzyme activity assay**

Ester substrate (1.0 mmol) was added to an aqueous solution (5 ml) of gum arabic (5% w/v) and calcium chloride (0.2 M), pH 7.5. The mixture was emulsified by sonication for 1 min. The enzyme was dissolved in 10 mM MOPS buffer, pH 7.5. The reaction was started by the addition of enzyme solution (5–10 µl) to the stirred, thermostatted (25°C) substrate solution (1.5 ml). The pH was maintained automatically with sodium hydroxide (10–100 mM), using a Radiometer pH-stat equipped with an ABU91 auto-burette (1 ml) connected to a VIT90 videotitrator. The reactions were run for 5 min under nitrogen.

**Batch hydrolysis of chiral esters**

The reactions were performed in a pH-stat as described for the enzyme activity assay but the reaction volume was 5 ml. The reaction was started by the addition of enzyme solution (50 µl) to the stirred, thermostatted (25°C) cuvette. The reactions were stopped by the addition of hydrochloric acid (1.0 M) until pH 1 was reached. The acidified reaction mixture (1 vol.) was extracted with diethyl ether (6×2 vol.). The pooled ether phase was dried (MgSO$_4$), filtered and evaporated to dryness.

Table III. Enantiomeric ratio ($V_m/K_m$)$_0$/$V_m/K_m$ and hydrolytic activity ($v_0$) of wild-type and Trp89Phe mutated *H. lanuginosa* lipase in the hydrolysis of 2-alky/decanoic acid esters at pH 7.5 and 25°C

<table>
<thead>
<tr>
<th>Substrate enzyme</th>
<th>($V_m/K_m$)$_0$/$V_m/K_m$</th>
<th>($v_0$) (µmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heptyl 2-methyldecanoate</td>
<td>Wild-type</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>Glu87Ala</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Hexyl 2-methyldecanoate</td>
<td>Wild-type</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Trp89Phe</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Heptyl 2-ethyldecanoate</td>
<td>Wild-type</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Trp89Phe</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>

The relative error in the $v_0$ values was estimated to be $\pm 10\%$, based on conducting each experiment in duplicate or triplicate. The standard deviation in $\varepsilon$ was calculated for a maximum experimental error in conversion of $\pm 2\%$ and in enantiomeric excess of the product of $\pm 1\%$ (van Tol et al., 1991).

Holmquist et al. (1993).
Role of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase

The catalytic triad (Ser146, His258 and Asp201), Glu87 and Trp89 (black) and the transition-state analogue (grey) are shown with a ball-and-stick model. (B) Stereochemistry of the active site (black) of the H.L.-C12 complex. This illustrates the transition-state analogue (grey) sandwiched between the side chain of Trp89 in the lid and the active site cleft.

Purification of 2-alkyldecanoic acid

The crude 2-alkyldecanoic acid was dissolved in chloroform (3 ml) and subjected to separation on a prewashed (2×8 ml of hexane) aminopropyl ion-exchange column (Mega Bond ElutTM, 2 g/12 ml, purchased from Analytichem International, Harbor City, CA). The ester and the alcohol were eluted with chloroform-propan-2-ol (2:1, 3×8 ml) and the acid was eluted with acetic acid-diethyl ether (2:98, 2×8 ml). Evaporation under reduced pressure yielded pure 2-alkyldecanoic acid.

Enantiomeric excess of 2-alkyldecanoic acid

The enantiomeric excess of 2-alkyldecanoic acid was determined according to the method described by Sonnet (1987). To a solution of 2-alkyldecanoic acid (10 μl, <0.05 mmol) in anhydrous diethyl ether (1 ml), dimethylformamide (10 μl), thionyl chloride (10 μl, 0.14 mmol) and enantiomerically pure (R)-1-phenylethylamine (20 μl, 0.16 mmol) were added. The mixture was diluted with diethyl ether and subsequently washed with water (1 ml), saturated sodium hydrogen carbonate solution (2×1 ml) and saturated sodium chloride solution (1 ml). The enantiomeric excess of 2-alkyldecanoic acid was determined by gas chromatography. The analyses were carried out with a Perkin-Elmer Model 5000 gas chromatograph equipped with a CP-Sil CB column (Chrompack; 25 m×0.32 mm i.d., dT = 0.32 mm). Helium was used as the carrier gas and temperature programming was employed.

Determination of enantiomeric ratio

The enantiomeric ratio \[ E = \frac{(V_A/K_m)_A}{(V_B/K_m)_B} \] where \( A \) and \( B \) denote the fast- and slow-reacting enantiomers, respectively]
was determined from the enantiomeric excess (ee) of the product and the conversion (c). The enantiomeric ratio was calculated according to Chen et al. (1982): 

\[ E = \ln[1 - c(1 + ee)]/\ln[1 - c(1 - ee)]. \]

All reactions were run to less than 15% conversion.

Results

Chain length specificity towards triglycerides

Wild-type *H. lanuginosa* lipase (HLL) showed higher hydrolytic activities with increasing chain length (C_2 - C_8) of the triglyceride substrate (Table I). With triolein (C18:1) HLL displayed the same activity as towards tripropionin. The Glu87Ala mutated lipase showed approximately one-third of the activity of the wild-type enzyme towards all triglycerides used.

The Trp89Phe mutant showed 51 and 33% of the activity of wild-type lipase towards triacetin and trioctanoin, respectively (Table I). Towards the other triglycerides the Trp89Phe mutant showed less than 20% of the activity of wild-type lipase. The decrease in activity of the lipase towards trioctanoin and tributyrin when replacing Trp89 with Phe, Leu or Gly correlated with the reduced size of the introduced residue (Table I).

The Trp89Glu mutant showed 1-4% activity compared to wild-type. All Trp89 mutations resulted in a greater loss in activity towards tributyrin than towards trioctanoin.

Probing the acylation and deacylation reactions with ethyl and vinyl esters

Wild-type HLL showed a 25-fold higher hydrolytic activity towards vinyl octanoate than ethyl octanoate (Table II). A 50-fold difference in activity was observed with the corresponding butyrate esters. The Glu87Ala mutant displayed slightly more than one-third of the activity of wild-type lipase towards vinyl and ethyl esters. The Trp89Phe mutation resulted in a fivefold decreased activity towards ethyl octanoate but only a 1.3-fold decreased rate towards vinyl octanoate. With ethyl and vinyl butyrate the Trp89Phe mutation resulted in a similar pattern as with the octanoate esters, even though the absolute effect was larger. In contrast to when triglycerides were used as substrates, the Trp89Leu mutant showed a higher activity than the Trp89Phe mutant towards vinyl and ethyl esters.

The Trp89Glu mutant showed 2-4% activity towards ethyl esters compared with wild-type. With vinyl ester substrates the mutant displayed 17-27% activity compared with wild-type.

2-Alkylalkanoic acid esters

The activity of wild-type HLL towards 2-methyl- and 2-ethyldecanoate esters was 40- and 2000-fold lower than that observed with the unbranched hexyl octanoate substrate (unpublished data). The Glu87Ala mutant showed 70% activity of wild-type lipase towards heptyl 2-methyldecanoate (Table III). In contrast, the Trp89Phe mutant showed a two- and threefold increased activity towards 2-methyl- and 2-ethyldecanoate esters, respectively, compared with wild-type.

The enantiomeric ratio, \( E \), defined as \( (V_{nu}/K_m)/(V_{nu}/K_m)_{nu} \), was 8.5 for wild-type HLL towards hexyl 2-methyldecanoate and 2.4 towards heptyl 2-ethyldecanoate (Table III). The Glu87Ala mutant showed higher specificity (\( E = 17 \)) towards heptyl 2-methyldecanoate than the wild-type (\( E = 10 \)) (Holmquist et al. (1993)). In contrast, the Trp89Phe mutant showed a lower enantiospecificity towards 2-alkyldecanoic acid esters (Table III).

Discussion

Chain length specificity towards triglycerides

Wild-type. The *H. lanuginosa* lipase displayed the highest activity towards trioctanoin of the triglycerides used (Table I). The three-dimensional structure of a *H. lanuginosa* lipase–inhibitor complex shows that the acyl chain of the tetrahedral intermediate is sandwiched between the side chain of Trp89 in the lid and the hydrophobic active site cleft (Figure 1) (Lawn et al., 1994). With a dodecyl phosphonate inhibitor approximately eight carbons of the alkyl chain are bound to the active site while the rest of the inhibitor points out into the solvent. This indicates that trioctanoin may utilize the acyl binding site more efficiently than the shorter triglycerides. However, it has been shown that tridodecanoin is the best substrate for HLL and a 1.5-fold higher activity compared with trioctanoin is displayed (Liu et al., 1973).
The Glu87Ala mutation did not change the acyl chain length specificity of the lipase (Figure 2). The side chain of Glu87 could affect the substrate recognition of the lipase by defining the position of the lid. In the crystal structure of the HLL-inhibitor complex the Glu87 residue points out into the solvent (Figure 1). However, when the lipase is adsorbed at a water/lipid interface, Glu87 will encounter a hydrophobic environment and electrostatic interactions between the carboxyl group of Glu87 and the surface of the protein would be more energetically favourable. The change of Glu to Ala prevents this interaction and the position of the lid may be altered. The lid conformation is important for activity as the lid interacts with the substrate and the complete oxynion hole is created as a result of the lid opening.

Trp89Phe, Leu or Gly. In contrast to the Glu87Ala mutation, the acyl chain length specificity was altered by the Trp89Phe mutation (Figure 2). The Trp89Phe mutant displayed a twofold lower activity than wild-type towards tributyrin. For C12, C14 and C6 triglycerides a six- to seven-fold decrease was observed. The structure of the lipase-inhibitor complex (Figure 1) indicates a close interaction between the acyl chain and Trp89. However, with the short acetyl acyl chain Trp89 may not be able to sandwich the substrate into the active site crevice. The mutation of Trp89 should thus have a smaller impact on the hydrolytic activity towards triacetin than towards triglycerides with longer acyl moieties.

The activity towards trioctanoin was less dependent on Trp89 than the shorter C3, C4 and C6 triglycerides (Figure 2). The activity towards trioctanoin was fourfold less affected by the Trp89Gly mutation than that towards tributyrin (Table II). Trioctanoin binds to a larger area of the acyl binding site of HLL than tributyrin (Figure 1). Thus, the interaction between Trp89 and the acyl chain constitutes a smaller contribution to the total binding energy for trioctanoin compared with the substrates (C3, C4 and C6) with shorter acyl moieties.

With triolein as a substrate the Trp89Phe mutant showed a fivefold decrease in activity compared with wild-type. The stereochemistry of the cis double bond at position 9 in the acyl moieties and the large portion of the acyl chain presumably not bound to the acyl binding site could counteract the binding of the first eight atoms of the substrate in a correct fashion for catalysis, thus making Trp89 more important for efficient hydrolysis of triolein than of trioctanoin.

In the R.delemar (RDL) lipase the importance of the size of the acyl binding site for triglycerides has been shown by the mutation Val209Trp (corresponding to Leu206 in HLL). The introduction of the large Trp residue in the acyl binding site was found to alter the chain length specificity towards triglycerides (Joerger and Haas, 1994). That lipase variant showed an increased tributyrin activity and reduced trioctanoin activity. The Ala89Trp mutation in RDL resulted in a 20–30% reduced hydrolytic activity towards triglycerides compared with wild-type. This shows that RDL has evolved to be an efficient enzyme without a Trp residue in the lid. There is no obvious residue in the RDL lid which may play a similar role to Trp89 in the HLL.

Probing the acylation and deacylation reactions with ethyl and vinyl esters

Kinetic studies including substrates with different leaving groups are a classical way of separately investigating the acylation and deacylation reaction rates of serine hydrolases (Fersht, 1985). Activity differences seen with substrates composed of different leaving groups originate from the differences in the acylation reaction as the deacylation reaction is the same. In homogeneous catalysis such studies can easily be performed, but with emulsified substrates data are more difficult to interpret, owing to possible differences in the physical properties of the substrates. However, by using two substrates with similar size such as vinyl and ethyl esters, the emulsions are probably similar in character.

Wild-type. The 25–50-fold higher activity observed with wild-type HLL towards vinyl esters as compared with ethyl esters (Table II) shows that the acylation reaction is rate determining for ethyl ester hydrolysis. The rate-determining step in the hydrolysis of vinyl esters is suggested to be deacylation controlled (see discussion below).

Glu87Ala. The Glu87Ala mutation resulted in the same decrease in activity towards all ester substrates used, even though the rate determining step differs between the substrates (Figure 3). This suggests that the Glu87Ala mutation affects the acylation and deacylation rates to the same extent in contrast to the Trp89 mutations where the acylation step is suggested to be exclusively affected (see discussion below).

Trp89Phe, Leu, Gly or Glu. Mutation of Trp89 had a wide-ranging effect on the hydrolytic activity towards the esters in Table II. Trp89 was more important in the hydrolysis of triglycerides and ethyl esters compared with vinyl esters (Figure 3). This is surprising as Trp89 does not interact with the leaving group of the substrate (Lawson et al., 1994). The effect of the mutation is best explained by a model in which the acylation reaction rate is reduced to a large extent, while the deacylation rate remains unaffected. It was assumed that the acylation rate was reduced to the same extent for all substrates. Using equations (1) and (2) the acylation and deacylation rates for wild-type and mutant lipase could be

Fig. 4. Transition-state model of (S)-methylalkanoic acid ester is illustrated as a 2-methyl analogue of the dodecylphosphonate inhibitor in the HLL-C12 complex (Lawson et al., 1994). The methyl group was added to the inhibitor without further energy minimization of the structure. The model illustrates unfavourable interactions between the methyl substituent of the substrate and the side chain of Trp89.
calculated from the experimental data for ethyl and vinyl octanoate and trioctanoin (Table II). Three substrates give a system of six equations that can easily be solved.

\[ \frac{1}{k_{cat,wt}^i} = \frac{1}{k_{a}^i} + \frac{1}{k_{d}^i} \]  

(1)

\[ \frac{1}{k_{cat,mutant}^i} = \frac{1}{k_{a,x}^i} + \frac{1}{k_{d}^i} \]  

(2)

The deacylation rate \( k_{d} \) is the same for ethyl and vinyl octanoate and trioctanoin. The acylation rate \( k_{a}^i \) is different for each substrate \( n \) denotes substrate). The acylation rate for the mutant equals \( k_{a,x}^i \), where \( x \) is the same for all substrates. This model showed that the acylation rate with vinyl octanoate was one order of magnitude faster (~200 000 \( \mu \text{mol/min} \cdot \text{mg} \)) than the deacylation rate (23 000 \( \mu \text{mol/min} \cdot \text{mg} \)) for wild-type HLL. Trioctanoin exhibited acylation and deacylation rates of the same magnitude, while ethyl octanoate had an acylation rate (990 \( \mu \text{mol/min} \cdot \text{mg} \)) 20-fold lower than the deacylation rate. The mutations resulted in a larger decrease in acylation rate towards the butyrate esters than towards the octanoate esters. This is consistent with the chain length specificity data obtained with triglycerides. The experimental data could not be fitted to a model in which the acylation and the deacylation rates were decreased to the same extent. Nor was a model in which only the deacylation rate was reduced feasible. The exclusive effect of the mutation on the acylation step in the catalysis suggests that Trp89 plays an important role in positioning the acyl part of the substrate optimally in the active site for catalysis. The role of Trp89 seems not to be important as soon as the acyl enzyme has been formed. With the assumption that the transition states in the acylation and deacylation reaction are similar, the most important feature of Trp89 is the binding of the substrate in a correct fashion in the Michaelis–Menten complex.

The Trp89Phe mutation resulted in an unaltered \( K_m \) of the substrates in transesterification reactions in cyclohexane (Holmquist et al., 1995). Thus, Trp89 influences the binding of substrates into the active site in a qualitative and not a quantitative way.

2-Alkylalkanoic acid esters

Wild-type. The low hydrolytic activities obtained towards hexyl 2-methyldecanoate and heptyl 2-ethyldecanoate are probably due to low chemical reactivity of the esters (March, 1985) and an unfavourable interaction between the methyl (ethyl) substituent of the substrate and the side chain of Trp89 in HLL (Figure 4).

Glu87Ala. The activity of the Glu87Ala variant towards heptyl 2-methyldecanoate and heptyl 2-ethyldecanoate are probably due to the alcohol part of the substrate as the activity towards hexyl octanoate displayed by the mutant was 60% of wild-type.

Trp89Phe. The Trp89Phe mutation resulted in an increased activity towards hexyl 2-methyl- and heptyl 2-ethyldecanoate (Table III). The replacement of Trp89 by a smaller residue increases the space in that area and probably reduces the unfavourable interaction between the substituent of the substrate and the side chain of Trp89 (Figure 4). Thus, even though Trp89 is important for binding the acyl chain of a natural substrate, the presence of Trp89 has a negative impact on the catalytic efficiency when substrates with more bulky acyl chains such as 2-alkylalkanoic acid esters are used.

Acknowledgements

The 2-alkylalkanoic acid esters were a kind gift from Dr Per Berglund. We thank the Nordic Industrial Fund (NIF), the Swedish Research Council for Engineering Sciences (TRF) and the National Swedish Board for Technical Development (NUTEK) for financial support.

References


Received September 12, 1995; revised February 19, 1996; accepted March 13, 1996