Stereoselectivity of *Mucorales* lipases toward triradylglycerols—A simple solution to a complex problem

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Abstract

The lipases from *Rhizopus* and *Rhizomucor* are members of the family of *Mucorales* lipases. Although they display high sequence homology, their stereoselectivity toward triradylglycerols (*sn*-2 substituted triacylglycerols) varies. Four different triradylglycerols were investigated, which were classified into two groups: *flexible* substrates with rotatable O'-C1' ether or ester bonds adjacent to C2 of glycerol and *rigid* substrates with a rigid N'-C1' amide bond or a phenyl ring in *sn*-2. Although *Rhizopus* lipase shows opposite stereopreference for flexible and rigid substrates (hydrolysis in *sn*-1 and *sn*-3, respectively), *Rhizomucor* lipase hydrolyzes both groups of triradylglycerols preferably in *sn*-1. To explain these experimental observations, computer-aided molecular modeling was applied to study the molecular basis of stereoselectivity. A generalized model for both lipases of the *Mucorales* family highlights the residues mediating stereoselectivity: (1) L258, the C-terminal neighbor of the catalytic histidine, and (2) G266, which is located in a loop contacting the glycerol backbone of a bound substrate. Interactions with triradylglycerol substrates are dominated by van der Waals contacts. Stereoselectivity can be predicted by analyzing the value of a single substrate torsion angle that discriminates between *sn*-1 and *sn*-3 stereopreference for all substrates and lipases investigated here. This simple model can be easily applied in enzyme and substrate engineering to predict *Mucorales* lipase variants and synthetic substrates with desired stereoselectivity.

Keywords: G-elbow loop; His gap; model; *Mucorales* lipase; protein engineering; stereoselectivity; triradylglycerol substrate

One important role of lipases (triacylglycerol hydrolases E.C. 3.1.1.3.) in nature is the hydrolysis of triacylglycerols (Schmid & Verger, 1998). Most lipases hydrolyze fatty acids in position *sn*-1(3) (Rogalska et al., 1993). *sn*-1(3) Stereoselectivity of substrate hydrolysis varies widely for the same triacylglycerol substrate (Rogalska et al., 1993), even among closely related lipases, like *Rhizopus* and *Rhizomucor* lipase. Earlier studies indicated that the chemical structure of the *sn*-2 substituent has a strong impact on the stereoselectivity of hydrolysis reactions in lipases (Stadler et al., 1995; Kovac et al., 1996). For *Rhizopus* lipase, a model was introduced (Haalck et al., 1997; Holzwarth et al., 1997) and verified by site-directed mutagenesis (Scheib et al., 1998) to explain its experimentally determined stereoselectivity. According to this model, the glycerol substituents were supposed to bind to two

hydrophobic patches. The *hydrophobic crevice* binds the hydrolyzed *sn*-1 or *sn*-3 fatty acid chain (substrate in *sn*-1 or *sn*-3 orientation, respectively), while the *hydrophobic dent* binds the *sn*-2 substituent. The nonhydrolyzed *sn*-3(1) fatty acid chain does not bind to a defined region in *Rhizopus* lipase.

Sterical interactions between the C-terminal neighbor of the catalytic histidine, residue 258, and the *sn*-2 substituent adjacent to C2 of glycerol were identified as being mainly responsible for stereoselectivity in *Rhizopus* lipase. Modifying the size of residue 258 affected the stereoselectivity of ROL toward triradylglycerols of different chemical structure.

Rhizopus and *Rhizomucor* lipase are *Mucorales* lipases, and display both high similarities in sequence and structure but also major differences concerning their stereoselectivity. In this work, we generalize the previously described model for *Rhizopus* lipase to *Mucorales* lipases using molecular modeling techniques and hydrolysis experiments to thoroughly understand the structure–function relationships in lipases.

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Results

Stereoselectivity of *Mucorales* lipases—

Experimental evidence

Rhizopus and *Rhizomucor* lipases display various differences in the stereoselectivity of triacylglycerol hydrolysis. Both lipases hydrolyze flexible substrates preferably in *sn*-1, while for rigid substrates *Rhizopus* lipase shows *sn*-3, *Rhizomucor* lipase *sn*-1 stereopreference (Table 1; Fig. 1). In *Rhizopus* lipase, flexibility correlates with stereoselectivity (Table 1). Enantioselectivity of the flexible ether and ester substrates decreases from $E = 4$ (highly flexible ether substrate) to $E = 1$ (less flexible ester substrate), while the ee-values decrease from 61 to 19% ee *sn*-1. A switch in stereopreference occurs for the rigid amide and phenyl substrate. *sn*-3 Stereoselectivity is even increased, as rigidity increases: the amide analog displays $E = 5$ (63% ee *sn*-3), while the phenyl substrate has $E = 8$ (77% ee *sn*-3).

In *Rhizomucor* lipase, for both types of triacylglycerols (flexible and rigid, respectively) ee-values and enantiomeric ratio hardly vary. For the ether and ester substrate, $E = 6$ and 7 (69 and 73% ee *sn*-1), respectively, while for the amide and phenyl substrate, $E = 4$ and 6 (56 and 68% ee *sn*-1), respectively.

Molecular modeling studies were performed to investigate the effects responsible for the observed differences in stereoselectivity.

The structure of the binding site

Rhizopus niveus and *oryzae* lipases are identical in sequence (Kazlauskas & Bornscheuer, 1998). The *Mucorales* lipases have 56% identical and an additional 14% similar residues (Fig. 2). *R. niveus*

lipase was crystallized only in the closed conformation, while for *R. oryzae* lipase no crystal structure is available. The structural model of *R. oryzae* lipase that was used for this study is similar to the lipase from *R. niveus*, except the position of the lid. From crystal structures of *Rhizomucor* lipase in both closed and open form (PDB entry 3tgi and 4tgi, respectively), we concluded for *Mucorales* lipases that structural variations between both closed and open conformation do not influence the position of the *G*-elbow.

The substrate fatty acid chains were supposed to bind to two putative hydrophobic patches. The scissile fatty acid is supposed to bind to the hydrophobic crevice, the *sn*-2 substituent to the hydrophobic dent. The deep hydrophobic crevice consists of T83, A89, I93, F95, F112, L146, P178, V206, V209, P210, and F216 in *Rhizopus* lipase, of S82, W88, L92, F94, F111, L145, P177, V205, L208, P209, and F215 in *Rhizomucor* lipase. The shallow hydrophobic dent is formed by I205, T252, L254, and L258 in *Rhizopus* lipase and I204, T252, V254, and L258 in *Rhizomucor* lipase.

Besides the residues of the catalytic triad (S145, D204, and H257 in *Rhizopus* lipase, S144, D203, and H257 in *Rhizomucor* lipase), hydrophobic crevice, and hydrophobic dent directly contact the substrate. Although the residues of the hydrophobic dent differ only in one residue, L254 in *Rhizopus* and V254 in *Rhizomucor* lipase, respectively, the hydrophobic crevice varies in four. Additionally, the bulky W88 in the lid of *Rhizomucor* lipase is substituted by A89 in *Rhizopus* lipase and is located near the *sn*-2 substituent of triacylglycerol substrate.

For the bound substrate, the *sn*-2 substituent adjacent to glycerol C2 is located in the *His gap* between the active site H257 and L258 (Scheib et al., 1998). Due to the opposite configuration of the (pro-)chiral C2 of glycerol, in *sn*-1 orientation, the functional group of the *sn*-2 substituent points into the *His gap* and interacts steri-

Fig. 1. A: Stereoselective hydrolysis of triacylglycerols to *sn*-1,2- or *sn*-2,3-diracylglycerols. **B:** *sn*-2 Substituents attached to the (pro-)chiral C2 of glycerol with trivial (top) and systematic (bottom) substrate names. Ether and ester analogs of triacylglycerols are classified as flexible, amide and phenyl analogs as rigid substrates.

ROL	1	S-DGGKVVAATTAQIQEFTKYAGIAATAY	CRSVVPGNKWDCVQCQKWVPDGKIIT
RML	1	SIDGG-IRAATSQEINELTYTTLSANSY	CRTVIPGATWDCIHCD-ATEDLKIHK
consensus	1	* * * * *	* * * * *
ROL	55	TFTSLSDTNGYVLRSDKQKTIYLVFRG	NSFRSITDVFNFSDYKPVKGAKVH
RML	54	TWSTLIYDTNAMVARGDSEKTIYVFRG	SSIRNITADTVVPVSYPPVSGTKVH
consensus	55	* * * * *	* * * * *
ROL	110	AGLSSYEQVVNDYFFVQEQLTAPTYKVI	VTGHS
RML	109	KGGLDSYGEVQNELVATVLDQFKQYPSY	KVAVTGHS
consensus	110	* * * * *	* * * * *
ROL	165	LSPKNLSIFTVGG	RVGNPTFAYYVESTGIPFQRTVHKRD
RML	164	LSSSNLFLYTQGG	RVGDPAFANYVSTGIPYRRTVNERD
consensus	165	* * * * *	* * * * *
ROL	220	GVESWIKSG-TSNVQICTSEIETKDCSNS	IVPFF
RML	219	GEEYWITDNSPETVQVCTSDLETSDCS	NSIVPFF
consensus	220	* * * * *	* * * * *

Fig. 2. Sequence alignment of the mature *Mucorales* lipases (*R. oryzae* (ROL) and *R. miehei* (RML)). ROL and RML have 56% identical ("*"), and 70% identical or similar residues ("*" and "*"). The catalytic triad is highlighted in black (e.g., **S145**). Residues of the hydrophobic crevice are marked in dark gray (e.g., **R83**), of the hydrophobic dent in light gray (e.g., **L258**). Residues 265–267 of the G-elbow loop are medium gray (e.g., **G266**), the cysteine residues forming the C- to N-terminal disulfide bond are marked (e.g., **C268**). The lid reaches from N84 and S83 in ROL and RML to F97 and P96, respectively.

cally with L258, which was identified to exhibit the main sterical interaction with the *sn*-2 substituent. In *sn*-3 orientation, the functional group of the *sn*-2 substituent points in the opposite direction reducing the sterical repulsion between the substrate and residue 258. L258, which was identified to exhibit the main stereoselectivity-determining interaction with the substrate in ROL, is conserved in *Mucorales* lipases. Therefore, it does not account for the different stereoselectivities toward rigid substrates. G266 is located in a

three-residue loop that is exposed to the binding site of *Rhizopus* and *Rhizomucor* lipase, close to the substrate at C2 of glycerol (Fig. 3). It is flanked by nonhomologous residues leading to a different loop structure in the otherwise homologous binding pockets of *Mucorales* lipases (Fig. 2). The position of G266 varies in *Rhizopus* and *Rhizomucor* lipase (C_{α} – C_{α} distance = 1.3 Å). In *Rhizopus* lipase, residues 265 and 267 are glutamate and serine, respectively, in *Rhizomucor* lipase threonine and leucine. While

Fig. 3. Ester substrate in *sn*-1 orientation in *Rhizopus* lipase (ROL, light gray) and *Rhizomucor* lipase (RML, dark gray). Only the amino acid side chains are displayed. The position of the G266 varies in both lipases (C_{α} – C_{α} distance = 1.3 Å) due to the different position of E265 in ROL and T265 in RML, respectively (horizontal arrow). Therefore, in ROL, the substrate binds deeper into the binding site than in RML ($C2_{RML}$ – $C2_{ROL}$ = 0.9 Å). Hence, sterical interactions between the *sn*-2 substituent and L258 are more pronounced in ROL rather than RML (vertical arrow).

T265 in *Rhizomucor* lipase forms an H-bond from its side-chain hydroxyl group to N₈ of the H143 imidazol ring, no such bond is formed by E265 in *Rhizopus* lipase. H144 in *Rhizopus* lipase (H143 in *Rhizomucor* lipase) is the N-terminal neighbor of the catalytic serine, which is part of the highly conserved GX SXG pentapeptide motif.

The C-terminus in *Rhizopus* and *Rhizomucor* lipase is further fixed by a disulfide bond from C268 to the N-terminal C29.

Φ_{O3-C3} —A probe for stereopreference

The investigated triradylglycerols were classified as flexible and rigid substrates, respectively, depending on the chemical structure of the *sn*-2 substituent (Scheib et al., 1998; Fig. 1). Flexible substrates contain a rotatable O'-C1' ether (ether substrate) or ester bond (ester substrate) adjacent to C2 of glycerol. In rigid substrates, the *sn*-2 substituent binds with a rigid N'-C1' amide bond (amide substrate) or an aromatic ring (phenyl substrate) to the glycerol. The substrate can bind to the lipase in two orientations (*sn*-1 and *sn*-3 orientation, respectively) resulting in an opposite configuration at the (pro-)chiral C2. The different geometries of the bound substrates can be monitored by one characteristic torsion angle Φ_{O3-C3} . Experimentally determined stereoselectivity and the value of torsion angle Φ_{O3-C3} correlate (Table 1). Both *Mucorales* lipases hydrolyze the flexible ether and ester substrates in *sn*-1, indicated by values for torsion angle $\Phi_{O3-C3} > 150^\circ$. Stereopreferences between *Rhizopus* and *Rhizomucor* lipase vary for the rigid amide and phenyl substrates. *Rhizopus* lipase hydrolyzes rigid triradylglycerols in *sn*-3 indicated by $\Phi_{O3-C3} < 150^\circ$ (amide:

$\Phi_{O3-C3} = 117^\circ$, phenyl: $\Phi_{O3-C3} = 118^\circ$), *Rhizomucor* lipase in *sn*-1 with $\Phi_{O3-C3} > 150^\circ$ (amide: $\Phi_{O3-C3} = 166^\circ$, phenyl: $\Phi_{O3-C3} = 173^\circ$).

Discussion

Model

To date, no X-ray structure has been published for *Rhizopus* lipase in the open conformation, while two structures of the open form of *Rhizomucor* lipase are accessible in the Protein Data Bank (Brzozowski et al., 1991; Derewenda et al., 1992). *Rhizopus* and *Rhizomucor* lipase display 70% sequence homology, which is sufficient for creating a structural model. From comparing this structural model of *Rhizopus* lipase with the X-ray structures of both the open form of *Rhizomucor* lipase and the half-open structure of *Rhizopus* lipase (Derewenda et al., 1994), we concluded that the opening of the lid does not significantly influence the positions of the relevant residues in the binding pocket. Also, substrate binding does not dramatically affect the lipase structure. Interactions between the substrate and the enzyme occur predominantly with the side chains of the residues rather than the backbone. Performing molecular dynamics simulations, these interactions were taken into account. Both the protein side chains and the substrate were allowed to move. Recently published studies of X-ray structures of *Pseudomonas cepacia* lipase complexed with triacylglycerol analog phosphonate inhibitors also indicated that the influence of an inhibitor binding to the lipase is negligible (Lang & Dijkstra, 1998).

Although *Rhizopus* and *Rhizomucor* lipase are highly homologous, their stereoselectivity toward triradylglycerols varies considerably. The stereoselectivity of *Rhizopus* lipase strongly depends on the *sn*-2 substituent of the substrate: flexible ether and ester substrates are preferably hydrolyzed in *sn*-1, rigid amide and phenyl substrates in *sn*-3. In contrast, *Rhizomucor* lipase hydrolyzes all four triradylglycerols preferentially in *sn*-1. Different stereoselectivities of homologous lipases toward triglyceride substrates have been previously published for the *Pseudomonas* lipases (Rogalska et al., 1993; Stadler et al., 1995).

To date, there is no general rule to explain the *sn*-1(3) stereoselectivity of lipases, while for secondary alcohols such a rule was developed (Kazlauskas et al., 1991). Thus, finding a simple method to explain and predict stereoselectivity of lipases toward triacylglycerols and analogs is of fundamental interest for the design and engineering of stereoselective catalysts in organic synthesis.

Previously, a simple method was introduced to explain the stereoselectivity of *Rhizopus* lipase. After docking a triradylglycerol substrate into the binding site of the enzyme and minimizing the energy of the resulting complex structure, the value of a single torsion angle Φ_{O3-C3} could be used to monitor stereopreference of the enzyme wild-type toward five different substrates and to predict stereoselectivity properties of mutants (Scheib et al., 1998).

This model holds for *Rhizomucor* lipase without exception. Thus, differences in geometry of the bound substrate in *sn*-1 and *sn*-3 orientation determine in which of the two orientations the substrate will preferably be hydrolyzed.

Torsion angle Φ_{O3-C3} indicates that for *Rhizopus* lipase, rigid and flexible substrates bind with different conformation. As a consequence, the substrates are hydrolyzed at opposite stereopreference. For *Rhizomucor* lipase, however, both rigid and flexible substrates bind similarly into the binding site. Hence, hydrolysis occurs at the same stereopreference. Two stereoselectivity deter-

Table 1. Stereoselectivities of *Rhizopus* and *Rhizomucor* lipase toward selected flexible and rigid triradylglycerols^a

Substrate	Experimentally obtained stereopreferences	ee-Value (%) ^c	E-value ^d	Predicted stereopreference	Φ_{O3-C3}
ROL ^b					
Ether	<i>sn</i> -1	61 (± 2)	4	<i>sn</i> -1	164
Ester	<i>sn</i> -1	19 (± 5)	1	<i>sn</i> -1	170
Amide	<i>sn</i> -3	63 (± 6)	5	<i>sn</i> -3	117
Phenyl	<i>sn</i> -3	77 (± 3)	8	<i>sn</i> -3	118
RML					
Ether	<i>sn</i> -1	69 (± 4)	6	<i>sn</i> -1	163
Ester	<i>sn</i> -1	73 (± 3)	7	<i>sn</i> -1	169
Amide	<i>sn</i> -1	56 (± 2)	4	<i>sn</i> -1	166
Phenyl	<i>sn</i> -1	68 (± 2)	6	<i>sn</i> -1	173

^aFor both flexible and rigid substrates in *Mucorales* lipases, experimentally determined and by molecular modeling predicted stereopreferences correlate. As for *Rhizopus* lipase, torsion angle Φ_{O3-C3} indicates stereopreference: for $\Phi_{O3-C3} > 150^\circ$, hydrolysis of triradylglycerols preferably occurs in *sn*-1, for $\Phi_{O3-C3} < 150^\circ$ in *sn*-3. *Rhizopus* lipase is abbreviated as ROL, *Rhizomucor* lipase by RML.

^bScheib et al. (1998).

$$^c ee = \frac{[A] - [B]}{[A] + [B]} \times 100.$$

$$^d E = \frac{\ln(1 - c(1 + ee_p))}{\ln(1 - c(1 - ee_p))}, \text{ determined at conversion } c = 10\%.$$

mining regions in *Mucorales* lipases were identified to explain this behavior.

His gap

In *Rhizopus* lipase, molecular modeling studies indicated that the *sn*-2 substituent of the bound substrate is placed into the His gap, a cleft between the active site H257 and L258 (Scheib et al., 1998). The *sn*-2 substituent directly contacts L258. Interactions between the *sn*-2 moiety and residue 258 are sterical. Thus, residue 258 discriminates between *sn*-1 and *sn*-3 stereopreference. According to this model, stereoselectivity can be affected by either the rigidity of the *sn*-2 substituent or the size of residue 258. The more intense the interactions between substrate and residue 258, the more selective the lipase toward *sn*-3. In *Mucorales* lipases, the His gap motif is conserved as well as all other residues of the hydrophobic dent, except position 254, which is leucine in *Rhizopus* and valine in *Rhizomucor* lipase. However, earlier studies with respect to substituting L254 by valine in *Rhizopus* lipase did not affect the stereoselectivity toward triacylglycerols and analogs (Scheib et al., 1998). Hence, additional interaction sites must exist that mediate stereoselectivity and are different in *Mucorales* lipases.

G-elbow

Because all residues in contact with the substrate are either conserved in *Mucorales* lipases or do not contribute to the stereoselectivity toward triacylglycerols, indirect effects on the substrate binding have to be taken into account, which result from different residues neighboring the binding pocket: G266 is located close to C2 of glycerol in a short loop. In *Rhizomucor* lipase, this G-elbow loop is further exposed into the binding site than in *Rhizopus* lipase. Both neighboring residues to G266 vary in *Mucorales* lipases. While the C-terminal neighbor (L267 in *Rhizomucor* lipase, S267 in *Rhizopus* lipase) is located far apart from the substrate and the catalytic machinery, the N-terminal neighbor (T265 in *Rhizomucor* lipase, E265 in *Rhizopus* lipase) interacts with H143 (in *Rhizomucor* lipase, H144 in *Rhizopus* lipase). Beer et al. (1996) postulated that H144 in *Rhizopus* lipase plays a key role in the positioning of the active site H257. Replacing E265 by aspartate resulted in a breakdown of the active site geometry, supposedly due to a new H-bond between H144 and the carboxyl group of D265 (Beer et al., 1996). Also, packing of the aspartate side chain close to H144 may increase sterical hindrance between both residues. Therefore, in *Rhizopus* lipase, the side chain of E265 points away from the corresponding H144 into the lipase, due to sterical reasons (Fig. 3). In *Rhizomucor* lipase, T265 is small enough to orientate its side chain toward H143, which is a prerequisite for the formation of an H-bond between the hydroxyl of T265 and N_δ of H143 imidazol. Hence, T265 in *Rhizomucor* lipase is located closer to H143 than E265 in *Rhizopus* lipase. Therefore, the positions of this residue and of its neighbor 266 differ in both lipases (Fig. 3, horizontal arrow). Additionally, in *Mucorales* lipases, the C-terminal part of the G-elbow loop is fixed by a disulfide bond from C268 to C29.

Thus, the different positions of the G-elbow in *Mucorales* lipases result from residues that do not directly contact the substrate, but have an indirect impact on the binding of triacylglycerols into the substrate binding site. In *Rhizomucor* lipase, G266 is more exposed into the binding site; hence, the substrate binds less deeply (Fig. 3, horizontal arrow). This may explain the reduced sterical

interactions between the *sn*-2 substituent and L258 in *Rhizomucor* lipase. As a consequence, L258 in *Rhizomucor* lipase does not discriminate between flexible and rigid substrates which, therefore, bind with a similar geometry and are both hydrolyzed with *sn*-1 stereopreference.

Lid

A89 in *Rhizopus* lipases is part of the lid; the corresponding residue in *Rhizomucor* lipase is W88. W88 in the X-ray structure of the open form of *Rhizomucor* lipase is close to the *sn*-2 substituent of the substrate and, therefore, could have a sterical influence on stereoselectivity. However, replacing A89 in *Rhizopus* lipase by tryptophane did not affect stereoselectivity of the lipase toward triacylglycerols (data not shown). Hence, the stereoselectivity of *Rhizopus* lipase toward both flexible and rigid triacylglycerols is not influenced by residue 89 from the lid. The lid is a highly flexible secondary structure element in lipases which, upon undergoing interfacial activation, exhibits the substrate binding site to the lipid (Derewenda et al., 1994). Modifying lid residues in lipases was performed with *Humicola lanuginosa* lipase (Martinelle et al., 1996), but the impact on stereoselectivity toward triacylglycerols was not investigated.

Specific lipase activity and stereoselectivity can be influenced by various factors such as, for example, solvent, water activity, or quality of the interface (for an overview: Kazlauskas & Bornscheuer, 1998). However, our results indicate that the main effect on lipase stereoselectivity toward triacylglycerols is the sterical interaction between the lipase and the substrate as the first tetrahedral intermediate of ester hydrolysis in hydrolases. This has been proven by engineering *Rhizopus* mutants (Scheib et al., 1998).

Therefore, our model explains and predicts stereopreferences of *Mucorales* lipases toward the hydrolysis of triacylglycerols. Further research will focus on generalizing this model to other lipases.

Materials and methods

Substrates

The preparation of 1,3-dioleoyl-2-*O*-hexadecylglycerol (ether substrate), 1,3-dioleoyl-2-deoxy-2-palmitoylamino-glycerol (amide substrate), and 1,3-dioleoyl-2-deoxy-2-phenylglycerol (phenyl substrate) was carried out as described previously (Stadler et al., 1995; Scheib et al., 1998). The ester substrate was triolein.

Lipase assay

Hydrolysis reactions were carried out with triacylglycerol emulsions in buffer (50 mM Tris/HCl, pH 7.0, 0.9% NaCl, 37 °C) without the addition of detergents, according to the procedure described by Kovac et al. (1996) and Scheib et al. (1998). The enantiomeric diacylglycerols produced during lipolysis were derivatized with chiral R-(+)-phenylethylisocyanate and the corresponding diastereomeric carbamates were separated by HPLC (Stadler et al., 1995). Calculation of enantiomeric excess values (% ee) and of enantiomeric ratio (*E*) provided a quantitative measure of lipase selectivity toward one of the primary ester groups of triacylglycerols (*sn*-1 or *sn*-3 orientation, respectively). The enantiomeric ratio was calculated from ee-values of the hydrolysis

products at a conversion (c) of 10% according to Chen et al. (1982). For $c < 10\%$, E is independent from the reaction time and can be calculated from ee-measurements. Possible effects due to differences in emulsion that might arise as a consequence of distinct hydrophobicity of the various substrates had to be considered. To exclude this possibility, experiments carried out with single substrate emulsions were repeated using substrate emulsions consisting of equimolar mixtures of two substrates and, essentially, identical results were obtained (Stadler et al., 1995).

Hard- and software

Molecular modeling was performed on a Silicon Graphics Workstation Indigo2/R10000. Sequences were extracted from the SWISS-PROT sequence database (Appel et al., 1994) and aligned using *Clustal W* (Higgins et al., 1996). X-ray structures were from the Protein Data Bank (PDB) (Bernstein et al., 1977). The binding sites of the lipases were investigated using the methyl, dry, and water probe of the *Grid* program (Goodford, 1985), and visualized with the graphical interface of the *InsightII* software package (Molecular Simulations, Inc., San Diego, California). Energy minimizations and molecular dynamics simulations were carried out with Sybyl 6.3 (Tripos, St. Louis, Missouri) using the Tripos force field (Clark et al., 1989).

Molecular modeling

The sequences of the lipases from *Rhizopus oryzae* and *Rhizomucor miehei* were aligned and analyzed (Fig. 2). For molecular dynamics studies, a structural model of *Rhizopus* lipase (Beer et al., 1996) built from the crystal structure of the open form of *Rhizomucor* lipase (PDB entry 4tgl; Derewenda et al., 1992) was used.

The lipase from *R. niveus* is identical to *R. oryzae* lipase (Kazlauskas & Bornscheuer, 1998). The crystal structure of *R. niveus* lipase (PDB entry 1lgy; Kohno et al., 1996) was determined in closed conformation. Therefore, for molecular modeling studies including substrate, this structure cannot be used. The structure of the G-elbow in the *R. oryzae* lipase model is similar to the structure of *R. niveus* lipase, but different from *Rhizomucor* lipase (RMS $C_\alpha = 0.7 \text{ \AA}$).

The binding sites of *Rhizopus* and *Rhizomucor* lipase were studied using *Grid* software identifying both hydrophobic (methyl and dry probe) and hydrophilic (water probe) binding regions. As in *Rhizopus* lipase, in *Rhizomucor* lipase two hydrophobic patches were identified to which the scissile *sn*-1(3) fatty acid (hydrophobic crevice) and the *sn*-2 substituent (hydrophobic dent) putatively bind. Kovac et al. (1996) demonstrated that the stereoselectivity remains unaffected by the chemical structure of the nonhydrolyzed *sn*-3(1) fatty acid chain. In molecular modeling, triolein, in hydrolysis experiments triolein derivatives were investigated. According to Stadler et al. (1995), the stereoselectivity of *Mucorales* lipases is essentially independent from fatty acid chain length.

Manual substrate docking and molecular dynamics simulations were performed as previously described (Scheib et al., 1998). During an equilibration phase of 16 ps the lipase–substrate complexes were heated to 100 K. Subsequently, during a 2-ps production phase 50 coordinate sets were collected to create an average structure of the lipase–substrate complex. The average structures were further analyzed. For the substrate in *sn*-3 orientation, torsion angle Φ_{O3-C3}

describes the torsion of the O3–C3 bond connecting the *sn*-3 fatty acid chain ester and C3 of glycerol.

Conclusion

Stereoselectivity of *Mucorales* lipases is determined by two interactions: (1) *His gap*: L258 discriminates between *sn*-1 and *sn*-3 stereopreference. Increasing the size of residue 258 and the rigidity of the substrate increases *sn*-3 stereoselectivity. (2) G-elbow: The G-elbow loop adjusts the position of the substrate in the binding site. In *Rhizopus* lipase, the binding site is deep, the substrate binds close to the *His gap*. Rigid and flexible substrates are hydrolyzed at opposite stereopreference. In *Rhizomucor* lipase, the binding site is less deep, the substrate is pushed away from the *His gap* and interacts less with L258. Rigid and flexible substrates are hydrolyzed at the same *sn*-1 stereopreference.

Stereopreference can be monitored by the value of torsion angle Φ_{O3-C3} of the bound substrate in *sn*-3 orientation. For $\Phi_{O3-C3} > 150^\circ$, the substrate is preferentially hydrolyzed in *sn*-1, for $\Phi_{O3-C3} < 150^\circ$ in *sn*-3. This prediction holds without exception for wild-type *Rhizopus* and *Rhizomucor* lipase.

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