Activity of Lipases and Esterases towards Tertiary Alcohols: Insights into Structure–Function Relationships**

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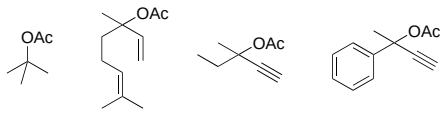
Dedicated to Professor Rolf D. Schmid on the occasion of his 60th birthday

Hydrolytic enzymes are versatile biocatalysts and find increasing applications in organic synthesis.[1] A considerable number of industrial processes using these enzymes have been commercialized.[2] Within this class, lipases (E.C. 3.1.1.3) and carboxy esterases (E.C. 3.1.1.1) are frequently used as they accept a broad range of non-natural substrates, are usually very stable in organic solvents, and exhibit good to excellent stereoselectivity. However, the vast majority of (chiral) substrates are secondary alcohols, followed by carboxylic acids and primary alcohols. Chiral tertiary alcohols (TA) and their esters (TAEs) are a group of important compounds, which can be found in a number of natural products (e.g., α-terpineol, linalool) and represent a group of useful building blocks.[3] Unfortunately, only very few hydrolases accept them as substrates.[4] Furthermore, reaction rates and/or enantioselectivities were quite low and consequently applications are rather unlikely. Structural reasons for the lack of activity—except for the expected lower chemical reactivity compared to secondary alcohols—and enantioselectivity of hydrolases towards these compounds have not been revealed so far, although the 3D structures of several lipases and esterases have been known for more than a decade.

Initially, we checked 25 commercially available enzyme preparations for their activity towards the four model acetates **1**–**4** (Scheme 1). In accordance with the literature, we found that only a lipase from *Candida rugosa* (CRL)[4c,d] and lipase A from *Candida antarctica* (CAL-A)[4e] are active.



Scheme 1. Esters of tertiary alcohols (TAEs) used as model substrates.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

The protein structure of CAL-A is unknown and sequence analysis shows no major similarity to known protein structures.[5] Thus, further investigations concentrated on the well-characterized CRL. Comparison of the 3D structure of CRL with those enzymes not active towards TAEs revealed that the alcohol binding pocket in CRL is 1.5–2 Å wider (Table 1), which thus allows the binding of the nearly spherical TA moiety. The amino acid sequence of CRL contains a GGGX motif that is positioned in the active center of the enzyme.[6] This pattern of amino acids, located on a protein loop near the binding site of the substrate ester's carboxylic group, is involved in the formation of the so-called oxyanion hole. This involvement stabilizes the anionic carbonyl oxygen atom of the tetrahedral intermediate during the catalytic cycle of ester hydrolysis by use of two H bonds which are provided by two amide groups of the protein backbone. In contrast, most commercial lipases and esterases have a GX motif in this particular part of the active site.[6] Here a bulky residue (labeled X) forms the H-bond. So far, the GX and GGGX motifs and their occurrence in α/β-hydrolases have been reported upon only as phenomena, but no direct linkage to catalytic properties of these enzymes has been identified.

Table 1. Comparison of the distances between the proton at the active-site histidine and the carbonyl oxygen atom at the indicated oxyanion hole residue ($N_{\varepsilon,\ His}$ H-$O_{Carb.}$) for various GX and GGGX-type α/β hydrolases.

Hydrolase	PDB entry[a]	Type	Oxyanion hole residues	$N_{\varepsilon,\ His}H\text{-}O_{Carb.}$ [Å]
PCL	4LIP	GX	G **L17**	5.88
PAL	1EX9	GX	G **M15**	6.00
CVL	1CVL	GX	G **L17**	4.06
CAL-B	1TCA	GX	G **T65**	6.29
HLL	1TIB	GX	G **S83**	6.44
CRL	1LPM	GGGX	FGG **G134** F	7.76
BsubpNBE	Homology model	GGAX	FGG **A107** F	7.67
hAChE	1CLJ	GGGX	YGG **G122**F	7.48
eeAChE	1C2B	GGGX	YGG **G121** F	7.83
BAChE	Homology model	GGGX	YGG **G123** F	7.25

[a] PDB = protein data bank (http://www.resb.org/pdb/).

Based on the structural differences of the GGGX and GX-type α/β-hydrolases, a computer model was developed (see Supporting Information for details). A thorough comparison of structural data of CRL with that of inactive GX hydrolases (e.g. lipase from *Pseudomonas cepacia*; PCL) revealed that in GX-type hydrolases the backbone carbonyl oxygen atom of the X residue (leucine in PCL) is orientated into the binding pocket at a location incorporating the C_α atom of the alcohol moiety of the substrate (Figure 1a). In the case of a quaternary C_α atom, which is present in TAEs, a repulsive interaction occurs between the carbonyl oxygen atom and the spherically arranged substituents of this C_α atom. In CRL, the GGGX loop should provide a most flexible conformation, because of the small hydrogen residue in glycine, (Figure 1b). Moreover, the carbonyl oxygen atoms are arranged parallel to the binding pocket, thus providing sufficient space at this critical part of the enzyme structure for the bulky tertiary alcohol group. This finding was verified first by computer simulation of substrate–enzyme complexes with GX and

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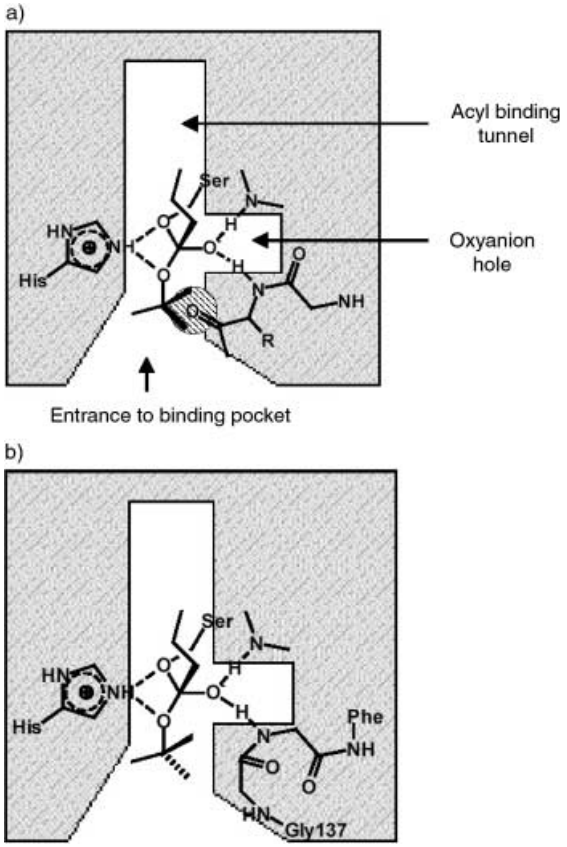

Figure 1. a) Schematic view into the binding pocket of GX-type hydrolases. The carbonyl oxygen atom of the oxyanion hole residue faces in, towards the binding pocket of the alcohol moiety. Thus interaction of this oxygen atom with the quaternary C_α atom of a TAE disables an appropriate binding of the substrate. b) Schematic view into the binding pocket of GGGX-type hydrolases (i.e. CRL). The carbonyl backbone of the flexible triple glycine motif is parallel to the limiting wall of the binding pocket. This yields an increased capability to incorporate space-demanding substrates.

GGGX-type esterases and lipases followed by experimental confirmation using a range of GGGX-type α/β-hydrolases, which were identified by amino acid sequence comparison using internet-based public databases.

Thus discovered, acetylcholine esterases from three species (electric eel (*Electrophorus electricus*; eeAChE), banded krait snake (*Bungarus fasciatus*, bAChE), and human (hAChE)), carboxylesterases (*p*-nitrobenzylesterase from *B. subtilis* (BsubpNBE) and pig liver esterase (PLE)), and an additional lipase (from *Geotrichum candidum* (GCL)) were checked for activity in the hydrolysis of model acetates **1**–**4**. All PLE and eeAChE were used as commercial-grade lyophilized powders; GCL,[7] bAChE, and hAChE[8] were expressed in the methylotrophic yeast *Pichia pastoris*. The gene encoding BsubpNBE was cloned from genomic DNA from *B. subtilis* DSM 402 and expressed in high yields in *E. coli* (see Supporting Information for details).

With the exception of GCL, all these GGGX type α/β-hydrolases hydrolyzed the model esters (Table 2). Only eeAChE showed no measurable activity towards *tert*-butyl acetate (**1**), but this was also a poor substrate for the other biocatalysts. In contrast, substrates **2**–**4** were converted at high rates (up to 77% conversion; Table 3). The activity of BsubpNBE towards **4** was 3890 mU mg^{-1} protein; the activity of the PLE preparation Chirazyme E-1 was 1640 mU mg^{-1} protein, which is thus 17 and 20 times more active towards **4** than towards **1**. Although activities and conversions were high, enantioselectivities of all hydrolases towards **2**–**4** never exceeded $E = 10$ (bAChE) and were mostly in the range of $E = 1.5$–4 (data not shown).

Table 2. Activity of hydrolases towards **1** compared to natural substrates. Activity was determined by pH-stat assay at pH 7.5 and 40 °C (eeAChE: 25 °C).

Hydrolase		Specific activity (natural substrate) [mU mg^{-1} Protein]	Specific activity towards **1** [mU mg^{-1} Protein]
CRL	(Amano AYS)	44 000[a]	10
GCL-B		83 000[a]	–[e]
EeAChE		200 000[b]	–[e]
CAL-A	(Chirazyme L5, lyo)	117 000[c]	50
PLE	(Chirazyme E1, lyo)	157 000[d]	80
	(Chirazyme E2, lyo)	103 000[d]	160
BsubpNBE		170 000[c]	230

[a] Triolein. [b] Acetylthiocholine. [c] Tributyrin. [d] Ethyl butyrate. [e] No activity.

An additional hint that the GGGX motif is crucial for activity towards tertiary alcohols are our unsuccessful experiments to create activity by directed evolution[9] experiments based on GX-type esterases. For this, enzyme libraries of two genes encoding esterases from *Pseudomonas fluorescens*[10] and *Bacillus stearothermophilus*[11] were built using error-prone polymerase chain reaction (PCR)[12] and DNA shuffling[13] methods, and the proteins were then expressed in *E. coli* using a strong rhamnose-inducible promoter.[14] However, no active mutants were identified after screening a library of approximately 15 000 esterase variants.

Table 3. Activity of GGGX-type hydrolases towards substrates **2**–**4**. Reactions were carried out at pH 7.5, 40 °C (AChEs 25 °C).

Enzyme[a]	Substrate					
	2		**3**		**4**	
	t [h]	Conversion [%]	*t* [h]	Conversion [%]	*t* [h]	Conversion [%]
PLE, Chirazyme E-1, 390 U	2	53	8	48	0.5	50
PLE, Chirazyme E-2, 270 U	2	69	8	72	n.b.[b]	n.b.[b]
CRL-CLEC, 10 mg	2	56	8	23	0.5	77
hAChE, 37.5 U	24	17	48	5	4	39
eeAChE, 100 U	24	26	48	10	4	50
bAChE, 100 U	8	57	48	6	4	66
CAL-A, 480 U	24	41	48	71	2	59
BsubpNBE, 350 U	0.8	50	0.6	50	0.3	50

[a] Units refer to the activity towards the natural substrates, see Table 2. [b] Not determined.

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We have provided strong evidence that the GGGX motif described for some lipases not only plays a role in stabilizing the tetrahedral intermediate during catalysis, but also governs activity towards TAs. With the exception of GCL, all tested esterases and lipases bearing the GGGX motif hydrolyzed the model acetates. In the case of GCL, we assume that this lipase will accept other TAEs.

The overall amino acid sequence similarity between these hydrolases originating from bacteria, yeast, and mammalian sources is rather low and only small regions around the GGGX motif and the active serine residue are conserved. This structure dissimilarity is also reflected by the large differences between the substrates normally accepted by these hydrolases,[15] which means, in turn, that knowledge about their usual biocatalytic activity would not suggest that these enzymes would have activity towards TAs in common.

Furthermore, our computer model also allows the prediction of mutants with enhanced or even inverse stereoselectivity in the resolution of the model acetates of TAs.[16]

Experimental Section

All experimental details can be found in the Supporting Information.

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$^{1}_{\infty}[HgGe_9]^{2-}$—A Polymer with Zintl Ions as Building Blocks Covalently Linked by Heteroatoms**

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Dedicated to Professor Dr. Gottfried Huttner on the occasion of his 65th birthday

Homoatomic-element polyhedra of the type E_n^{x-} (E = Group 14 element) are promising candidates for the formation of complex structures with interesting electronic properties. The polyhedra E_n are stable with different charges so they can be employed as electron reservoirs and suitable coupling of the clusters can lead to unexpected properties. The alkali-metal fulleride phases are a particularly impressive example of this. They contain C_{60}^{3-} ions and become superconducting at relatively high critical temperatures.[1] The structural characterizations of the linear polymer anions with C_{70}[2] and Ge_9[3] as building blocks show new possibilities for obtaining complex structures by covalent linking of the polyhedra. Recently we have pointed out parallels between fullerides and homoatomic polyhedra of the heavier elements of Group 14, the latter are known as Zintl ions.[4] Such homoatomic Zintl ions have been known since 1891 when there formation by dissolving elementary tin or lead in a solution of sodium in liquid ammonia was reported,[5] however, there is still little known about their chemical reactivity.[6]

During our examinations of semiconductors and metal clusters as building blocks for ordered structures, we successfully coupled Ge_9 clusters with Hg atoms for the first time.[7] The compound $[K(crypt)]_2[HgGe_9](en)_2$ (**1**) can be prepared by the reaction of a solution of the binary alloy K_4Ge_9 in ethylenediamine (en) with elementary mercury in the presence of [2.2.2]cryptand (crypt).[8] As expected the Hg atoms appear as bridges between the E_9 clusters forming a linear polymer of the composition $^{1}_{\infty}[HgGe_9]$.

Single-crystal structure determination of **1** shows that the Hg atoms are connected to two Ge atoms situated opposite of the open rectangular face of the Ge_9 cluster.[9] The shortest Hg–Ge separations (Figure 1) are 2.543(1) Å (Hg–Ge3) and 2.606(1) Å (Hg–Ge4). There are significantly longer separations from the Hg atom to two more sets of Ge atoms 3.049(1) Å (Hg–Ge5) and 3.120(1) Å (Hg–Ge6) as well as 4.096(1) Å (Hg–Ge7) and 4.043(1) Å (Hg–Ge8). The shorter distances to the atoms Ge5 and Ge6 means that the Ge_9 cluster is tilted with respect to the Hg–Hg vector.

The bond angle Ge3-Hg-Ge4 is 172.2(3)° and points to a coordination number of two for the Hg atom and thus to a covalent interaction between Hg and the Ge atoms. The

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