Enhancement of the enantioselectivity of carboxylesterase A by structure-based mutagenesis

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\textbf{ABSTRACT}

Previously studied Bacillus subtilis carboxylesterases (CesA and CesB) have potential for the kinetic resolution of racemic esters of 1,2-O-isopropylideneglycerol (IPG). CesA exhibits high activity but low enantioselectivity towards IPG-butyrate and IPG-caprylate, while the more enantioselective CesB does not process IPG-butyrate and exhibits several-fold lower activity than CesA towards IPG-caprylate. A sequence and structure comparison allowed us to identify active site residues that may cause the difference in (enantio)selectivities of CesA and CesB towards these IPG esters. This structure-based approach led to the identification of two active site residues in CesA (F166 and F182), as promising candidates for mutagenesis in order to enhance its enantioselectivity. Mutagenesis of positions 166 and 182 in CesA yielded novel variants with enhanced enantioselectivity and without significant loss of catalytic activity. For IPG-butyrate, a CesA double mutant F166V/F182C (ee\textsubscript{S} = 13) was generated showing a ~13-fold increased enantioselectivity as compared to wild-type CesA (ee\textsubscript{S} = 1). For IPG-caprylate, we designed a CesA double mutant F166V/F182Y (ee\textsubscript{S} = 9) displaying a ~5-fold increased enantioselectivity as compared to the wild-type enzyme (ee\textsubscript{S} = 2). These findings, combined with the results of molecular docking experiments, demonstrate the importance of residues at positions 166 and 182 for the enantioselectivity of CesA, and may contribute to the development of efficient biocatalysts.

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1. Introduction

Carboxylesterases (EC 3.1.1.1.) represent a class of hydrolytic enzymes that is highly useful for the preparation of optically pure compounds. These enzymes are widespread in nature and have been isolated from several organisms, including microbises, plants and animals. Their broad substrate acceptance, high stability, enantioselectivity, and the fact that they do not require cofactors for activity, make them attractive biocatalysts for use in organic synthesis (Bornscheuer, 2002a,b; Bornscheuer and Kazlauskas, 1999). For example, carboxylesterase NP of B. subtilis That-1-B has been exploited for kinetic resolution of the racemic methyl ester of the non-steroidal anti-inflammatory drug naproxen [2-(6-methoxy-2-naphthyl)propionic acid] (Quax and Broekhuizen, 1994; Smeets and Kieboom, 1992). Two closely related carboxylesterases from B. subtilis 168, carboxylesterase CesA (gene name “nap”) and carboxylesterase CesB (gene name “yb/f”), have also been investigated for the enantioselective hydrolysis of naproxen methyl ester. Like NP, CesA (ee\textsubscript{S} = 99%) exhibits high enantioselectivity towards naproxen methyl ester, but CesB (ee\textsubscript{S} = 85%) has only modest enantioselectivity (Dröge et al., 2005).

CesA and CesB share 60% sequence identity, and show 98% and 64% sequence identity to NP, respectively. These carboxylesterases (NP, CesA and CesB) belong to the \alpha/\beta-hydrolase fold superfamily of proteins and as such have the characteristic canonical \alpha/\beta-hydrolase fold (Dröge et al., 2005). The active site contains the typical catalytic triad (nucleophile-His-acid) present in all members of this superfamily. In addition to the triad, a characteristic consensus sequence motif (GXSXG) is present around the catalytic serine (nucleophile). The superfamily carboxylesterases catalyze the hydrolysis of esters via a two-step nucleophilic substitution mechanism similar to that of serine proteases. In the first step, the catalytic serine attacks the substrate’s carbonyl carbon, resulting in the formation of a tetrahedral intermediate that is stabilized by at least two backbone amide atoms from residues that form the oxyanion hole. Collapse of the tetrahedral intermediate results in
the release of the alcohol product and the formation of an acyl-
enzyme complex. In the second step, this acyl-enzyme complex is 
hydrolized, which yields the fatty acid product and regenerates 
the free enzyme (Bornscheuer and Kazlauskas, 1999; Holmquist,
2000).

We are interested in developing a biocatalytic process for the
kinetic resolution of racemic 1,2-O-isopropylideneglycerol (IPG)
esters (Dröge et al., 2006a). In the ideal case, a biocatalyst con-
verts only the R-enantiomer of the substrate, yielding the optically 
pure product (S)-IPG, which is an important building block for the
synthesis of β-blockers, prostaglandins, and leukotrienes (Jurczak
et al., 1986; Magoni et al., 1990). Activity assays using racemic
IPG-butyrate (1) and IPG-caprylate (3) (Fig. 1) revealed that car-
boxylesterase CesA can efficiently hydrolyse both esters of IPG,
but exhibits very low enantioselectivity towards these substrates
(Dröge et al., 2005). CesB, on the other hand, has been reported to
hydrolyse 3 with the desired preference towards the R-enantiomer.
This enzyme, however, does not hydrolyse 1 and it exhibits much
lower catalytic activity towards 3 when compared to CesA (Dröge
et al., 2005).

Herein, we present a comparison of the crystal structure of CesA
and a homology model of the CesB active site, which led to the
identification of two active site residues in CesA, F166 and F182,
which are replaced by Y166 and Y182 in CesB. We hypothesized
that these active site differences may underlie the difference in selec-
tivities observed for CesA and CesB towards substrates 1 and 3.
Based on these findings, rational and random mutagenesis of posi-
tions 166 and 182 in CesA was performed. The results obtained may
contribute to the development of efficient hydrolytic enzymes that
have potential for the kinetic resolution of IPG esters.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade unless specified otherwise.
The p-nitrophenyl butyrate was purchased from Sigma-Aldrich.
(R)-IPG-butyrates, (S)-IPG-butyrates, (R)-IPG-caprylates and (S)-IPG-
caprylates were synthesized according to literature procedures
(Mezzetti et al., 2003; Straw et al., 1989). Ingredients for buffers
and media were obtained from Duchefa Biochemie (Haarlem, The
Netherlands). Enzymes and reagents used in the molecular biology
procedures, including PCR reagents, T4 DNA ligase, DNA ladders,
restriction enzymes and protein molecular weight standards, were
obtained from Invitrogen Corp. (Carlsbad, CA, USA), Promega Corp.
(Madison, WI, USA), Finnzymes (Espoo, Finland) or New England
Biolabs (Ipswich, MA, USA). Oligonucleotides for DNA amplification
were synthesized by Operon Biotechnologies (Cologne, Germany).

Fig. 1. Esterase-catalyzed kinetic resolutions of IPG esters.

PCR purification, gel extraction, and Miniprep kits were provided
by Qiagen (Hilden, Germany).

2.2. General methods, plasmids, bacterial strains and media

E. coli strain BL21 (DE3) (Stratagene, La Jolla, CA) was used in
combination with the pET15b vector (Novagen) for recombinant
protein production. E. coli cells were grown in concentrated (2×)
Luria-Bertani (LB) media containing Bactotrypton (1.6%, w/v), Bacto
yeast extract (1%, w/v) and sodium chloride (0.5%, w/v). When
required, ampicillin (Ap; 100 μg mL⁻¹), Difo agar (15 g L⁻¹), and
IPTG (1 mM) were added to the medium. DNA sequence analy-
thesis was performed by Macrogen (Seoul, Korea). Recombinant DNA
techniques were performed as described by Sambrook and Russell

2.3. Construction of the CesA expression vector

The CesA gene was amplified from pCANTABSpG3pCesA vec-
tor (Dröge et al., 2006b) using two synthetic primers. The forward
primer (5’-GGC GCC CAG CCG CAT ATG GCA CAA AAC-3’) contains
a Ndel restriction site (in bold) and the reverse primer (5’-AC CTC
GAC GGA TCT TA TGA AGT CGG-3’) contains a BamHI restric-
tion site (in bold). The resulting PCR product and the pET15b vector
were digested with Ndel and BamHI restriction enzymes, purified,
and ligated by using T4 DNA ligase. The ligation mixture was
used to transform competent E. coli BL21 (DE3) cells. To verify
the presence of the insert, transformants were selected from LB/Ap
plates and analyzed by colony PCR. Positive clones were grown
overnight on LB/Ap medium and the plasmid DNA was isolated.
The cloned cesA gene was sequenced to verify that no mutations
had been introduced during the amplification and cloning of the
gene. The newly constructed expression vector for CesA was named
pET15bCesAwt.

2.4. Generation of site-directed CesA mutants

Mutations at positions F166 and F182 were introduced by
megaprimer PCR, using plasmid pET15bCesAwt as the template,
according to Miyazaki and Takenouchi (2002). All mutant genes
were sequenced to confirm that only the intended mutations had
been introduced.

2.5. Expression and purification of CesA wild-type and mutants

The pET expression system was used for the production of
CesA wild-type and mutants. E. coli BL21 (DE3) cells containing
the appropriate expression vector were inoculated in 2× LB/Ap
medium (5 mL) and grown overnight at 37 °C. A sufficient quan-
tity of the culture was used to inoculate 2× LB/Ap medium (1 L)
to an initial A₅₀₀ of 0.05. Cultures were grown at 37 °C until an
A₅₀₀ of ~0.5, after which the cells were induced with IPTG
(1 mM). The culture was then further incubated at 20 °C. After
16 h of growth, cells were harvested by centrifugation and resus-
pended (3 mL/g of wet cells) in binding buffer (50 mM Tris–HCl,
500 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol, 0.14% (v/v) β-
mercaptoethanol, 1% Tween 20, pH 7.5). Cells were disrupted by
sonication, and the resulting extracts were clarified by centrifuga-
tion (50 min, 40,000 × g). The supernatant was filtered with the aid
of a cellulose acetate filter (0.2 μm) and subsequently loaded
on a nickel–charged HiTrap HP (5 mL) column (GE Healthcare).
After washing the column with binding buffer, elution of retained
proteins was performed with a linear gradient from 0 to 1 M imi-
dazole in elution buffer (50 mM Tris–HCl, 500 mM NaCl, 10% (v/v)
glycerol, 0.14% (v/v) β–mercaptoethanol, pH 8). Fractions were ana-
lized by SDS-PAGE and those that contained purified CesA were
pooled and concentrated using a Vivaspin centrifugal concentrator equipped with a 5 kDa cut-off membrane (Sartorius Stedim Biotech S.A., France). Subsequently, the buffer was exchanged against 50 mM Tris–HCl (pH 8.0), containing 10% (v/v) glycerol, by using a dialysis cassette with a 10 kDa cut-off membrane (Pierce Rockford, IL, USA). Purified protein solutions were flash frozen in liquid nitrogen and stored at −80 °C until further use.

2.6. Spectrophotometric assay for esterase activity

Esterase activity towards pNP-C4 (p-nitrophenyl-butyrate) ester was determined at 30 °C by monitoring the increase in product (p-nitrophenol) absorbance at 410 nm (ε = 1.5 × 10^4 M⁻¹ cm⁻¹) (Boersma et al., 2008). Assay mixtures (900 μL) contained purified enzyme (100 μg) and pNP-C4 ester (0.5 mM) in phosphate buffer (50 mM, pH 8), containing Triton X100 (0.36%, v/v) and gua Arabic (0.1%, w/v). Stock solutions of pNP-C4 ester (10 mM) were prepared in absolute methanol. One unit (U) of activity was defined as the amount of enzyme that produces 1 μmol of p-nitrophenol per min under the assay conditions (Gerritse et al., 1998). For each measurement, the nonenzymatic rate of hydrolysis of substrate was subtracted from the enzymatic rate of hydrolysis.

2.7. Chiral GC assay for esterase activity

The enantioselectivity of CesA wild-type and mutants was determined by performing a kinetic resolution of racemic 1 (2 mM) or 3 (4 mM) in 0.5 mL of MOPS buffer (70 mM, pH 7.5) at 30 °C. Stock solutions of 1 and 3 were made in MOPS buffer (70 mM, pH 7.5), containing Tween 80 (14.3%, v/v). Reactions were started by the addition of a suitable amount of purified enzyme (250–500 μg). Control samples for analyzing the nonenzymatic rate were prepared in the same way, but now no enzyme was added. Reaction mixtures and control samples were incubated for either 7 h (1) or 2 h (3). Subsequently, saturated NaCl solution (500 μL) was added and the aqueous solution was extracted twice with ethyl acetate (1 mL), containing cis-3-hexene-1-ol (2 mM) as internal standard. The samples were analyzed by chiral GC on a Hewlett Packard 5890 series II gas chromatograph, as described by Drögé et al. (2003). The non-enzymatic hydrolysis of 1 and 3 was negligible under the conditions used. The enantiomeric excess (ee) of products was calculated according to Chen et al. (1982). E-values were calculated by using the program Selectivity (K. Faber, H. Hoenig; ftp://borg185.kfunigraz.ac.at/pub/enantio/).

2.8. Library construction and activity screening

Saturation mutagenesis at positions 166 and 182 in CesA was performed by megaprimer PCR, using pET15bCesAwt as the template (Miyazaki and Takenouchi, 2002). The resulting plasmids harboring the mutant cesA genes were transformed into electro-competent BL21 (DE3) cells. Transformants were selected on LB/Ap plates. Colonies were picked with sterile toothpicks into 96-well plates (flat bottom, Greiner Bio-one) containing LB/Ap medium (200 μL per well). Cells were grown overnight at room temperature with agitation. ZYP-5052 medium (Studier, 2005) (1.3 mL) containing ampicillin was then added to the wells and the plates were incubated for 2 days at room temperature with agitation to allow protein production. Plates were then duplicated by transferring 50 μL of culture from each well into a new plate. The cells of the remaining culture were harvested by centrifugation (2000 × g, 4 °C, 15 min). The cell pellet was then resuspended in Bug-buster solution (Novagen, Merck, Germany) (300 μL) and the plates were incubated at room temperature with agitation for 30 min. Subsequently, 200 μL of phosphate buffer (50 mM, pH 8) was added to each well and centrifugation was performed (2000 × g, 4 °C, 1 h) to remove unbroken cells and cell debris. Aliquots (30 μL) of the cell free extracts were transferred into new 96-well plates containing phosphate buffer (200 μL, 50 mM, pH 8) and pNP-C4 ester (0.5 mM). Product formation was followed by measuring absorbance values at 410 nm directly after mixing and after 30 min. Cell free extracts prepared from BL21 (DE3) cells transformed with empty pET15b vector were used as references.

Clones with pronounced esterase activity were transferred from the master plate to LB/Ap medium (3 mL) and grown overnight at 37 °C and 250 rpm. A sufficient quantity of the overnight culture was used to inoculate 2 × LB/Ap medium (5 mL) to an initial A_600 of 0.05. Cultures were grown at 37 °C till an A_600 of ~0.5, after which the cultures were induced with IPTG (1 mM) and further incubated at 25 °C. After 16 h of growth, cells were harvested by centrifugation and A_600 was determined. Then, cells were resuspended in Bug-buster solution (500 μL) and incubated at 4 °C for 20 min. Unbroken cells and cell debris were removed by centrifugation at 4 °C (20 min, 2000 × g). The cell free extract (100 μL) was incubated with racemic 1 (500 μL of a 2 mM solution in 0.07 M MOPS pH 7.5, 14.3% (v/v) Tween 80) and incubated for 4 h at 30 °C. After incubation, the extraction procedure and chiral GC analysis was performed as described above. Mutant enzymes of which the enantiaselectivity was potentially increased were selected and purified for further characterization, and the corresponding genes were sequenced.

2.9. Determination of the kinetic parameters of the wild-type and mutant enzymes

The kinetic parameters K_m and k_cat were determined by measuring the initial rates of product (2 or 4) formation at different concentrations of substrate (1 or 3) in MOPS buffer at 30 °C. The concentrations of (R)-1, (S)-1, (R)-3, and (S)-3 in the assay ranged from 1 to 100 mM. Stock solutions of substrate were prepared as described above. Samples were withdrawn at different time intervals, extracted, and analyzed by chiral GC as described above. All enzyme assays were carried out at least twice.

2.10. Homology modeling and molecular docking

A structural model of CesB was built by using the program Coot (Emsley and Cowtan, 2004). The crystal structure of CesA (Protein Data Bank accession code 2RI1) was used as the template for homology modeling. To obtain a structural view of the differences in the active sites of CesA and CesB, an overlay was made of the CesA crystal structure and the CesB model, using Discovery Studio 2.5 (Accelrys, San Diego, CA, USA). Two active site residues in CesA, F166 and F182, were mutated in silico to the positionally equivalent residues in CesB, V166 and Y182. The mutant enzyme model was energy minimized using the CHARMM forcefield (Brooks et al., 1983). Minimization was done using a dielectric constant of 1 and a non-bonded cutoff distance of 12 Å. The wild-type CesA structure was then refined using the same parameters to account for any differences in the modeled structures. The RMSD calculated for the minimized CesA crystal structure relative to the CesA crystal structure (Protein Data Bank accession code 2RI1) is 1.1 Å.

Substrates (R)-3 and (S)-3 were constructed and molecular docking simulations were performed in wild-type CesA, the structural model of the CesA mutant (F166V/F182Y), and the structural model of wild-type CesB using the grid-based approach CDocker (Mackerell et al., 1998; Erickson et al., 2004). All structures were further energy minimized using CHARMM. The enzyme was fixed and the atoms of the substrates (R-3 and S-3) were allowed to

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1 Joint Center for Structural Genomics, doi:10.2210/pdb2r11/pdb, to be published.
move. In subsequent rounds of minimization, the constraints on the amino acids forming the active site were removed and the structures containing the substrate were refined by energy minimization consisting of 100 steps of steepest descent followed by 500 iterations of the adopted basis–set Newton–Raphson algorithm using an energy tolerance of 0.01 k cal mol⁻¹ Å⁻¹.

3. Results

3.1. Rationale for mutagenesis

Carboxylesterase CesA can efficiently hydrolyse both esters of IPG while exhibiting very low enantioselectivity towards these substrates (Dröge et al., 2005). CesB, on the other hand, hydrolyses 3 with the desired preference towards the R-enantiomer (Dröge et al., 2005). In addition, CesA can hydrolyse 1, which is not accepted as substrate by CesB (Dröge et al., 2005). To identify residues influencing the substrate selectivity of these two carboxylesterases, the amino acid sequences of CesA and CesB were aligned (Fig. 2A). Examination of this alignment, guided by the crystal structure of CesA₁, suggested that most active site residues of CesA are conserved in CesB. However, two active site residues in CesA, F166 and F182, are replaced by V166 and Y182 in CesB. These active site differences may underlie the different substrate selectivities of CesA and CesB.

The proposed substrate selectivity residues were further examined by comparing the three-dimensional structures of CesA and CesB. As a structure of CesB is not available yet, a structural model of CesB was built by using the program Coot. The crystal structure of CesA (PDB accession code 2R11) was used as the template for homology modeling since CesA shares 60% sequence identity with CesB. This CesB model was compared to the available CesA structure (Fig. 2B), which indicates that V166 and Y182 in CesB are structurally equivalent to F166 and F182 in CesA. Docking experiments using the CesB model and substrate 3 further suggest that (for the most energetically favorable binding modes of the substrate) V166 interacts with the IPG moiety of 3, whereas Y182 is in close proximity to the aliphatic side chain of 3 (data not shown). Hence, these two presumed substrate-binding residues may contribute to the enantioselectivity of CesB. These observations suggest that changing F166 to a valine and F182 to a tyrosine in CesA could affect the enantioselectivity of this enzyme towards substrates 1 and 3.

3.2. Characterization of site-directed mutants of CesA

To investigate the importance of F166 and F182 for the activity and selectivity of CesA, four single-site and four double-site mutants were constructed (Table 1). The wild-type enzyme and the CesA mutants were overexpressed in E. coli BL21 (DE3) and purified to >90% homogeneity, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One of the double mutants constructed (F166A/F182A) showed no soluble expression and, hence, was excluded from further studies. The esterase activities of the CesA mutants were measured using p-nitrophenyl butyrate (pNP-C4 ester) as the substrate and compared to that of wild-type CesA (Table 2). Whereas the activities of mutants R1 and R3 are comparable to that of the wild-type enzyme, those of mutants R2, R5, R6, and R7 are somewhat lower than those obtained for CesA. Under the conditions of the assay, mutant R4 showed no detectable activity. Notably, the replacement of F182 by a tyrosine has no significant effect on activity, whereas the replacement of F182 by the shorter alanine completely abolishes the activity of CesA. This suggests that, at position 182, a residue with a large (aromatic) side chain is needed for optimal binding and/or hydrolysis of pNP-C4 ester. It is important to emphasize that mutating the residues F166 and F182 in CesA to the positionally conserved ones in CesB has only minor effects on the esterase activity level of CesA (mutants R1, R3 and R7 in Table 2).

Table 1

<table>
<thead>
<tr>
<th>Nomenclature of CesA mutants.</th>
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</thead>
<tbody>
<tr>
<td><strong>Definition</strong></td>
</tr>
<tr>
<td>Rational design</td>
</tr>
<tr>
<td>R1</td>
</tr>
<tr>
<td>R2</td>
</tr>
<tr>
<td>R3</td>
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<tr>
<td>R4</td>
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<tr>
<td>R5</td>
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<tr>
<td>R6</td>
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<tr>
<td>R7</td>
</tr>
<tr>
<td>R8</td>
</tr>
<tr>
<td>Directed evolution</td>
</tr>
<tr>
<td>D1</td>
</tr>
<tr>
<td>D2</td>
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<tr>
<td>D3</td>
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</table>

Table 2

<table>
<thead>
<tr>
<th>Specific activities of CesA wild-type and designed mutants towards the substrate pNP-C4. Specific activities were determined in 50mM phosphate buffer (pH 8) containing Triton X100 (0.35%, v/v) and gum Arabic (0.1%, w/v) at 30°C. ND, not determined.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme</strong></td>
</tr>
<tr>
<td>CesAwt</td>
</tr>
<tr>
<td>R1</td>
</tr>
<tr>
<td>R2</td>
</tr>
<tr>
<td>R3</td>
</tr>
<tr>
<td>R4</td>
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<tr>
<td>R5</td>
</tr>
<tr>
<td>R6</td>
</tr>
<tr>
<td>R7</td>
</tr>
</tbody>
</table>

To determine the effect of the mutations on the enantioselectivity of CesA, the E-values of the designed mutants were determined by performing kinetic resolution experiments with substrates 1 and 3 (Table 3). For substrate 1, only mutant R5 (F166V/F182A) showed a significant enhancement (~3-fold) in enantioselectivity when compared to wild-type CesA. The single mutant R1 (F166V) had no enhanced enantioselectivity towards 1, whereas the single mutant R2 (F182A) was not able to convert substrate 1 under the assay conditions. Taken together, this data suggests that the observed increase in enantioselectivity of mutant R5 towards 1 is due to the additive effect of both mutations. For substrate 3,
mutant R7 showed a ∼4-fold enhancement in enantioselectivity when compared to wild-type CesA (Table 3). The combination of mutations F166V and F182Y in mutant R7 resulted in an E-value that was higher than that of the single mutants, indicating that the effect of the mutations was again additive.

3.4. Enhancing enantioselectivity by directed evolution

The preceding results show that several mutations at positions F166 and F182 in CesA enhance the enantioselectivity of the enzyme towards substrates 1 and 3. To identify additional beneficial mutations, a focused saturation mutagenesis library was generated at these two positions. To reduce the screening effort, the library was prepared with the help of degenerate NNS primers, comprising 32 codon variants, which offers close to natural distributions for all amino acids while minimizing the occurrence of stop codons. The resulting library was first screened for active mutants exhibiting esterase activity. This was achieved by growing individual clones expressing the mutant genes in 96-wells plates, after which cells were washed, lysed and assayed for their ability to hydrolyse pNP-C4 ester. In this way, the more time-consuming screening for enantioselectivity towards 1 and 3 could be restricted to mutants that have significant esterase activity. Out of 824 mutants screened, 69 mutants had pronounced esterase activity. These active mutants were then screened for improved enantioselectivity by following the time course of the conversion of 1 by chiral GC analysis. In this way, three mutants were found of which the enantioselectivity for substrate 1 was potentially increased.

The three selected mutant genes were overexpressed in E. coli BL21. The corresponding proteins were purified to homogeneity (>90% as assessed by SDS-PAGE) and used to perform kinetic...
resolutions with racemic 1 and 3. The results indicate that the three mutant enzymes (D1-D3, Table 1) have increased enantioselectivities towards both substrates 1 and 3 when compared to wild-type CesA (Table 3). Both D1 and D3 are double mutants, containing a valine at position 166 (Table 1). Mutant D2 contains a single mutation (F182W). The enantioselectivities of the three evolved mutants, however, are, at best, comparable (substrate 3) or only slightly higher (substrate 1) than those of the initially designed mutants.

3.5. Kinetic analysis of the best CesA mutants

To establish whether the enhanced enantioselectivity of the best CesA mutants is caused by changes in binding affinity and/or turnover rate of the individual enantiomers, kinetic parameters for the enzyme-catalyzed conversion of (R)-1, (S)-1, (R)-3 and (S)-3 were determined (Tables 4 and 5). For substrate 1, the 13-fold enhanced enantioselectivity of the best mutant (D1) was due to a 4.6-fold increase in catalytic efficiency for (R)-1 combined with a 2.3-fold decrease in catalytic efficiency for (S)-1, when compared to wild-type CesA. The enhanced $k_{cat}/K_m$ for the preferred enantiomer is due to both a lower $K_m$ and a higher $k_{cat}$.

For substrate 3, the enhanced enantioselectivity of the best CesA mutant R7, which contains the CesB residues in positions 166 and 182, was mainly due a 2.3-fold increase in $K_m$ combined with a 4.7-fold decrease in $k_{cat}$ for (S)-3, resulting in a 9-fold decrease in $k_{cat}/K_m$. The catalytic efficiency for (R)-3 was lowered only ~2-fold in comparison to that of wild-type CesA. Although the $E$-values determined by kinetic parameters are slightly higher than the $E$-values determined by kinetic resolution, they demonstrate the enhancement of CesA variants enantioselectivity towards IPG esters.

### Table 4

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>$E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CesAwt</td>
<td>(R)-1</td>
<td>100 ± 42*</td>
<td>0.4 ± 0.09*</td>
<td>4.0*</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(S)-1</td>
<td>38.8 ± 5.6</td>
<td>0.15 ± 0.01</td>
<td>13.6</td>
<td>9</td>
</tr>
<tr>
<td>R5</td>
<td>(R)-1</td>
<td>8.8 ± 3.2</td>
<td>0.12 ± 0.03</td>
<td>15.6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(S)-1</td>
<td>31.9 ± 6.6</td>
<td>0.05 ± 0.004</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>(R)-1</td>
<td>33.7 ± 4.4</td>
<td>0.02 ± 0.04</td>
<td>18.4</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(S)-1</td>
<td>13.9 ± 2.6</td>
<td>0.02 ± 0.003</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>(R)-1</td>
<td>27.9 ± 2.3</td>
<td>0.42 ± 0.016</td>
<td>57.5</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>(S)-1</td>
<td>10.3 ± 2.9</td>
<td>0.03 ± 0.002</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>(R)-1</td>
<td>11.6 ± 2.5</td>
<td>0.25 ± 0.024</td>
<td>21.6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(S)-1</td>
<td>12.8 ± 2.1</td>
<td>0.04 ± 0.004</td>
<td>3.1</td>
<td></td>
</tr>
</tbody>
</table>

* Saturation with substrate was not achieved and the values reported are estimations obtained from curve fitting with the Grafit program.

### Table 5

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>$E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CesAwt</td>
<td>(R)-3</td>
<td>4.5 ± 1.2</td>
<td>0.04 ± 0.004</td>
<td>8.9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(S)-3</td>
<td>4.1 ± 0.4</td>
<td>0.02 ± 0.002</td>
<td>4.9</td>
<td>2</td>
</tr>
<tr>
<td>D2</td>
<td>(R)-3</td>
<td>5.4 ± 0.7</td>
<td>0.07 ± 0.004</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(S)-3</td>
<td>4.8 ± 0.6</td>
<td>0.01 ± 0.005</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>(R)-3</td>
<td>3.9 ± 0.5</td>
<td>0.09 ± 0.004</td>
<td>23.1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(S)-3</td>
<td>3.3 ± 0.2</td>
<td>0.02 ± 0.001</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>R7</td>
<td>(R)-3</td>
<td>4.2 ± 0.7</td>
<td>0.02 ± 0.001</td>
<td>4.8</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(S)-3</td>
<td>9.0 ± 1.9</td>
<td>0.005 ± 0.001</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

### 3.6. Characterization of wild-type CesA and mutant R7 by using molecular modeling

To further explain the enhanced enantioselectivity of mutant R7 (F166V/F182Y), the R and S enantiomers of 3 were modeled into the active site of this mutant and that of wild-type CesA. We analyzed the most favorable binding modes for both (R)-3 and (S)-3 in wild-type and mutant CesA with respect to the distance between the Oy of the catalytic nucleophile (S130) and the carbonyl carbon of the substrate. Notably, (R)-3 appears to bind in a favorable position for nucleophilic attack by S130 in the active site of both wild-type CesA and mutant R7, with an observed distance between the substrate carbonyl carbon and the Oy (catalytic serine) of 2.9 Å in both cases (Fig. 3A). Interestingly, whereas substrate (S)-3 cannot bind in an optimal orientation in the active site of wild-type CesA (3.2 Å distance between Oy and (S)-3 carbonyl carbon), its binding is unproductive in the active site of mutant R7 (Fig. 3B). The distance of 5.7 Å between Oy and the carbonyl carbon of the substrate in the energetically most favorable binding mode (Fig. 3B) is too far for nucleophilic attack. These observations may explain the reduced catalytic efficiency of mutant R7 towards this enantiomer and, hence, its increased enantioselectivity.

The mutation F166V contributes most to the enhanced enantioselectivity of R7. It is important to note that F166 seems to play an important role in substrate positioning. According to the models, this residue likely has more extensive hydrophobic interactions with (S)-3 than with (R)-3. These interactions are reduced upon mutation of F166 to a valine, causing (S)-3 to reorient quite dramatically, but without affecting the productive conformation of (R)-3 (Fig. 3B). Notably, the F182Y mutation alone has no significant effect on the enantiopreference of CesA (Table 3). However, this mutation seems to act in concert with F166V, as shown by the enhanced selectivity of R7 (F166V/F182Y), when compared to the single mutants R1 (F166V) and R3 (F182Y) (Table 3). This additive (or perhaps synergistic) effect is observed for substrate 3, but not for 1, and indicates that the residue (Tyr182) in mutant R7 (compared to the Phe182 in mutant R1) affects the spatial arrangement of the long alkyl chain of (S)-3, not allowing it to accommodate properly in the binding cleft of CesA mutant R7 (Fig. 3B).

### 4. Discussion

The current interest in industry in the use of esterases instead of lipases in organic synthesis processes is mainly due to the fact that esterases lack interfaced activation, exhibiting high activity towards soluble substrates. Several hydrolases have been previously screened for hydrolytic activity towards IPG esters (Liu et al., 2001; Monti et al., 2008; Jans et al., 1998) and some of these hydrolases showed activity towards IPG esters but with modest enantioselectivity. Lipase from the fungus Rhizopus oryzae showed the highest enantioselectivity (Ek = 11.3) towards IPG-butyrate and the carboxylesterase A from Bacillus coagulans was reported to have good enantioselectivity (Ek = 43) towards IPG-caprylate with low activity, however. Bacillus subtilis CesA was recently characterized as an esterase capable to convert IPG esters of different chain lengths with low enantioselectivity but with higher activity than its homologue CesB (Dröge et al., 2005). Making use of the available information concerning these two carboxylic esterases homologous
and the recent elucidation of CesA crystal structure, we decided to perform a structure-based approach to identify active site residues that could be responsible for CesA enantioselectivity. This approach led to the identification of two active site residues that to some extent, affected CesA enantioselectivity in the conversion of IPG esters. In this way we were able to generate two CesA variants which exhibit higher enantioselectivity than the wild-type enzyme towards these set of compounds, with a preference towards the R-enantiomer substrate. Although, the obtained E values of these variants may not be high enough for practical applications, these mutants are a good starting point for additional mutagenesis studies. Thus, to further optimize the enantioselectivity, other residues directly interacting with the substrate could be subjected to mutagenesis. A possible strategy would pass by applying the method of iterative saturation mutagenesis, a strategy recently described by Reetz and coworkers to improve enzyme enantioselectivity (Reetz and Carballera, 2007; Reetz, 2011), with the advantage of starting with a better template than the wild-type CesA and the possibility to combine with a selection strategy (Boersma et al., 2007). In addition, immobilization techniques may be applied to these CesA variants in order to increase their enantioselectivity or their stability (Mateo et al., 2007; Hernandez and Fernandez-Lafuente, 2011).

In summary, the enantioselectivity of carboxylesterase A from B. subtilis towards butyrate and caprylate esters of 1,2-O-isopropylideneglycerol has been improved up to 13-fold by structure-guided mutagenesis. Enhanced enantioselectivity could be obtained in various CesA variants without loss of catalytic activity. These results contribute to the understanding of mechanism underlying enantioselectivity and to the development of efficient biocatalysts that have potential for the kinetic resolution of IPG esters.

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References


