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Altering the Interfacial Activation Mechanism of a Lipase by Solid-Phase Selective Chemical Modification

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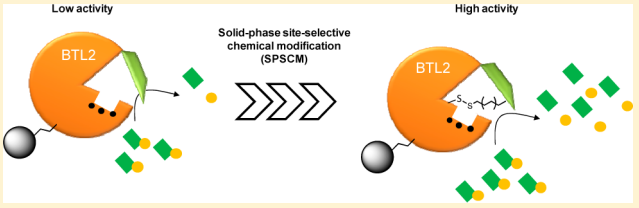
Supporting Information

ABSTRACT: This study presents a combined protein immobilization, directed mutagenesis, and site-selective chemical modification approach, which was used to create a hyperactivated semisynthetic variant of BTL2. Various alkane chains were tethered at three different positions in order to mimic the lipase interfacial activation exogenously triggered by detergents. Optimum results were obtained when a dodecane chain was introduced at position 320 by solid-phase site-selective chemical modification. The resulting semisynthetic variant showed a 2.5-fold higher activity than the wild-type nonmodified variant in aqueous conditions. Remarkably, this is the maximum hyperactivation ever observed for BTL2 in the presence of detergents such as Triton X-100. We present evidence to suggest that the endogenous dodecane chain hyperactivates the enzyme in a similar fashion as an exogenous detergent molecule. In this way, we also observe a faster irreversible enzyme inhibition and an altered detergent sensitivity profile promoted by the site-selective chemical modification. These findings are also supported by fluorescence studies, which reveal that the structural conformation changes of the semisynthetic variant are different to those of the wild type, an effect that is more pronounced in the presence of detergent. Finally, the optimal immobilized semisynthetic variant was successfully applied to the selective synthesis of oxiran-2-yl butyrate. Significantly, this biocatalyst is 12-fold more efficient than the immobilized wild-type enzyme, producing the S-enantiomer with higher enantiospecificity (ee = 92%).



The design of novel biocatalysts with new properties by tailoring their catalytic sites with non-natural molecules has been exploited in recent decades. Such techniques have allowed researchers to tune enzyme selectivity or specificity in order to better accommodate unnatural substrates.[1−3] This technology represents an alternative to the directed evolution of enzymes to improve their performances in nonconventional applications. The synthetic groups introduced in the protein core may act to modulate or enhance a desirable property of the enzyme in the same fashion as *in vitro* genetic evolution.[4]

The introduction of unnatural groups in the protein structure may be carried out in either a nonspecific or a specific approach. The nonspecific approach mainly involves modification of aspartate, glutamate, or lysine residues on the protein surface.[1] Most examples of random chemical modification involve the improvement of enzyme stability, although chemical modification may also be used to improve the activity and even the selectivity of an enzyme.[5] One limitation of this technique is that it is difficult to predict the effect of the chemical modifications, due to its unspecific nature. On the other hand, site-selective chemical modification allows researchers to fine-tune a protein by rational design. The main residue selected to chemically tether a synthetic molecule to a protein scaffold is cysteine due to the selectivity and versatility of the thiol chemistry. This approach is not suitable for proteins containing native cysteine that cannot be replaced since those mutations would drive to the inactivation or destabilization of the resulting variant. The incorporation of non-natural amino acids would be the alternative for such proteins.[6]

Luckily, the low abundance of cysteine in the protein primary sequences favors the extensive use of this residue for site-selective chemical modification.[7] However, the cysteine is not always located at the position required to tune the enzyme properties; therefore a combinatorial approach of site-selective chemical modification and protein engineering is often applied. The increase in availability of structural data for many enzymes has facilitated the rational design of novel semisynthetic biocatalysts.

A number of studies have been published in which immobilized enzymes have been site-selectively chemically modified with synthetic groups; however, few of these cases

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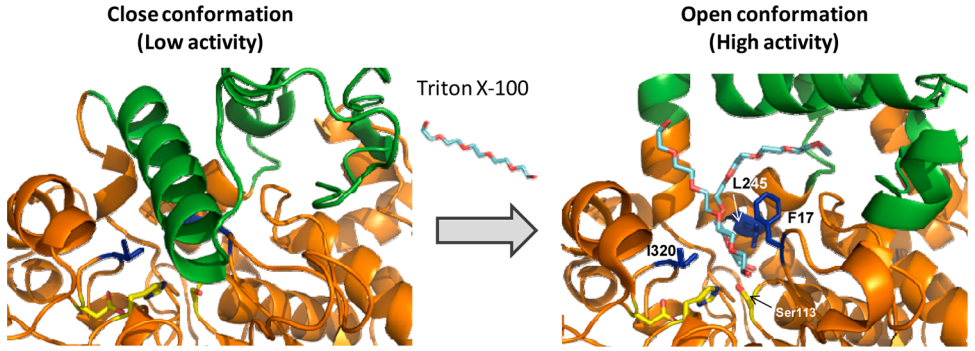

Figure 1. Interfacial activation of lipase 2 from *G. thermocatenolatus*. The figure shows the crystal structures of both open (PDB 2W22) and closed (model based on structure 1JI3) conformations of BTL2.[15] The interfacial activation in the presence of Triton X-100 promotes a large conformational change. Such structural movement mainly involves 64 residues (176−240) from the lipase lid (green). The remainder of the tertiary structure is mostly unaltered during the interfacial activation. In the open conformation, two molecules of Triton X-100 (cyan sticks) are found inside the binding cavity. The catalytic triad (S114, D318, H359) (yellow sticks) is located at the bottom of the substrate binding cavity. We propose three positions surrounding the catalytic triad (F17, L245, and I320 colored in blue sticks) to be mutated to cysteine residues in order to tether thiolated alkanes. Structures were analyzed and images created with Pymol 0.99v software.

involved modifications within the catalytic cavity.[8,9] Chemical modification in solid phase simplifies the modification protocol since the tedious dialysis steps are eliminated, avoiding protein inactivation by aggregation under the sometimes harsh chemical modification conditions. Moreover, once the immobilized enzyme is chemically modified, it is ready to be used as a heterogeneous biocatalyst.

Lipases have been extensively used as biocatalysts in industry for the last four decades thanks to their versatility, selectivity, and robustness.[10] They are serine/threonine hydrolases, as well as proteases, and have been successfully used as the template to create semisynthetic enzymes with improved properties.[11,12] The catalytic mechanism of the vast majority of lipases relies on an interesting structural reorganization known as interfacial activation.[13,14] In the presence of hydrophobic interfaces, an important structural domain, known as the "lid", undergoes a conformational change that exposes the catalytic cavity to the media, consequently increasing the lipase activity (Figure 1).[15−18] The equilibrium between closed and open conformations of the lid is dependent upon the media conditions. The lid acts to control access of the substrate to the lipase active center. In this study, we have used a lipase from *Geobacillus thermocatenulatus* (BTL2) to tune its performance by site-selective chemical modification. This lipase has recently been crystallized in its open form, revealing two molecules of Triton X-100 interacting with the inner face of the lid and thus stabilizing the open and more active conformation.[15] Using this open form structure as our model, we have selectively introduced a highly hydrophobic alkane chain to artificially freeze an open and highly active conformation of BTL2. The site-selective chemical modification has been carried out in solid-phase and different positions of the active center have been tested. The resulting semisynthetic heterogeneous biocatalysts have been applied to different industrially relevant reactions.

EXPERIMENTAL PROCEDURES

Materials. Cyanogen bromide 4B Sepharose was from GE Healthcare (Uppsala, Sweden). Triton X-100, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), dithiotreitol (DTT), *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl caprylate (*p*-NPC), *p*-nitrophenyl palmitate (*p*-NPP), 1-dodecanethiol, 1-octanethiol, *rac*-glycidol, methyl mandelate were purchased from Sigma Chem. Co (St. Louis, MO, USA). Other reagents were of analytical grade.

Bacterial Strains, Plasmids and Enzyme Expression. *Escherichia coli* strain DH5α (laboratory stock) was used for routine cloning procedures. Overproduction of BTL2 variants was carried out using BL21 (DE3) (laboratory stock). The *E. coli* strains were routinely cultured at 37 °C in Luria−Bertani (LB) broth using ampicillin (150 μg/mL) as a resistance marker. The overexpression and purification of BTL2 variants were carried out as previously described by Godoy et al.[19]

Site-Directed Mutagenesis of BTL2. All site-directed mutagenesis experiments were carried out by PCR using mutagenic primers. The resulting PCR products were further digested with endonuclease *Dpn*I to eliminate template DNA. Three single mutations, F17C, L245C, and I320C, were made using as template the plasmid pT1BTL2(WTCL), which codes for a BTL2 gene lacking two native cysteines.[19] The primer pairs for each mutation are described in Table S1, Supporting Information. The resulting mutated plasmids were validated by sequencing.

Enzymatic Activity Assays. Esterase activity of BTL2 variants was spectrophotometrically assayed using as substrates *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl caprylate (*p*-NPC), or *p*-nitrophenyl palmitate (*p*-NPP).[20] The substrate concentration was 0.25 mM in 25 mM sodium phosphate buffer at pH 7 and 25 °C.

Protein Immobilization on Cyanogen Bromide 4B Sepharose. The immobilization was carried out by adding 2 g of matrix to 20 mL of enzyme solution (5−60 U/mL) dissolved in 25 mM sodium phosphate buffer at pH 8. The suspension was maintained for 1 h with gentle stirring at 25 °C. Afterward, the support was filtered and washed with 25 mM sodium phosphate buffer at pH 8 and incubated for 2 h in 1 M ethanolamine at pH 8 to block the remaining cyanogen bromide groups. Finally, the immobilized preparation was washed with distilled water.

Solid-Phase Site-Selective Chemical Modification (SPSCM) of Monocysteine BTL2 Mutants. The immobilized preparation of each BTL2 variant was reduced in a 1:10 (w/v) ratio with a solution containing 5 mM DTT and 0.02% Triton X-100 at pH 8 and 25 °C for 30 min. Then, the derivatives were exhaustively washed, using vacuum filtration, with distilled water and finally equilibrated with 25 mM sodium

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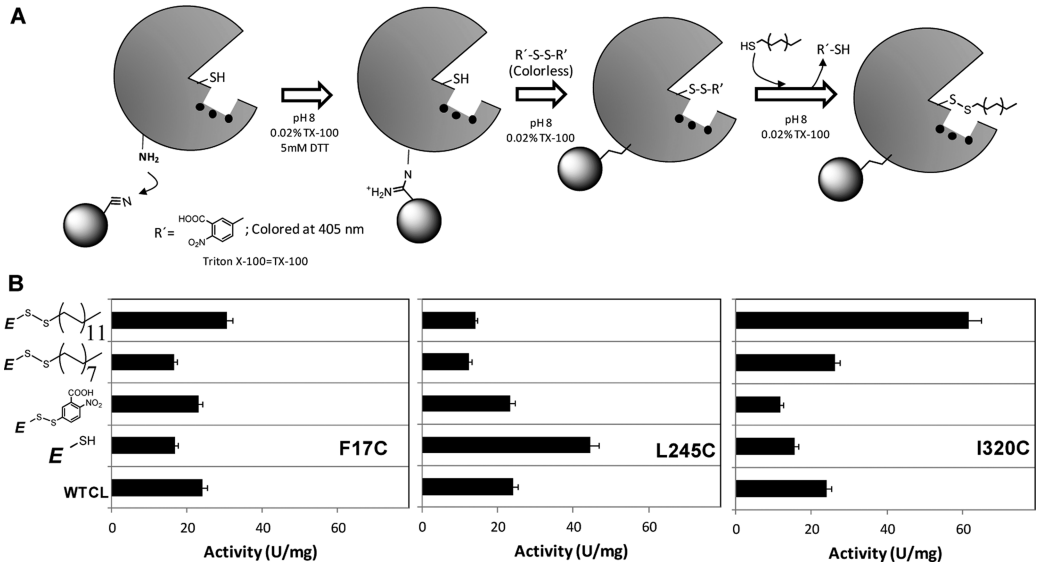

Figure 2. Solid-phase chemical modification of monocysteine BTL2 mutants with thiol-alkanes (A). The BTL2 mutant with one single cysteine at the active center is primarily immobilized through its N-terminus on agarose beads activated with cyanogen bromide groups (CNBr-Ag). The resulting immobilized protein is reduced in the presence of DTT in order to ensure that all the cysteine residues are in their fully reduced state. Then, the thiol group of the cysteine is activated with Ellman's reagent (DTNB) forming a disulfide bridge. This bond finally reacts with the thiol-alkane, leaving the protein modified with the non-natural group at its active site. All steps are carried out in presence of Triton X-100 to facilitate the access of thiolated synthetic group to the cysteine located at the active center. The modification in the solid phase enables a simpler protocol because washing steps may be carried out under vacuum filtration. Moreover, this methodology is quantitative since both activation and tethering processes release 2 mol of 5-mercapto-2-nitrobenzoid acid (colorimetrically monitored at 405 nm) per mol of cysteine modified. Hydrolytic activity of different BTL2 immobilized and site-selectively modified with different alkanes (B). Three different monocysteine mutants (F17C, L245C, and I320C) were first activated with DTNB and then tethered with two different thiol-alkanes, octadecane-1-thiol and dodecane-1-thiol. The effect of the synthetic group tethered to BTL2 on the hydrolytic activity depends on the location of the cysteine. BTL2 wild-type without cysteines (WTCL) immobilized on CNBr-Ag was used as a reference in all graphs. The hydrolytic activities were measured at 25 °C using 0.5 mM pNP-butyrate at pH 7 as substrate solution. One IU is defined as the hydrolysis of 1 μmol of pNPB per minute. Each point in the plot is the main value of a triplicate experiment, the standard deviation being no higher than 5% of the mean value.

phosphate at pH 8. The reduced derivatives were activated by thiol-exchange with a 1 mM DTNB solution prepared in 25 mM sodium phosphate and 0.02% Triton X-100 at pH 8 and 25 °C. This incubation was carried out with gentle stirring for 1 h. Afterward, the absorbance of the supernatants was measured in 96-well plates at 412 nm in order to determine the moles of 5-thiol-2-nitrobenzoic acid (ε = 14150 $M^{-1}\cdot cm^{-1}$) concomitantly released to the moles of cysteine activated with DNTB. Following the activation step, the immobilized samples were washed with distilled water and finally equilibrated in 17.5 mM Tris-HCl buffer with 0.02% Triton X-100 and 60% of acetonitrile. At this point, the immobilized and activated enzymes were ready to tether with the different alkane-thiols. A solution of 1 mM alkane-thiol (1-dodecanethiol or 1-octanethiol) was prepared in 17.5 mM Tris-HCl buffer with 0.02% Triton X-100 and 60% of acetonitrile at pH 8, and this solution was incubated with gentle stirring at 25 °C with the immobilized BTL2 variant already activated in a ratio 1:10 (w/v) for 2 h. A supernatant sample was withdrawn and spectrophotometrically measured at 412 nm to quantify the concomitant release of 5-thiol-2-nitrobenzoic acid to the activation of the cysteine with the corresponding alkanes. Finally, the BTL2 variants modified with the alkanes were exhaustively washed with distilled water and equilibrated with 25 mM Tris-HCl at pH 7. All samples were subsequently stored at 4 °C.

Determination of the Hyperactivation Profiles of BTL2 Variants. The activity of the immobilized derivatives was determined as previously described at different concentrations of Triton X-100 (0−1%) in 25 mM sodium phosphate at pH 7 and 25 °C.

Fluorescence Spectroscopy. Fluorescence measurements were performed in a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies), monitoring the intrinsic tryptophan fluorescence of immobilized BTL2 variants, using an excitation wavelength of 280 nm with excitation and emission bandwidths of 5 nm and recording fluorescence emission spectra between 300 and 500 nm. All spectroscopic measurements were made in 25 mM sodium phosphate at pH 7 with or without 0.02% Triton X-100.

Irreversible Inhibition of BTL2 Variants with Paraoxon. The activity of different immobilized BTL2 variants was measured in the presence of 1 mM of paraoxon in short intervals (100 s). The initial pNPB hydrolytic rate was determined for each short interval.

Kinetically Controlled Synthesis of *S*-Oxiran-2-yl Butyrate. Different immobilized BTL2 variants (0.1 g) were incubated with 2 mL of a reaction mixture consisting of 90% *rac*-glycidol in 10 mM Tris-HCl buffer and 50 mM methyl butyrate at pH 7. The reaction was carried out with gently stirring at 25 °C, and samples of supernatants were withdrawn at different times. The samples were analyzed by HPLC (Spectra Physic SP 100 coupled with a Diodos detector Spectra Physic SP 8450) using a Kromasil C8 column (15 cm ×0.46 cm) supplied by Análisis Vínicos (Spain). The mobile phase was 30% acetonitrile−70% water with 0.025% of acetic acid at pH 3.7. The analyses were performed at a flow of 0.7 mL/min by recording the absorbance at two wavelengths of 225 and 210

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nm. The enantiomeric excess (ee) of the different enantiomers was also determined by HPLC analysis on a chiral column (OD-H) using a mixture 98:2 hexane/isopropanol as mobile phase. The analyses were performed at a flow of 0.4 mL/min by recording the absorbance at 210 nm.

RESULTS

Chemical Modification of Immobilized BTL2 Monocysteine Mutants with Thioalkanes. Recent structural studies have revealed that the poly(ethylene oxide) chain of Triton X-100 molecules appears to stabilize the open conformation of BTL2 (Figure 1). This information enabled us to design semisynthetic lipases with medium-long aliphatic carbon chains tethered to their active sites, in an attempt to affect the lipase's interfacial activation mechanism in a similar fashion to detergent.[15] We therefore set out to create semisynthetic BTL2 variants with only one cysteine located at their active center. Three monocysteine BTL2 variants were created in this study, with a single cysteine located at positions 17, 245, and 320 (Figure 1). These positions surround the catalytic serine (S113) and are located higher in the active site cavity than the catalytic residues. Such active site architecture enabled us to utilize this unique position to tether alkane-thiols and therefore direct the open conformation of the active site lid. Additionally, we had previously created a BTL2 cysteine-less variant (WTCL),[18] which was shown to have retained activity. Therefore, using WTCL as template, residues Phe17, Leu245, and Ile320 were replaced by cysteine residues by directed mutagenesis to create the three monocysteine variants.

The monocysteine variants were chemically modified with two alkane-thiols, 1-dodecanethiol and 1-octanethiol. Before the chemical modification, the monocysteine variants were immobilized via their N-termini on CNBr-activated Sepharose gel (Figure 2A). The immobilized proteins were chemically modified in three steps: (1) reduction of the β-thiol of the cysteine, (2) activation of the reduced cysteine with DTNB, and (3) tethering of the alkane chain to the protein by thiol−disulfide exchange between the thiol group from the alkane and the disulfide bond from the protein. The process was carried out in the presence of Triton X-100 to maintain an open BTL2 conformation, thereby ensuring the accessibility of any reagent to the cysteine residue at the active center. The efficiency of the process could be determined because two molecules of 5-thiol-2-nitrobenzene were released per molecule of cysteine modified. The first molecule is released in the activation step, and the second molecule is released during the tethering step. 5-Thiol-2-nitrobenzene is colored and can be spectrophotometrically quantified. Derivatives loaded with 56 ± 4 nmol of I320C mutant per gram of support released 62 ± 4 mol of 5-thiol-2-nitrobenzene in both the activation and tethering steps. A similar observation was recorded for the F17C and L245C mutants. Therefore, the chemical modification of each of the monocysteine variants yielded an immobilized catalyst where one molecule of alkane was tethered to each lipase molecule.

Effect of the Directed Chemical Modification on the Lipase Activity. The specific activities in fully aqueous media of six different semisynthetic lipases tethering either 1-octanethiol (C_8) or 1-dodecanethiol (C_{12}) (F17C-C_8, L245C-C_8, I320C-C_8, F17C-C_{12}, L245C-C_{12}, I320C-C_{12}) were compared with those observed for the wild-type cysteine-less lipase (WTCL); for the monocysteine mutants with the free cysteine (nontethered) (F17C, L245C, and I320C), and for the monocysteine mutants activated with DTNB (F17C-DTNB, L245C-DTNB, and I320C-DTNB) (Figure 2B). Whereas the monocysteine variants F17C and I320C were slightly less active than WTCL, the L245C mutant had roughly 2-fold higher activity than WTCL. In the semisynthetic variants, the tethering position determined their final activities as summarized in Figure 2B. The introduction of alkanes at position 245 diminished the enzyme activity by more than 2-fold compared with L245C, whereas the introduction of alkanes at position 320 increased the specific activity of the resulting semisynthetic variants. I320C-C_{12} displayed 4- and 2.5-fold higher specific activity than its corresponding monocysteine mutant (I320C) and WTCL respectively. However, tethering of an octane molecule resulted in a specific activity that was only 1.6 times higher than the monocysteine mutant and similar to WTCL. On the other hand, in the case of position 17, tethering of a dodecane molecule resulted in a slightly higher specific activity compared with WTCL.

Michaelis−Menten constants for both immobilized WTCL and I320C-C_{12} toward the substrate *p*-nitrophenyl butyrate (pNPB) were calculated. K_m values of I320C-C_{12} and WTCL were 0.17 ± 0.07 and 0.36 ± 0.01 mM, respectively.

Effect of the Site-Selective Chemical Modification on the Response of BTL2 to Detergents. Figure 3 shows the lipase activity for each BTL2 variant at different detergent (Triton X-100) concentrations. The activity profile, with respect to the Triton X-100 concentration, was affected by both position and type of the chemical modification. Both the immobilized WTCL variant and the monocysteine variant F17C underwent a 2.7-fold hyperactivation. However, when 1-octanethiol was tethered to position 17, the resulting semisynthetic lipase was 4.5-fold hyperactivated. For position 245, the monocysteine mutant was 1.4-fold hyperactivated, resulting in a variant less sensitive to detergent. The sensitivity to detergent of this particular monocysteine mutant could be recovered by tethering an alkane-thiol, an effect that appeared to be independent of carbon chain length. In the case of position 320, the mutant I320C, in contrast to L245C, underwent a hyperactivation of 3.9-fold. However, when this variant was modified with alkanes, the lipase became less sensitive to detergent, and this insensitivity was more pronounced with longer alkanes (I320C-C_{12}) (Figure 3).

The irreversible inhibition of the different BTL2 variants is summarized in Figure 4 and Table S2, Supporting Information. Among the semisynthetic lipases, I320C-C_{12} was the only variant in which the inhibition rate did not increase when Triton X-100 was added to the reaction (Table S2, Supporting Information). As can be seen in Figure 4, the inhibition of the I320C-C_{12} variant was consistent, regardless of the presence of detergent in the inhibition media, while the inhibition rate for WTCL increased in the presence of Triton X-100, as one might expect.[15]

Fluorescence Studies of Immobilized I320C-C_{12} and WTCL Preparations. To better understand the effect of the introduced C_{12} aliphatic chain on the interfacial activation of semisynthetic BTL2, we carried out fluorescence analysis of the immobilized I320C-C_{12} and WTCL variants. Fluorescence was measured under two conditions: (1) in buffer solution, in which the lid of the lipase is expected to shield the active site and (2) in a 0.02% Triton X-100 solution in which we expect the lipase to be interfacially activated and therefore the catalytic residues to be exposed to the media (further details are described in the Experimental Procedures). Since the excitation wavelength used for the fluorescence spectroscopy was 280 nm, the

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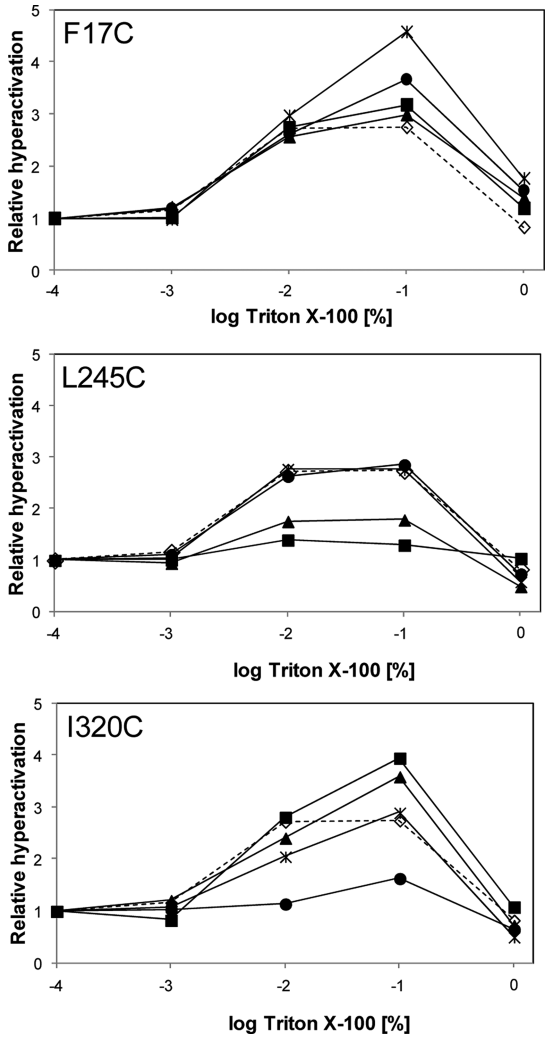

Figure 3. Hyperactivation profiles of different BTL2 variants immobilized and site-specifically modified with different alkanes. The hyperactivation profiles of different BTL2 variants were evaluated by increasing the Triton X-100 concentration in the reaction media. A higher concentration of detergent promotes an increase in hydrolase activity due to the underlying interfacial activation of the enzyme. Each monocysteine BTL2 mutant is indicated in the upper left corner of each graph. The traces represent untethered monocysteine mutant (■), monocysteine mutant activated with DTNB (▲), and monocysteine mutant tethered to octane-1-thiol (×) or dodecane-1-thiol (●). BTL2 cysteineless (WTCL) was plotted as a reference (◇, – – –). The hydrolytic activities were measured at 25 °C using 0.5 mM pNP-butyrate at pH 7 as substrate solution and different concentrations of Triton X-100 (0.001–1%). Each point in the plot is the main value of a triplicate experiment, the standard deviation being no higher than 5% of the mean value.

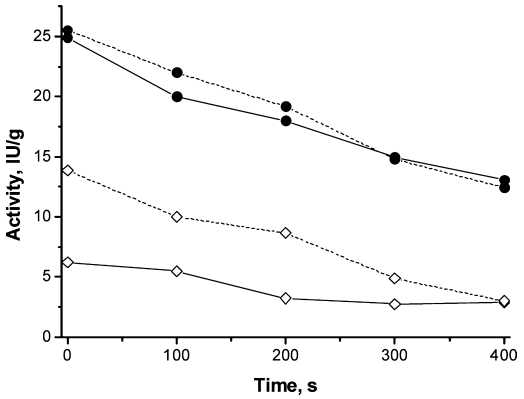

Figure 4. Irreversible inhibition of immobilized I320C-C_{12} (●) and WTCL (◇) under different conditions. The immobilized preparations of the different BTL2 variants were incubated with 1 mM paraoxon (irreversible inhibitor of serin/threonine hydrolases). The hydrolytic rate was monitored over time in the presence of the inhibitor. The rates were calculated in 100 s intervals. The irreversible inhibition was determined without (—) and with 0.1% Triton X-100 (– – –) at pH 7 and 25 °C. One IU is defined as the hydrolysis of 1 μmol of pNPB per minute. Each point in the plot is the main value of a triplicate experiment, the standard deviation being no higher than 5% of the mean value.

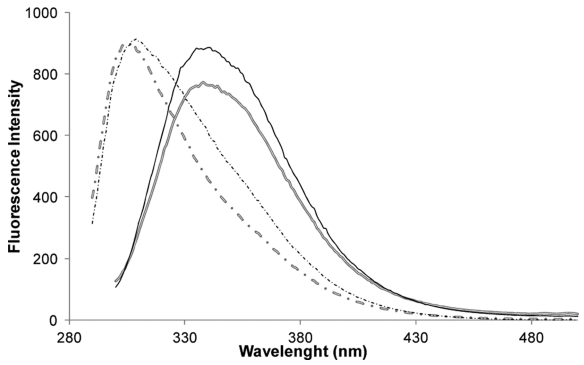

Figure 5. Fluorescence spectra of immobilized WTCL and I320C-C_{12} under different conditions The fluorescence spectra were recorded without Triton X-100 (gray solid line for the WTCL and black solid line for I320C-C_{12}) and with 0.02% Triton X-100 (dashed gray line for the WTCL and dashed black line for I320C-C_{12})). Fluorescence intensity of I320C-C_{12} does not vary upon addition of Triton X-100 to the reactions, while the fluorescence intensity of WTCL does. In the case of the λ_{max}, there was a more significant shift observed for WTCL than for I320C-C_{12}. These fluorescence data suggest that the insertion of a dodecane chain into the binding pocket of BTL2 reduced the structural rearrangements induced by detergents and measured by fluorescence spectroscopy.

differences in fluorescence are due mainly to any conformational changes in Trp9 and Tyr19 residues under the different measurement conditions.

The corresponding fluorescence emission spectra for both immobilized I320C-C_{12} and WTCL variants under different media conditions are shown in Figure 5. The detergent (Triton X-100) had a different effect on the fluorescent behavior of each variant. The WTCL variant exhibited higher total fluorescence intensity in the presence of detergent than when Triton-X100 was not in the media. However, the fluorescence spectrum of I320C-C_{12} was unaffected by the presence or lack of detergent. The wavelength for the maximum fluorescence emission (λ_{max}) of immobilized WTCL variant was shifted from 338 to 304 nm in presence of the detergent; representing a 34 nm shift. However, in the case of the immobilized I320C-C_{12} variant, the detergent promoted a smaller shift, 26 nm, of its λ_{max} (Table S3, Supporting Information).

Reversibility of Site-Directed Chemical Modification. We studied different conditions to optimally remove the tethered group and confirmed the efficiency of its removal by analyzing the lipase activity of the immobilized I320C-C_{12} at different detergent concentrations. After incubation of immobilized I320C-C_{12} with 50 mM DTT and 0.2% Triton X-100, the hyperactivation profile of the immobilized derivative was

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similar to that of the nonmodified I320C variant (Figure 6). However, lower concentrations of both reducing agent and

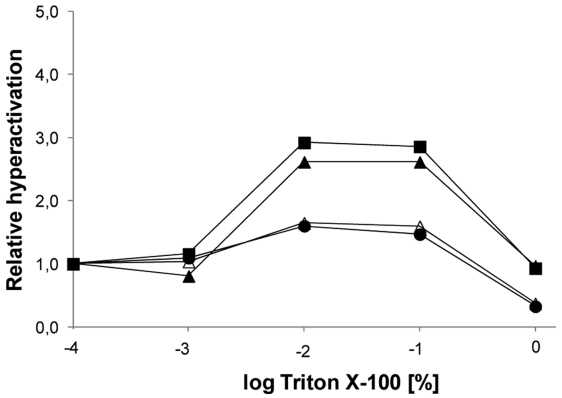

Figure 6. Hyperactivation profile of monocysteine I320C mutant unmodified and modified with dodecane-1-thiol incubated with reducing agents. The immobilized semisynthetic variant I320C-C_{12} was incubated for 24 h in buffer 25 mM sodium phosphate at pH 8 (●), in 1 mM DTT, 0.01% Triton X-100 dissolved in 25 mM sodium phosphate at pH 8 (△), or in 50 mM DTT, 0.2% Triton X-100 dissolved in 25 mM sodium phosphate at pH 8 (▲). The immobilized monocysteine mutant I320C (■) is plotted as a reference. The chemical modification only could be reverted when the semisynthetic variant was incubated with both 0.2% Triton X-100 and 50 mM DTT. Each point in the plot is the main value of a triplicate experiment, the standard deviation being no higher than 5% of the mean value.

detergent were not sufficient to reduce the disulfide bridge between the Cys320 and the dodecane chain. This result indicates that the disulfide bond between the alkane and the protein only can be broken if the lipase active site is accessible and if the environment is sufficiently reduced.

Substrate Specificity toward Different Aliphatic Esters with Differing Chain Length. Activity of the semisynthetic lipase I320C-C_{12} and WTCL were assayed in the presence of different *p*-nitrophenol esters in a very hydrophilic media. A low concentration of the esters (0.25 mM) was used in order to avoid any interfacial activation effect induced by either the substrate or the media. Table 1 summarizes the observed activities for each lipase variant toward each ester. The hydrolytic activity of the semisynthetic lipase was reduced from 4.2-fold higher than WTCL toward a short-chain ester like pNPB to 3.1-fold lower than WTCL toward a long-chain ester like p-NPP. Therefore, the C_{12} chain tethered to the lipase

Table 1. Relative Activity of WTCL and I320C-C_{12} Variants toward Different p-NP Esters with Different Side Chain Lengths

	relative activity[a]		
enzyme derivative	pNP-butyrate (C_4)	pNP-caprylate (C_8)	pNP-palmitate (C_{16})
WTCL[b]	1	0.19	0.03
I320C-C_{12}[b]	4.2	0.12	0.01

[a]The relative activity is defined as the activity of each sample divided by the activity of the WTCL derivative toward p-NP butyrate (C_4). [b]All the enzymes (tethered and nontethered with synthetic groups) were used in their immobilized forms. The relative activities are the mean value of three separate experiments with standard deviation no higher than 5%.

active site may be causing steric hindrances that reduce its hydrolytic activity toward long-chain esters when compared with the activities observed for the WTCL variant.

Application of the Immobilized Semisynthetic Catalyst I320C-C_{12} to Different Biotransformations. The semisynthetic lipase I320C-C_{12} was used as a heterogeneous biocatalyst in two industrially relevant kinetic resolutions of secondary alcohols by hydrolysis of *rac*-1-phenyl acetate **5** (Table S4, Supporting Information) and synthesis of oxiran-2-yl butyrate **3** (Table 2). Activity and selectivity of the catalyst were analyzed for both biotransformations. In the case of the hydrolysis of **5**, the insertion of the C_{12} chain yielded a more active biocatalyst; however it lacked selectivity (Table S4, Supporting Information). In contrast, for the synthesis of *S*-(**3**), the semisynthetic I320C-C_{12} variant displayed excellent catalytic behavior, being 12-fold more active and more enantioselective than the immobilized WTCL variant. Immobilized I320C-C_{12} synthesized preferentially *S*-(**3**) with an enantiomeric excess of 92%. Moreover, because of the kinetically controlled character of the reaction, the semisynthetic lipase presented a roughly 6 times higher synthesis/hydrolysis ratio than WTCL. Therefore, the synthase activity was improved relative to the esterase activity by introducing the alkane chain in the active center.

■ DISCUSSION

Solid-Phase Site-Selective Chemical Modification (SPSCM) as a Tool To Alter Enzymatic Mechanisms. Altering enzymes to improve their catalytic properties is one of the current demands in biocatalysis.[2,3] In this study, we have established a robust procedure to site-selectively modify the active center of enzymes with non-natural chains in order to improve their catalytic properties. We have successfully developed and optimized a highly efficient protocol for solid-phase site-selective chemical modification (SPSCM) (Figure 2A). This approach enables the straightforward *in vitro* production of a heterogeneous semisynthetic, selectively modified biocatalyst in only four steps: immobilization, reduction, activation, and site-selective tethering (Figure 2A).

Mimicking the Natural Interfacial Activation of Lipases by SPSCM. The interfacial activation of BTL2, as well as other lipases,[16−18] is induced by hydrophobic interfaces (oil−water mixture, organic solvents), by hydrophobic surfaces,[21] or by detergents.[22] In the case of detergents (e.g., Triton X-100), adding them to the media drives the lipase to an open conformation resulting in a hyperactivated enzyme (Figure 1). This hyperactivation is dependent upon the detergent concentration in the media.[22,23] This response of lipase activity to Triton X-100 concentration is known as the hyperactivation profile, and the hyperactivation factor can be described as the ratio between the activity at a given detergent concentration and the activity without detergent. Hyperactivation factors higher than 1 result in lipase activation, while hyperactivation factors lower than 1 result in lipase inhibition.

The open 3D structure of BTL2 revealed two molecules of Triton X-100 bound to its active center. Using this information, we have mimicked the action of the detergent molecules by selectively tethering alkane chains to specific positions of an immobilized BTL2 preparation. The effect of the selective chemical modification is reliant not only upon the position to which the synthetic group is tethered but also upon the nature of the group. The highest increase in activity was obtained by

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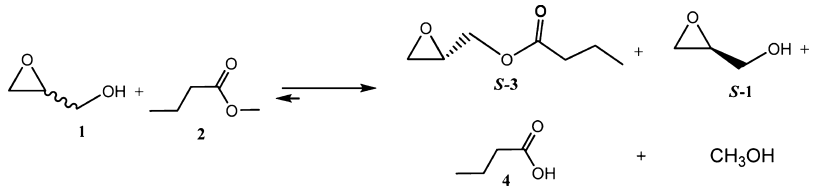
Table 2. Kinetically Controlled Synthesis of *S*-Oxiran-2-yl Butyrate[a]



	V_{TOT}[b] (U/mg)	V_S[c] (IU/mg)	V_H[d] (IU/mg)	V_S/V_H	ee$_{(3)}$ (%) (*S*)[e]
WTCL	0.032	0.028	0.004	7	79
I320C-C_{12}	0.34	0.33	0.008	41.25	92

[a]The molecules depicted in the reaction are (**1**) glycidol, (**2**) methyl butyrate, (**3**) oxiran-2-yl butyrate, and (**4**) butyric acid. [b]V_{TOT} = total velocity defined as the sum of both hydrolysis and synthesis velocity. [c]V_S = synthesis rate where 1 IU is the production of 1 μmol of **3** per minute. [d]V_H = hydrolysis rate where 1 IU is the production of 1 μmol of **4** per minute. [e]ee$_{(3)}$ (enantiomeric exces of **3**) (%) = $\{([S_3] - [R_3])/([S_3] + [R_3])\} \times 100$. The numbers in the table are the mean value of three separate experiments with a standard deviation no higher than 5%. All the enzymes (tethered and nontethered with synthetic groups) were used in their immobilized forms. The reaction conditions were pH 7 and 25 °C (more details are described in Experimental Procedures).

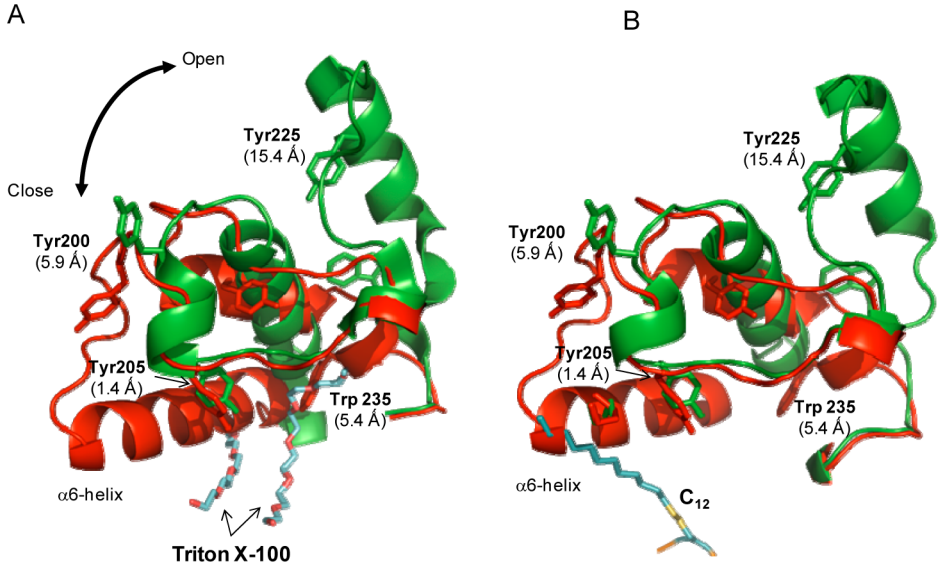


Figure 7. Underlying structural changes in WTCL and I320C-C_{12} variants during the interfacial activation mechanism. The structural rearrangement of the lid during the transition from the closed (red) to the open (green) conformation mainly involves the residues Tyr200, Tyr205, Tyr225, and Trp235. The dragging distance for each residue is indicated between parentheses. These aromatic residues are located in the lid of BTL2 (176−240). The spatial positions of the remaining tryptophans and tyrosines forming part of the primary sequence of BTL2 do not change noticeably during the structural transition triggered by the interfacial activation (see Figure S1, Supporting Information). (A) Conformational change exogenously induced by Triton X-100 (cyan). Detergent molecules enable the displacement of the α6-helix to unveil the binding site. (B) Structural model of semisynthetic I320C-C_{12} variant adopting an open conformation. The dodecane chain (cyan) is not expected to be located at the same position as detergent molecules. Hence, the tertiary structure adopted by the chemically modified enzyme will likely be different from the unmodified protein structure in presence of detergents. More details about the structural model of the semisynthetic I320C-C_{12} are provided in the Supporting Information.

inserting a C_{12} aliphatic chain at position 320. The resulting immobilized variant I320C-C_{12} was 2.5-fold more active than the immobilized WTCL in the absence of detergents (Figure 2B). Remarkably, the maximum hyperactivation factor found for the WTCL lipase has been 2.5 in presence of 0.01% Triton X-100. These data suggest that the alkane chain promotes a similar hyperactivation to that promoted by Triton X-100. In functional terms, the tethering of the C_{12} chain at position 320 of BTL2 may be driving the lipase to an open conformation, in a similar effect to that observed for the wild-type variant in presence of detergent. The higher activity observed by insertion of an alkane chain at the active center of BTL2 is also supported by the lower apparent K_m values found for I320C-C_{12} variant toward pNPB. Hence, the 2-fold lower K_m determined for I320C-C_{12} suggests a higher substrate affinity of the chemically modified active site than its native counterpart. Furthermore, this semisynthetic variant was significantly insensitive to the hyperactivation triggered by detergents. Interestingly, such insensitivity to Triton X-100 may be associated with the higher specific activity in fully aqueous media.

On the other hand, immobilized I320C-C_{12} was irreversibly inhibited much more rapidly than the unmodified enzymes. The inhibitor would have better access to the catalytic serine of those enzymes whose active centers were more accessible to the media, as would be the case of the WTCL in the presence of Triton X-100 and the variant I320C-C_{12} under any condition. For this reason, the inhibition rate of the semisynthetic variant was not affected by the detergent, suggesting once again a more

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accessible active site in the site-selectively chemically modified enzyme.

Some Light on the Structural Rearrangements Induced by the Site-Selective Chemical Modification. The structural rearrangements induced by the interfacial activation of different immobilized BTL2 variants have been determined by monitoring their intrinsic fluorescence. Interfacial activation conditions were simulated by performing the fluorescence spectroscopy in the presence of 0.02% Triton X-100. X-ray crystallographic studies of soluble BTL2 with and without Triton X-100 have shown how the detergent induces a conformational change that mainly involves the lid region and drives the lipase to be hyperactivated (interfacial activation mechanism).[15] As one might expect, a similar conformational change was also observed for immobilized preparations of WTCL lipase through fluorescence spectroscopy. Low concentrations of Triton X-100 increase the maximum intrinsic fluorescence, and the λ_{max} is shifted to lower wavelengths. These spectroscopic changes are most likely due to the different exposures of mainly Tyr200, Tyr205, Tyr225, and Trp235 (lid region) in the open conformation compared with the closed conformation (Figure 7A). Nevertheless, in the case of the immobilized I320C-C_{12} variant, the intrinsic fluorescence was not affected by detergent, and the resulting λ_{max} shift is smaller. These data suggest that (1) the insertion of a C_{12}-alkane chain in the BTL2 active center modifies the tertiary structure of the enzyme in the absence of detergents and (2) the detergents trigger different structural rearrangements in the chemically modified enzyme compared with the unmodified and immobilized WTCL variant. If this was the case, it would also explain the higher activity (under aqueous conditions), the more rapid inhibition, and the lower detergent sensitivity (in terms of activity) observed for the chemically modified lipase (I320C-C_{12}).

Hence, the insertion of C_{12}-alkane chain at position 320 of BTL2 is likely mimicking the role of detergent molecules in the lipase active center. However, it would be unlikely that the dodecane chain would adopt the same position and conformation inside the BTL2 binding pocket as the Triton X-100 molecules, because both are chemically and sterically different. Nevertheless, the alkane chain could interact with the hydrophobic face of the lid forcing a pseudo-open conformation, in the same way as alkane chains (butyl, octyl, or decaoctyl) on the hydrophobic support surfaces trigger the interfacial activation of the lipase [21] by interaction with the hydrophobic face of the "lid".

In light of these functional and structural insights, we propose a molecular model of the semisynthetic BTL2 modified with a C_{12} chain located at position 320 (Figure 7B). This model reveals how the alkane chain might be located in the hydrophobic cavity where α6 helix would be accommodated in the closed conformation of the lipase (Figure 7B and Figure S2, Supporting Information). Hence, the C_{12} chain tethered to the active site could act as a "door stopper", making the structural transition from the open to the closed conformation difficult. In this scenario, the lipase would present a pseudo-open conformation stabilized by molecular interactions between the alkane chain and the amino acids in the binding cavity. This less shielded conformation would explain both the high activity, and the faster irreversible inhibition found in fully hydrophilic media where interfacial activation would normally not be possible. Therefore, the more active lipase conformation would be endogenously stabilized by the site-selective chemical modification rather than exogenously triggered by a hydrophobic substrate, an organic solvent, a detergent, or a hydrophobic surface.[14,15,20] (Figure 7)

Biotechnological Applications of the Immobilized I320C-C_{12} Variant. The chemical modification was shown to be reversible by adding both reducing agents and detergents to the media. The disulfide bond that tethers the dodecane thiol to the single cysteine is located in the vicinity of the catalytic triad; therefore the chemical modification could theoretically be reverted by simply incubation the semisynthetic lipase in reducing media. Detergent was required to ensure an open lipase conformation that would enable access of DTT molecules to the active center where the disulfide bridge is located. In this system, the chemical modification is reversible unlike other site-selective modification protocols where resulting bonds between the protein and the synthetic group are irreversible.[24−26] The reversibility of the site-directed chemical modification of immobilized enzymes would allow modification of the properties of the biocatalyst by the simple addition of reducing agents and detergents to the reaction media. This feature, together with the insoluble nature of the final preparation of the catalyst, is a highly valuable asset in modern biocatalysis because they provide the user with "the functional switch". Therefore, the reversible nature of this bond is most certainly an advantage. Moreover, this technology also enables the easy transformation of the disulfide bond to an irreversible thioeter bond, thereby allowing the user to create an irreversible semisynthetic biocatalyst when the case requires.[24] This solid-phase site-selective chemical modification may be also envisioned using maleimide or α-halocarbonyl-containing compounds that are able to directly react with cysteine forming an irreversible bond that cannot be cleaved under reducing conditions.[7]

Finally, we have evaluated the biotechnological potential of this semisynthetic enzyme in the synthesis of glycidyl esters. We found that our biocatalyst was more efficient and selective than the unmodified lipase. We have been able to alter the substrate selectivity of the semisynthetic lipase in a similar fashion to other semisynthetic proteins.[27−29] Moreover, this is one of the few examples where the rationalization of single site-selective chemical modification of an enzyme also improves its enantioselectivity.[27] This work therefore presents a completely novel strategy to rationally modify the catalytic mechanism of BTL2, and the resulting heterogeneous semisynthetic biocatalyst can be applied to an industrially relevant biotechnological process.[30] This is the first example of the manipulation of the interfacial activation mechanism of an immobilized lipase by a tailor-made chemical modification.

■ ASSOCIATED CONTENT

Supporting Information

Information about the primers used for directed mutagenesis to create the monocysteine variants of BTL2, inhibition constants of different immobilized BTL2 preparations tethered to different alkanes and untethered, maximum emission fluorescence peaks of immobilized WTCL and I320C-C_{12} variants, different parameters about the enzymatic hydrolysis of *rac*-1-phenyl acetate, and *in silico* structural studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

BTL2, lipase 2 from *Geobacillus thermocatenulatus*; SPSCM, solid-phase site-selective chemical modification

REFERENCES

(1) Davis, B. G. (2003) Chemical modification of biocatalysts. *Curr. Opin. Biotechnol. 14*, 379−386.

(2) Diaz-Rodriguez, A., and Davis, B. G. (2011) Chemical modification in the creation of novel biocatalysts. *Curr. Opin. Chem. Biol. 15*, 211−219.

(3) Sletten, E. M., and Bertozzi, C. R. (2009) Bioorthogonal chemistry: Fishing for selectivity in a sea of functionality. *Angew. Chem., Int. Ed. 48*, 6974−6998.

(4) Bommarius, A. S., Blum, J. K., and Abrahamson, M. J. (2011) Status of protein engineering for biocatalysts: How to design an industrially useful biocatalyst. *Curr. Opin. Chem. Biol. 15*, 194−200.

(5) Palomo, J. M., Fernández-Lorente, G., Guisán, J. M., and Fernández-Lafuente, R. (2007) Modulation of immobilized lipase enantioselectivity via chemical amination. *Adv. Synth. Catal. 349*, 1119−1127.

(6) Hoesl, M. G., and Budisa, N. (2012) Recent advances in genetic code engineering in *Escherichia coli*. *Curr. Opin. Biotechnol.*, DOI: 10.1016/j.copbio.2011.12.027.

(7) Chalker, J. M., Bernardes, G. J. L., Lin, Y. A., and Davis, B. G. (2009) Chemical modification of proteins at cysteine: Opportunities in chemistry and biology. *Chem.—Asian J. 4*, 630−640.

(8) Godoy, C. A., de las Rivas, B., Filice, M., Fernández-Lorente, G., Guisan, J. M., and Palomo, J. M. (2010) Enhanced activity of an immobilized lipase promoted by site-directed chemical modification with polymers. *Process Biochem. 45*, 534−541.

(9) Gutarra, M. L. E., Romero, O., Abian, O., Torres, F. A. G., Freire, D. M. G., Castro, A. M., Guisan, J. M., and Palomo, J. M. (2011) Enzyme surface glycosylation in the solid phase: Improved activity and selectivity of *Candida antarctica* lipase B. *ChemCatChem 3*, 1902−1910.

(10) Jaeger, K. E., and Reetz, M. T. (1998) Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol. 16*, 396−403.

(11) DeSantis, G., Berglund, P., Stabile, M. R., Gold, M., and Jones, J. B. (1998) Site-directed mutagenesis combined with chemical modification as a strategy for altering the specificity of the S1 and S1′ pockets of subtilisin *Bacillus lentus*. *Biochemistry 37*, 5968−5973.

(12) Plettner, E., DeSantis, G., Stabile, M. R., and Jones, J. B. (1999) Modulation of esterase and amidase activity of subtilisin *Bacillus lentus* by chemical modification of cysteine mutants. *J. Am. Chem. Soc. 121*, 4977−4981.

(13) Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S. A., and Thim, L. (1991) A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature 351*, 491−494.

(14) Derewenda, Z. S., and Sharp, A. M. (1993) News from the interface: The molecular structures of triacylglyceride lipases. *Trends Biochem. Sci. 18*, 20−25.

(15) Carrasco-López, C., Godoy, C., de las Rivas, B., Fernández-Lorente, G., Palomo, J. M., Guisán, J. M., Fernández-Lafuente, R., Martínez-Ripoll, M., and Hermoso, J. A. (2009) Activation of bacterial thermo alkalophilic lipases is spurred by dramatic structural rearrangements. *J. Biol. Chem. 284* (287), 4365−4372.

(16) Brzozowski, A. M., Savage, H., Verma, C. S., Turkenburg, J. P., Lawson, D. M., Svendsen, A., and Patkar, S. (2000) Structural origins of the interfacial activation in *Thermomyces (Humicola) lanuginosa* lipase. *Biochemistry 39* (49), 15071−15082.

(17) Martinelle, M., Holmquist, M., and Hult, K. (1998) On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim. Biophys. Acta, Lipids Lipid Metab. 1258* (1253), 1272−1276.

(18) Verger, R. (1997) 'Interfacial activation' of lipases: Facts and artifacts. *Trends Biotechnol. 15* (11), 32−38.

(19) Godoy, C. A., Rivas, B. D., Grazu, V., Montes, T., Guisan, J. M., and Lopez-Gallego, F. (2011) Glyoxyl-disulfide agarose: A tailor-made support for site-directed rigidification of proteins. *Biomacromolecules 12*, 1800−1809.

(20) Fernandez-Lorente, G., Godoy, C. A., Mendes, A., Lopez-Gallego, F., Grazu, V., de las Rivas, B., Palomo, J., Hermoso, J., Fernandez-Lafuente, R., and Guisan, J. (2008) Solid-phase chemical amination of a lipase from *Bacillus thermocatenulatus* to improve its stabilization via covalent immobilization on highly activated glyoxyl-agarose. *Biomacromolecules 9* (9), 2553−2561.

(21) Fernandez-Lorente, G., Cabrera, Z., Godoy, C., Fernandez-Lafuente, R., Palomo, J. M., and Guisan, J. M. (2008) Interfacially activated lipases against hydrophobic supports: Effect of the support nature on the biocatalytic properties. *Process Biochem. 43*, 1061−1067.

(22) Fernandez-Lorente, G., Palomo, J. M., Cabrera, Z., Fernandez-Lafuente, R., and Guisán, J. M. (2007) Improved catalytic properties of immobilized lipases by the presence of very low concentrations of detergents in the reaction medium. *J. Mol. Catal. B: Enzym. 10* (14), 385−393.

(23) Mogensen, J. E., Sehgal, P., and Otzen, D. E. (2005) Activation, inhibition, and destabilization of *Thermomyces lanuginosus* lipase by detergents. *Biochemistry 44*, 1719−1730.

(24) Bernardes, G. J. L., Grayson, E. J., Thompson, S., Chalker, J. M., Errey, J. C., El Oualid, F., Claridge, T. D. W., and Davis, B. G. (2008) From disulfide- to thioether-linked glycoproteins. *Angew. Chem., Int. Ed. 47*, 2244−2247.

(25) Koçer, A., Walko, M., Meijberg, W., and Feringa, B. L. (2005) Chemistry: A light-actuated nanovalve derived from a channel protein. *Science 309*, 755−758.

(26) Schierling, B., Noël, A. J., Wende, W., Hien, L. T., Volkov, E., Kubareva, E., Oretskaya, T., Kokkinidis, M., Römpp, A., Spengler, B., and Pingoud, A. (2010) Controlling the enzymatic activity of a restriction enzyme by light. *Proc. Natl. Acad. Sci. U.S.A. 107*, 1361−1366.

(27) Ivarsson, Y., Norrgård, M. A., Hellman, U., and Mannervik, B. (2007) Engineering the enantioselectivity of glutathione transferase by combined active-site mutations and chemical modifications. *Biochim. Biophys. Acta, Gen. Subj. 1770*, 1374−1381.

(28) Metanis, N., Keinan, E., and Dawson, P. E. (2005) A designed synthetic analogue of 4-OT is specific for a non-natural substrate. *J. Am. Chem. Soc. 127*, 5862−5868.

(29) Nakatsuka, T., Sasaki, T., and Kaiser, E. T. (1987) Peptide segment coupling catalyzed by the semisynthetic enzyme thiolsubtilisin. *J. Am. Chem. Soc. 109*, 3808−3810.

(30) Lozano, P., De Diego, T., Carrié, D., Vaultier, M., and Iborra, J. L. (2004) Synthesis of glycidyl esters catalyzed by lipases in ionic liquids and supercritical carbon dioxide. *J. Mol. Catal. A: Chem. 214*, 113−119.