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Influence of the Nucleophile on the *Candida antarctica* Lipase B-Catalysed Resolution of a Chiral Acyl Donor

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Dedicated to Professor Karl Hult on the occasion of his 65th birthday.

The resolution of methyl (±)-3-hydroxypentanoate catalysed by *Candida antarctica* lipase B has been performed by using ammonia and benzyl amine as nucleophiles. In all cases, the lipase reacts faster with the *R* enantiomer of the ester, but when benzyl amine is used, the enantiomeric ratio is approximately three times as high as that measured for ammonia. The analysis of the molecular dynamics simulations carried out over the corresponding deacylation transition state analogues indicated specular binding modes between enantiomers that vary greatly upon the nucleophile used. For the case of ammonia, an intramolecular hydrogen bond between the β-hydroxyl group and the protons of the nucleophile is established. However, the presence of the substituent in benzyl amine disrupts this interaction. Instead, the acyl chain binds to a more restrictive area of the protein where the higher number of contacts established with the side chains of Thr40, Gln157 and Ile189 have been identified as the reason for the higher enantioselectivity observed in the aminolysis reaction.

Introduction

Enzymes are efficient catalysts in the enantioselective preparation of chiral compounds.[1] Among all the enzymatic transformations available, the lipase-catalysed nucleophilic attack upon carboxylic acids and their derivatives is, by far, the most studied and applied.[2] In these processes, if any of the substrates are either racemic or *meso*, the transformation leads to optically active products, of which the enantiomeric excess depends on the degree of enantioselectivity shown by the lipase. Rationalisation of the enzymatic enantioselectivity in terms of protein–substrate interactions is a very useful approach because it usually allows for the formulation of simple rules that govern how substrates and/or enzymes can be engineered to attain high enantioselectivities. For instance, in the case of lipases and racemic secondary alcohols, an empirical rule has been formulated on the basis of the size of the substituents at the stereocentre.[3] This rule states, from a qualitative point of view, that the bigger the difference in size between substituents, the higher the enantiomeric ratios. Structural studies have revealed that this behaviour is due to the presence of two pockets of different size in the nucleophilic binding site of lipases.[4] As this pocket is highly conserved among members of this family,[5] the rule is also valid for the majority of them, and it can be extrapolated to other serine proteases and related nucleophiles such as disubstituted primary alcohols and isosteric primary amines.[6] Unfortunately, both the structural diversity of the acyl binding sites of lipases[7] and the lack of experimental 3D structures that contain mimic inhibitors of chiral acyl donors are responsible for the fact that rules explaining the chiral preference of lipases towards this kind of substrate have only been formulated for a few cases[8] and cannot be generalised.

Although the enantioselectivity displayed by an enzyme towards a series of substrates can be generally explained on the sole basis of the structure, this procedure is seriously limited in cases in which this is not the major contribution to enantioselectivity. One of the factors ignored by this approach is the role played by the other reagents that also participate in the reaction mechanism. Thus, for a given substrate or a series of substrates, the structure–enantioselectivity profile can be completely altered depending on the reagents employed. For instance, it has been demonstrated that, in the lipase-catalysed resolution of racemic nucleophiles, the enantioselectivity is considerably enhanced by using a "substrate matching" methodology between the incoming nucleophile and the leaving group;[9] the closer the structural similarity, the more efficient the resolution, even though the enantiodiscrimination of each nucleophile takes place during different steps of the catalytic mechanism (TI_1 and TI_2, Scheme 1). On the other hand, solvent molecules, apart from solvation/desolvation contributions to the energetics of enantioselectivity, can also bind to the active site of enzymes and play a role during enantiodiscrimination. For instance, it has been recently explained at the molecular level how the water present in the reaction medium enantiose-

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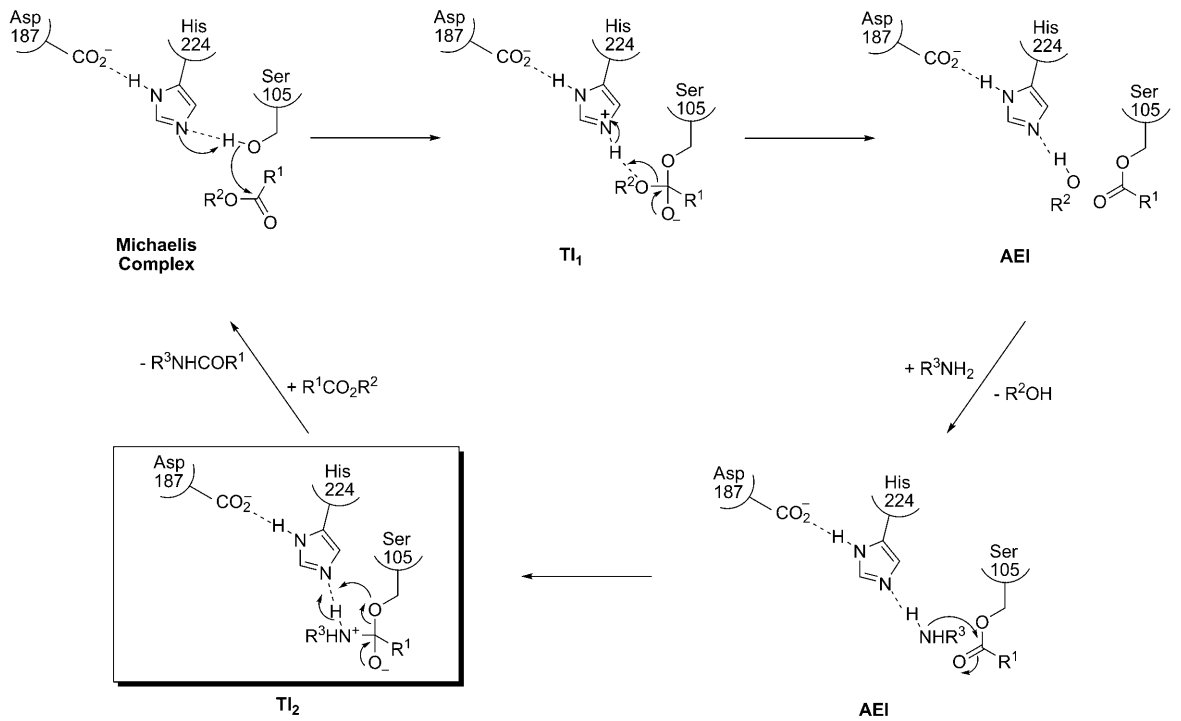

Scheme 1. Simplified mechanism for the serine hydrolase-catalysed ammonolysis and aminolysis of esters. Only the Michaelis complex, early tetrahedral intermediates (TI_1 and TI_2) and the acyl enzyme intermediate (AEI) are shown. The intermediate selected to carry out the MDS (TI_2) study is highlighted.

lectively inhibits the esterification of a secondary alcohol catalysed by *Candida antarctica* lipase B (CALB).[10]

Acyl donors have a dramatic influence on both the activity and the enantioselectivity displayed by lipases in the resolution/desymmetrisation of nucleophiles.[11] Accordingly, the opposite should also be true. However, little rationalisation has been done in this field,[12] which has been mainly left to a trial and error process. We have been traditionally interested in the CALB-catalysed, enantioselective preparation of β-hydroxyesters by means of aminolysis and ammonolysis processes.[13] In general, we have observed that ammonia tends to be a less effective nucleophile than a primary amine (Scheme 2), the best

Scheme 2. Examples of racemic β-hydroxyesters for which the CALB-catalysed ammonolysis shows a lower enantioselectivity than the corresponding aminolysis with benzyl amine.[13a,d]

of which depends on the β-hydroxyester employed. In order to elucidate the role played by the nucleophile in the enantiorecognition mechanism of this type of chiral acyl donor, we report herein a study on the dependence of the enantioselectivity of the resolution of methyl (±)-3-hydroxypentanoate, selected as a model β-hydroxyester, with two nitrogenated nucleophiles: ammonia and benzyl amine. First, the resolutions were carried out under standard experimental conditions. Second, suitable intermediates were selected to carry out the computational study. According to the accepted mechanism for serine hydrolases,[14] the different enantioselectivity displayed by CALB depending on the nucleophile used should stem from those steps corresponding to the deacylation of the catalytic serine (TI_2, Scheme 1). Moreover, and due to the higher nucleophilicity of amines as compared to alcohols, early TI_2s were assumed to best represent the enantioselectivity-determining step.[15] Accordingly, the corresponding molecular dynamics simulations (MDS) were performed. The analysis of the obtained trajectories allowed us to examine the key interactions responsible for the experimental behaviour observed. Representative snapshots of the major conformations were energy-minimised and used to illustrate the hypotheses.

Results and Discussion

Enzymatic kinetic resolution of methyl (±)-3-hydroxypentanoate [(±)-1]:

For both ammonia and benzyl amine, CALB reacts faster with the *R* enantiomer of the hydroxyester [(*R*)-**1**], and thus affords the corresponding *R* amides [(*R*)-**3 a**,**b**] and the *S* hydroxyester [(*S*)-**1**] as the major products and substrate, respectively (Scheme 3). Due to the uncertainty in the concentration of ammonia (**2 a**) in the reaction medium, it is not possible to determine which reaction is actually faster. However, the enzyme is approximately three times more enantioselective when benzyl amine (**2 b**) is used as the nucleophile (Table 1).

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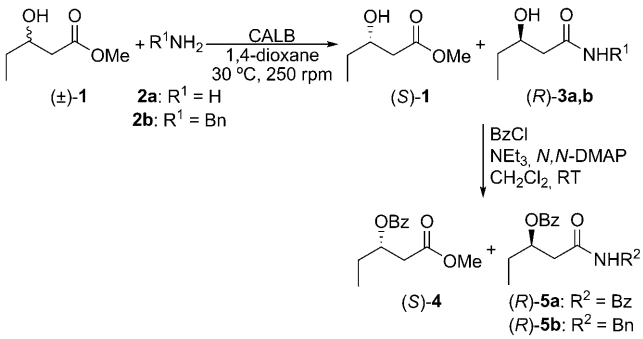


Scheme 3. CALB-catalysed aminolysis and ammonolysis of (±)-**1** and subsequent derivatisation for the *ee* measurement.

Table 1. CALB-catalysed resolution of (±)-**1**.

Entry	R^1	conversion (*c*) [%][a]	enantioselectivity (*E*)[b]
1	H	31	15.1±0.3
2	benzyl	49	41.9±2.2

[a] Reaction time: 40 min. [b] Fitted from data obtained at reaction times of 10, 20, 30 and 40 min.

CALB structure

CALB (Uniprot[16] ID: P41365) is a triacylglycerol hydrolase (EC 3.1.1.3) comprised of 342 amino acids. The enzyme belongs to the α/β-hydrolase superfamily[17] and shows no interfacial activation. Many 3D structures of this enzyme, either in the apo form or complexed with different inhibitors, have been determined in recent years.[18] These structures have revealed that the binding site of CALB (Figure 1) consists of a catalytic triad (Ser105, His224 and Asp187) and the following pockets: one for the nucleophile, also termed the stereospecificity pocket, one for the acyl chain and one for the oxyanion hole, formed by the backbone amide groups of Gln105 and Thr40 and the side chain hydroxyl group of the latter. The starting structures for each of the tetrahedral intermediates studied here (Scheme 4) were built so that the nucleophile was allocated in the stereospecificity pocket, the acyl chain in the acyl binding site and the oxyanion in the oxyanion hole (see the Experimental Section for details). Subsequently, 1 ns MDS were carried out, and the last 0.9 ns of the trajectories were used for analysis.

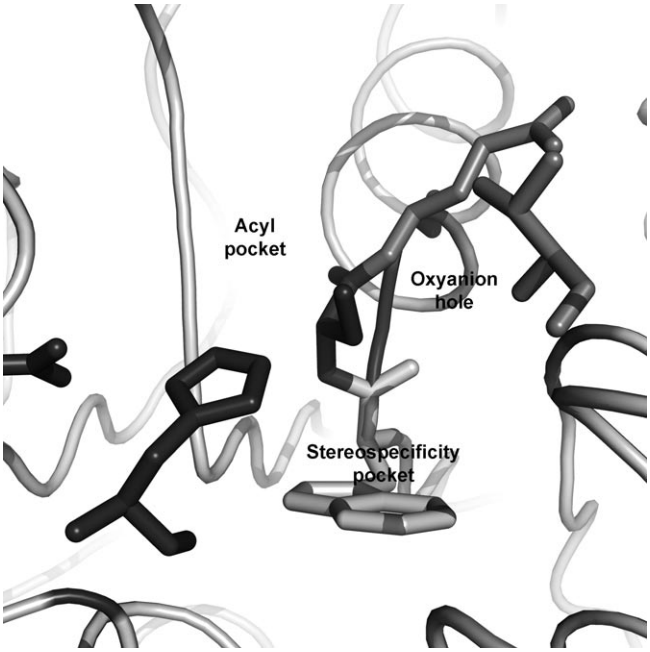
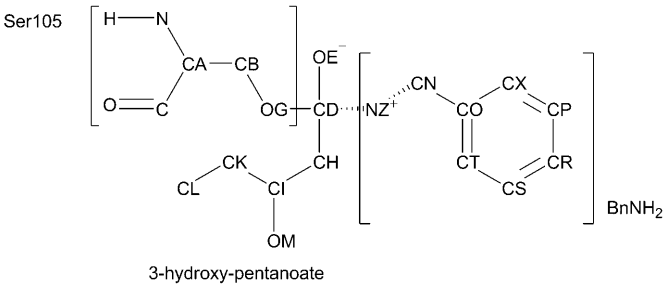


Figure 1. Active site of CALB. The catalytic triad (dark grey), the amino acids of the oxyanion hole (grey) and Trp104 (light grey), the "floor" of the active site, are shown in stick mode.



Scheme 4. Atom names of the deacylation tetrahedral intermediates with benzyl amine [TI_2-(*R/S*)-**3 b**].

Catalytic hydrogen bonding

The average geometries of the catalytic hydrogen bonds were calculated and analysed for the four diastereomeric tetrahedral intermediates corresponding to the two nucleophiles studied here (Table 2). The distances between the nucleophilic nitrogen atoms and the Nε of His224 are, on average, slightly longer for the fast- (Table 2, Entries 1 and 5) than for the slow-reacting enantiomers (Table 2, Entries 3 and 7). However, as both the magnitude of these differences and the fact that the trend is the same regardless of the nucleophile used, we conclude that this interaction is not responsible for the different enantioselectivity observed (Table 1). It is worth noting that when ammonia is employed as the nucleophile, all the hydrogens on the incoming nucleophilic nitrogen can establish a hydrogen bond with His224 equally well by rotation around the CD-NZ axis. However, for benzyl amine, this equivalence is disrupted, and only the *pro-R* hydrogen of the nucleophilic nitrogen is able to bind to the catalytic histidine. Concerning the stabilisation of the oxyanion, we observed that the hydrogen bond with Gln106 is the weakest one and, for the case of ammonia, the one that shows a largest difference between the fast- (Table 2, Entry 1) and the slow-reacting (Table 2, Entry 3) enantiomers. If we assume that the three hydrogen donors of the oxyanion hole of CALB can equally stabilise the oxyanion, it is logical to think that a weaker hydrogen bond can be compensated by a stronger one. Therefore, for the oxyanion, we decided to compare average distances and angles rather than individual hydrogen bonds. As can be seen in Table 2, for ammo-

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Table 2. Catalytic hydrogen-bonding distances (Å) and angles (°).

	Intermediate		Nucleophilic nitrogen (NZ) His224 (Nε2)	oxyanion (OE) Gln106 (H)	Thr40 (H)	Thr40 (Hγ1)	Average[a]
1	TI_2-(*R*)-**3a**	distance [Å]	3.10 ± 0.16	2.27 ± 0.20	2.00 ± 0.15	1.86 ± 0.17	2.04
2		angle [°]	–[b]	158.5 ± 11.7	163.0 ± 8.7	164.8 ± 8.3	162.1
3	TI_2-(*S*)-**3a**	distance [Å]	3.05 ± 0.15	2.47 ± 0.24	2.01 ± 0.15	1.92 ± 0.21	2.13
4		angle [°]	–[b]	160.9 ± 11.1	163.8 ± 8.7	162.8 ± 10.5	162.5
5	TI_2-(*R*)-**3b**	distance [Å]	3.20 ± 0.21	2.14 ± 0.18	1.92 ± 0.11	1.90 ± 0.18	1.99
6		angle [°]	–[b]	162.2 ± 10.4	164.8 ± 8.3	164.2 ± 9.1	163.7
7	TI_2-(*S*)-**3b**	distance [Å]	3.17 ± 0.18	2.15 ± 0.18	1.91 ± 0.12	1.92 ± 0.20	1.99
8		angle [°]	–[b]	158.2 ± 11.7	163.5 ± 9.0	163.2 ± 10.2	161.6

[a] Average distance/angle per bond. [b] Angles were not measured because more than one equivalent hydrogen was involved in the hydrogen bond.

nia, although the oxyanion is slightly less stabilised in the slow- than in the fast-reacting enantiomer, the difference is too small (≈ 0.1 Å) to be regarded as significant. When benzyl amine is the nucleophile, there is practically no difference between enantiomers, and the oxyanion is slightly better stabilised than it is in the ammonolysis reaction. Therefore, the results indicate that the catalytic hydrogen-bonding network should not play a role in the different enantioselectivity displayed by CALB towards (±)-**1** depending upon the nucleophile used.

Binding of the acyl chain

The other hydrogen-bonding group of the substrate is the 3-hydroxyl group of the acyl chain. Due to the hydrophobic nature of the acyl binding site of CALB, there are not so many hydrogen-bonding partners in its vicinity. These are limited to the oxygen of the side chain amido group of Gln157 (Oε1), the nitrogen of which is in turn interacting with the side-chain oxygen of Thr40, and the carboxylic acid moiety of Asp134. However, when ammonia is the nucleophile, the 3-hydroxyl group prefers to accept one of the protons of the incoming ammonium group; it thus forms an intramolecular six-membered ring, which makes the acyl chain adopt a hairpin-like conformation (Figure 2). This interaction is longer and sharper for the slow- (2.38 ± 0.29 Å and 128.1 ± 9.4°) than for the fast-reacting diastereomeric, tetrahedral intermediates (2.08 ± 0.17 Å and 136.5 ± 10.8°). Moreover, in this conformation, the hydroxyl hydrogen of TI_2-(*R*)-**3a**, but not that of TI_2-(*S*)-**3a**, establishes an additional (but weak) hydrogen bond with the backbone carbonyl oxygen of Ala281 (2.83 ± 0.94 Å, 113.4 ± 26.3°). Conversely, the employment of benzyl amine leads to a completely different scenario (Figure 3). Although there is also one proton from the nucleophilic nitrogen available for interaction with the 3-hydroxyl oxygen of the acyl donor, this interaction is not established. Alternatively, the 3-hydroxyl group remains mainly unpaired although it also shows, for both TI_2s, short-lived interactions with the Oγ of the catalytic serine, the oxyanion (OE) and the oxygens of the side chains of Gln157 and Asp134.

Intramolecular hydrogen bonds have previously been ascribed a key role in both enzymatic activity and selectivity.[19] In fact, the conformation adopted by the tetrahedral intermediate with ammonia (Figure 2) is qualitatively similar to the one proposed to account for the unusually high enzymatic acylation rate of amines when ethyl ethoxyacetate is used as the acyl donor.[19b] Although we cannot rule out the potential contribution of the different "strength" of this intramolecular interaction to the enantioselectivity measured for the ammonolysis of (±)-**1** (Figure 2), we lean towards ascribing to this interaction an "anchoring" effect that determines a different binding mode for the acyl chain during the catalytic mechanism. Thus, we hypothesise that the benzyl substituent of the amine hampers the required approach of the secondary 3-hydroxyl moiety towards the ammonium

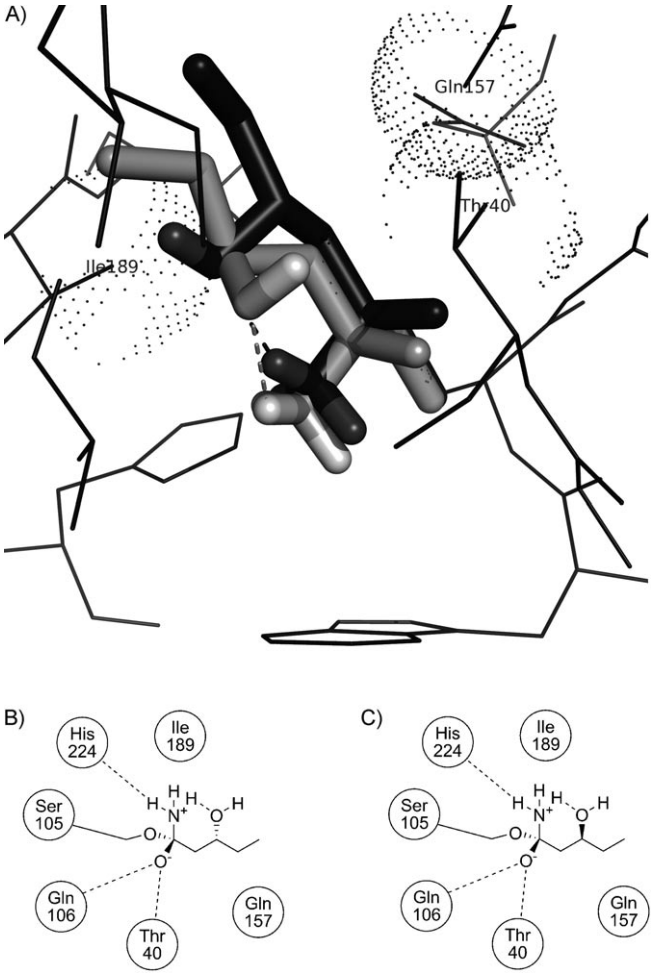

Figure 2. A) 3D, representative, energy-minimised conformations of (*R*)-(black) and (*S*)-configured (grey) deacylation tetrahedral intermediates corresponding to the ammonolysis of (±)-**1**. Only side chain polar hydrogens are shown. The corresponding 2D versions are depicted in B) and C), respectively.

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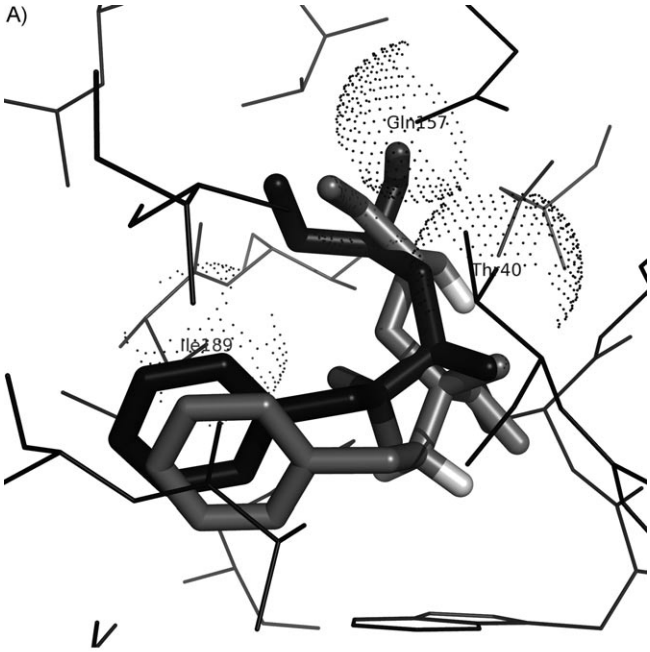

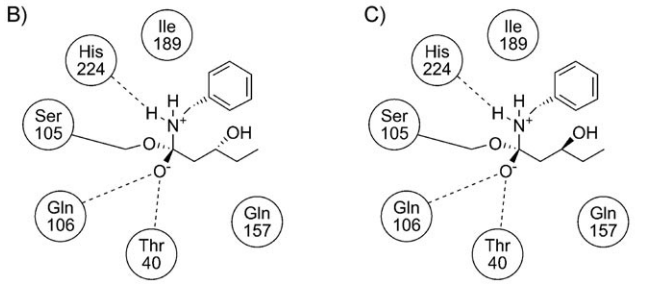

Figure 3. A) 3D, representative, energy-minimised conformations of the (*R*)-(black) and (*S*)-configured (grey) deacylation tetrahedral intermediates corresponding to the aminolysis of (±)-**1** with benzyl amine. Only side chain polar hydrogens are shown. The corresponding 2D versions are depicted in B) and C), respectively.

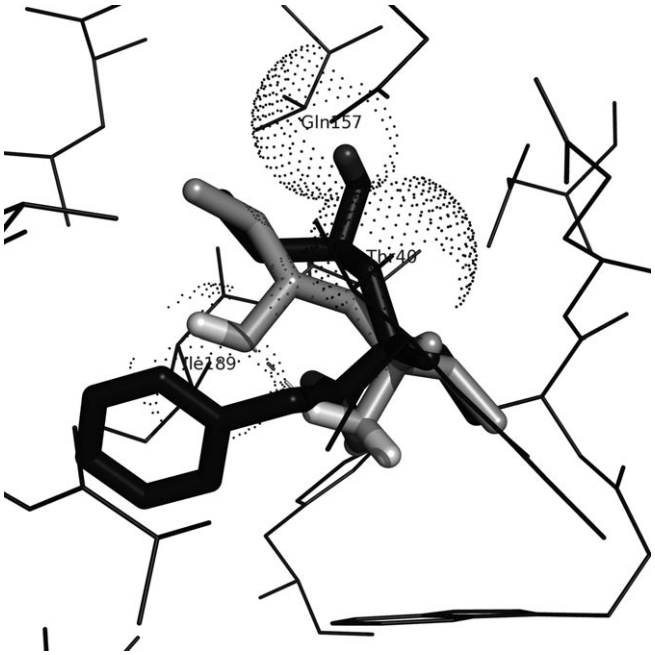

Figure 4. 3D, representative, energy-minimised conformations of the fast-reacting enantiomers of the deacylation tetrahedral intermediates corresponding to the aminolysis of (±)-**1** with benzyl amine (black) and ammonia (grey). Only side-chain polar hydrogens are shown.

group by "shielding" the hydrogens of the incoming NZ (Figure 3). Therefore, and conversely to the case described for ethyl ethoxyacetate,[19b] the intramolecular hydrogen bond is only possible when a sterically undemanding nucleophile, such as ammonia, is used (Figure 4). On the other hand, the presence or absence of the intramolecular hydrogen bond indeed causes the acyl chain of (±)-**1** to adopt two different conformations, and hence, to face two different binding environments in the CALB acyl pocket, depending upon the nucleophile used (compare Figures 2 and 3 and see Figure 4). Thus, when ammonia is employed (Figure 2), the acyl chain is located closer to the entrance of the binding site than it is when benzyl amine is employed (Figure 3).

Inspection of the representative conformations obtained for the four tetrahedral intermediates studied here (Figures 2 and 3) shows that, for each of the nucleophiles, mirror-image binding orientations are obtained. On the one hand, when ammonia is the nucleophile, the restraints imposed by the hydrogen-bonding network and the absolute configuration of the chiral centre make the reactive carbonyl group lie slightly outside of the oxyanion hole (Figure 2). These different locations occupied by the reactive carbonyl group would account for the aforementioned differences observed in the geometries of the catalytic hydrogen bonds of the enzymatic ammonolysis (Table 2, entries 1 and 3). On the other hand, when benzyl amine is employed (Figures 3 and 4), the acyl chain occupies a more "crowded" are of the acyl pocket. Accordingly, this location should be more restrictive than the one occupied by the acyl chain in the ammonolysis mechanism (Figures 2 and 4). In order to quantify this observation, we have counted the number of snapshots of the MDS trajectories containing both close[20,21] and "normal" contacts[21,22] between non-hydrogen atoms of the acyl chain and the amino acids lining the acyl binding site. The results obtained show that, for both binding modes (Figures 2 and 3), the acyl binding site offers enough space to accommodate the acyl chain of the substrate as is suggested by the lack of serious steric clashes. However, and as has been previously observed, when benzyl amine is the nucleophile, the binding location of the acyl chain is more crowded; this is reflected in the fact that for both TI_2s leading to amide **3 b**, the acyl chain presents 64 009 "normal" contacts, while those leading to amide **3 a** only present 44 343 (Tables S6 and S7 in the Supporting Information).

A more detailed inspection of the different number of normal contacts established by the atoms of the acyl chain of (±)-**1** in the four tetrahedral intermediates studied here was carried out. The aim was the identification, if possible, of the interactions between the acyl donor and the enzyme that more significantly contribute to the experimental enantioselectivity. To be more precise, a normal contact involving hydrophobic atoms was considered a van der Waals interaction and

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assumed to accelerate the reaction rate of the tetrahedral intermediates where it was present. Otherwise, when incompatible polarities were present, the contact was assumed to destabilise the corresponding intermediate. As a general feature, due to the fact that the binding modes obtained for both the aminolysis and the ammonolysis of (±)-**1** are specular, favourable and unfavourable interactions usually cancel out between enantiomers. However, the acyl binding site is not completely symmetric, and some interesting differential interactions have been identified. In the ammonolysis of (±)-**1** (Figure 5), Thr40, Gln157 and Ile189 turn out to be key amino acids.[23] Gln157

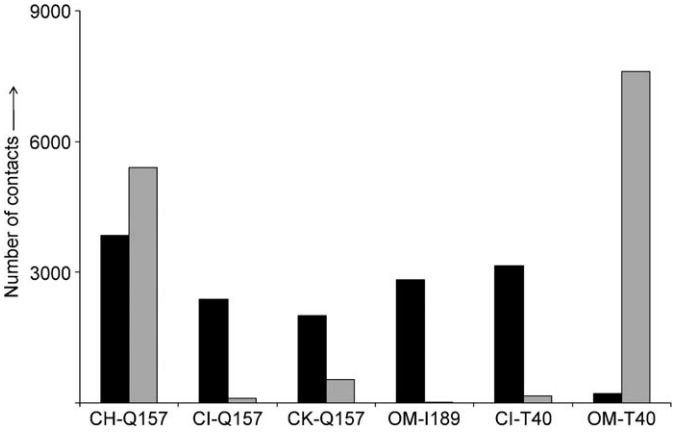

Figure 5. Number of contacts established between non-hydrogen atoms of the acyl chain of (*R*)-**1** (black) and (*S*)-**1** (grey) and the amino acids of the binding site of CALB during the ammonolysis reaction. All contacts involve atoms of unsuitable polarities.

interacts through its Oε1 atom with the carbon atoms CH, CI and CK of the acyl chain. Therefore, because of the polarities of the atoms involved, these contacts destabilise the tetrahedral intermediates where they occur. In particular, the interaction between Gln157 and CH favours the reactivity of the fast-reacting enantiomer. However, the effect is the opposite for CI and CK (Figure 5). Thus, if we assume that each of these contacts contribute equally to the energetics of the enantioselectivity, the net contribution of Gln157 is deleterious to the enantioselectivity of the ammonolysis of (±)-**1**; TI_2-(*R*)-**3a** and TI_2-(*S*)-**3a** present with the carbon atoms of the acyl chain a total of 8234 and 6040 contacts, respectively (Table S6). Additionally, Thr40 also disfavours the *R* enantiopreference shown by CALB, since its Oγ1 atom establishes more contacts with the CI atom of the acyl chain in TI_2-(*S*)-**3a** than it does in TI_2-(*R*)-**3a**. It is worth noting that the CL terminal carbon of the acyl chain does not seem to play any significant role in the enantiorecognition mechanism of the CALB-catalysed ammonolysis, because it does not establish a significant amount of interactions with the amino acids of the binding site. Finally, the 3-hydroxyl group (OM), in addition to its intramolecular interaction with NZ, also seems to be a key atom for the preference shown by CALB towards (*R*)-**1**. Although the interactions established with the Cγ2 atom of Ile189 preferentially stabilise the slow-reacting enantiomer, the large number of contacts established with the Oγ1 atoms of Thr40 in TI_2-(*S*)-**3a** strongly favour the preferential conversion of (*R*)-**1**.

For the tetrahedral intermediates of the aminolysis reaction, although the same amino acids participate in the most significant contacts,[23] the atoms of the acyl chain involved and the number of contacts present vary considerably (Figure 6). Thus,

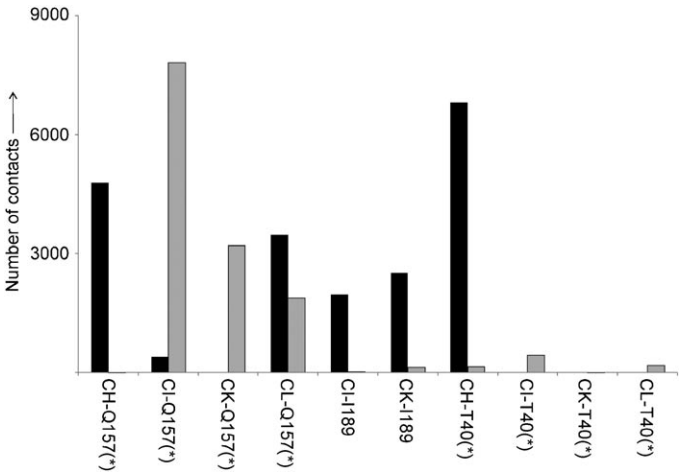

Figure 6. Number of contacts established between non-hydrogen atoms of the acyl chain of (*R*)-**1** (black) and (*S*)-**1** (grey) and the amino acids of the binding site of CALB during the aminolysis reaction with benzyl amine (**3b**). Contacts marked with an asterisk involve atoms of unsuitable polarities.

now the Oε1 of Gln157 establishes contacts with all the carbon atoms of the acyl chain. In particular, the interaction with CI and CK favour the preferential transformation of (*R*)-**1**, while the contacts with CH and CL play the opposite role. Globally, Oε1 of Gln157 establishes 8659 and 12896 contacts with the carbon atoms of the acyl chain of TI_2-(*R*)-**3b** and TI_2-(*S*)-**3b**, respectively (Table S7). On the other hand, the binding mode shown by the acyl chain in both TI_2-(*R*/*S*)-**3b** makes Ile189 preferentially stabilise the fast-reacting enantiomer by means of van der Waals interactions with CI and CK. Conversely to the ammonolysis reaction, the destabilisation of the 3-hydroxyl group by interactions with Thr40 is not observed. Finally, the Oγ1 atom of Thr40 interacts with the α-carbon of the acyl chain (CH) in the fast-reacting enantiomer; this makes it less stable than the slow-reacting one.

The interactions present in the four tetrahedral intermediates studied here were also grouped into three different classes based on an atomic criterion (Figure 7):[23] 1) C–C contacts or van der Waals interactions, 2) C–O and 3) O–O contacts, the last two of which are deleterious for the energy of the tetrahedral intermediate where they occur. As can be seen in Figure 7, van der Waals interactions are only present during the aminolysis reaction and clearly support the preferential transformation of (*R*)-**1**. Conversely, O–O contacts are only present in the ammonolysis, where they also favour the transformation of the fast-reacting enantiomer of the substrate. Finally, C–O contacts are present in both processes and, although they always favour the transformation of the slow-

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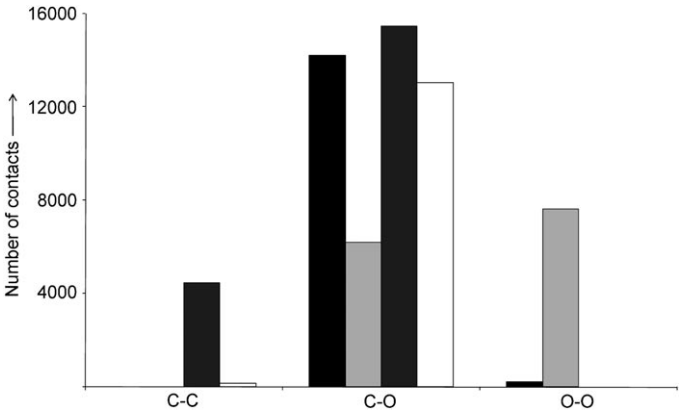

Figure 7. Number of non-hydrogen atomic contacts established between the acyl chain of (±)-**1** and the amino acids of the binding site of CALB during the ammonolysis and aminolysis with benzyl amine. (*R*)-TI_2-**3a**: black; (*S*)-TI_2-**3a**: light grey; (*R*)-TI_2-**3b**: dark grey; (*S*)-TI_2-**3b**: white.

reacting enantiomer of the substrate, this effect is more pronounced for the ammonolysis reaction.

Conclusions

Mirror-image binding modes have been proposed for each of the enantiomeric series of the tetrahedral intermediates corresponding to the deacylation of the catalytic serine during the CALB-catalysed ammonolysis and aminolysis with benzyl amine of methyl (±)-3-hydroxypentanoate. However, the binding modes significantly differ depending on the nucleophile used. This was explained on the basis of an intramolecular hydrogen bond between the 3-hydroxyl group of the acyl chain and the nucleophilic nitrogen atom; this bond would only be established in cases in which a low-steric-demand nucleophile, like ammonia, is used.

For the ammonolysis reaction, the *R* enantiopreference shown by the lipase has been ascribed to an imbalance in the nature and amount of contacts established between the acyl chain and the side chains of the amino acids surrounding it. Thus, the interactions between CH and the Oε1 of Gln157, and, especially, OM and the Oγ1 of Thr40 have been identified as the major contributions to the observed enantioselectivity. On the other hand, the bulkier benzyl amine would make the acyl chain bind to a more restrictive area. In this binding location, the preferential stabilisation of CI and CK of the *R* enantiomer of the substrate by means of van der Waals interactions with Ile189, and their destabilisation in the slow-reacting enantiomer by means of contacts with the Oε1 of Gln157, have been indicated as the reason for the higher enantioselectivity measured for the aminolysis process.

Experimental Section

General experimental methods: Lipase B from *Candida antarctica* (SP 435) was a gift from Novozymes (Bagsværd, Denmark). Methyl 3-oxopentanoate was purchased from Alfa Aesar (Karlsruhe, Germany, and benzyl amine and sodium borohydride were obtained from Aldrich. Amines were distilled prior to use. Dry ammonia was purchased from Carburos Metálicos (Barcelona, Spain). 1,4-Dioxane was purchased from Prolabo (Barcelona, Spain) and dried prior to use by refluxing over sodium by using benzophenone as an indicator. HPLC-grade hexane and propan-2-ol were purchased from Romil (Cambridge, United Kingdom). Flash chromatography was performed using silica gel 60 (230–240 mesh). High performance liquid chromatography (HPLC) analyses were carried out with a Hewlett Packard 1100 chromatograph equipped with a UV detector and a Daicel Chiralcel OD column (25 cm×4.6 mm, Chiral Technologies, Europe, Illkirch, France). ^{1}H, ^{13}C NMR and DEPT experiments were obtained with a DPX-300 (^{1}H, 300.13 MHz and ^{13}C, 75.5 MHz) Bruker spectrometer.

Preparation of racemic methyl 3-hydroxypentanoate [(±)-1]: Commercially available methyl 3-oxo-pentanoate (1.3 g, 10.0 mmol) was dissolved in methanol (20 mL), and sodium borohydride (265 mg, 7.0 mmol) was slowly added at 0°C. After 2 h at RT, the reaction was quenched with a saturated aq solution of ammonium chloride (5 mL), and the solvent was evaporated under reduced pressure. The resulting mixture was dissolved in water (5 mL), and HCl (3 N) was added until a pH value of 2 was reached. The aqueous phase was then extracted with methylene chloride (3×15 mL), the organic phases were combined and dried over Na_2SO_4, and the solvent was removed by distillation under reduced pressure. The corresponding crude material was purified by flash chromatography (10% EtOAc/hexane), and thus afforded the hydroxyester **1** in 56% yield.

Preparation of racemic 3-hydroxypentanamide [(±)-3a]: The ester (±)-**1** (100 mg, 0.76 mmol) was dissolved in a saturated solution of ammonia in methanol. The reaction mixture was stirred at 70°C for 48 h in a sealed tube. Afterwards, the solvent was evaporated at reduced pressure, and the resulting crude material was purified by flash chromatography using EtOAc as the eluent. The corresponding amide (±)-**3a** was obtained in 89% yield.

Preparation of racemic *N*-benzyl-3-hydroxypentanamide [(±)-3b]: The ester (±)-**1** (100 mg, 0.76 mmol) and the amine **2b** (166 μL, 1.51 mmol) were mixed in a sealed tube and stirred at 105°C for 16 h. The resulting crude material was then purified by flash chromatography using an eluent gradient (30 to 70% EtOAc/hexane). Amide (±)-**3b** was obtained in 87% yield.

Preparation of 1-substituted-1-oxopentan-3-yl benzoates (4 and 5a,b): Methyl 3-hydroxypentanoate (**1**) or the corresponding 3-hydroxyamide (**3a** or **b**, 0.38 mmol) was dissolved in methylene chloride (1 mL) and triethylamine (61 μL, 0.76 mmol), 4-*N,N*-dimethylaminopyridine (9.8 mg, 0.08 mmol) and benzoyl chloride (88 μL, 0.76 mmol) were successively added. After 4 h at RT, the reaction was stopped, the solvent was evaporated, and the resulting crude material was purified by flash chromatography (40% EtOAc/hexane) to yield the corresponding benzoylated derivatives **4** and **5a**,**b** in 91–97% yield.

Enzymatic aminolysis/ammonolysis of methyl (±)-3-hydroxypentanoate [(±)-1]: To a suspension of the ester (±)-**1** (50 mg, 0.38 mmol) and CALB (22.7 mg), 1,4-dioxane (1.5 mL) and the amine **3b** (42 μL, 0.38 mmol) were subsequently added under a nitrogen atmosphere. For the case of ammonia (**3a**), a dry, saturated solution of it in 1,4-dioxane was used instead. The resulting mixture was shaken at 30°C and 250 rpm for 10, 20, 30 and 40 min. Afterwards, the enzyme was filtered off, washed with methylene chloride, and the organic solvents were evaporated under reduced pressure.

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Determination of enantiomeric excesses and the enantiomeric ratio: The enantiomeric excesses of the substrate and products of the enzymatic reactions were determined by means of the HPLC analysis of their benzoylated derivatives (**4** and **5a**,**b**). A propan-2-ol/hexane mixture (10%) was employed as the eluent. The enantiomeric ratio was measured by means of the Rakels' method.[24]

General computational methods: All molecular dynamic simulations and energy minimizations (EMs) were performed on an iMac by using the molecular modeling package Molecular Operating Environment (MOE) 2007.09 (Chemical Computing Group, Inc., Montréal, Canada).[25] In all cases, the Amber94 force field[26] and the corresponding dictionary charges were used as implemented in MOE. Due to the lack of parameters for 1,4-dioxane compatible with the force field used, we opted for a continuum solvation method, an approach that has already afforded good results in similar studies.[10,12,19b] In particular, a distance-dependent relative dielectric constant of two was selected as a good compromise between the dielectric constant of 1,4-dioxane[27] and that of a globular protein matrix.[28] A nonbonded cut-off of 8 Å with a smoothing function acting in the 8–10 Å range were used. In all MDS, the *NVT* ensemble and the Nosé-Poincaré-Anderson (NPA) equations were selected. The initial and simulation temperatures were set to 0 and 300 K, respectively. No constraints were imposed on any bond, and a step size of 1 fs was used. For the 1 ns MDS, a heating period of 10 ps was used, and the temperature relaxation time was set to 0.1 ps. Snapshots of the trajectory were saved every 0.1 ps, and only those corresponding to the last 900 ps of the simulation were used for analysis. For the 1 ps MDS, the heating period duration and the temperature relaxation time were set to 1 ps and 10 fs, respectively. The convergence criterion of the EM was set to a gradient value of 0.01 kcal mol^{-1} when only parts of the protein–ligand complex were minimised. However, if the whole enzyme–substrate complex was minimised it was lowered to a value of 0.001 kcal mol^{-1}. High-quality pictures of representative structures were generated with PyMOL 0.99.[29]

Preparation of the enzyme model: The enzyme starting structure to be used in all computations was prepared in a similar way to that described by Raza et al.[12] Thus, the coordinates of CALB were retrieved from the Brookhaven Protein Data Bank[30] (ID: 1TCA[18a]) and used to build the tetrahedral intermediates of the slow- and fast-reacting enantiomers, respectively, of 3-hydroxypentanoate with ammonia [TI$_2$-(*R/S*)-**3a**] and benzyl amine [TI$_2$-(*R/S*)-**3b**]. In all cases, the two sugar units (*N*-acetyl-D-glucosamine) were removed, the carboxy- and amino-terminal groups of the protein backbone were changed to carboxylate and ammonium, respectively, and Asp134 was protonated.[4b] In order to improve the initial orientation of the hydrogens, a series of 1 ps MDS (heating period of 1 ps) and EMs were carried out as follows.[12] First, only water hydrogens and, subsequently, hydrogens of the side chains of certain amino acids (Ala, Cys, Ile, Leu, Lys, Met, Ser, Thr, Tyr and Val) were allowed to move. Next, subsequent EMs were carried out over the water molecules and the side chains of all the amino acids. At this stage, any crystallographic water molecule not establishing any hydrogen bond with either the protein or another water molecule was removed by means of the LigX tool within MOE. Finally, subsequent EMs over the remaining water molecules, the side chains of the amino acids and the ligand, and the whole protein–ligand complex were carried out.

Preparation and 1 ns MDS of the tetrahedral intermediates: The transition states responsible for the enantioselectivity displayed by CALB in the aminolysis and ammonolysis of methyl (±)-3-hydroxypentanoate were approximated to the tetrahedral intermediates of the deacylation step of the catalytic serine.[31] They were built manually by the modification of the side chain of Ser105 in the enzyme model as follows: 1) a tetrahedral carbon covalently bonded to the Oγ of Ser105 and to aliphatic carbon, nitrogen and oxygen atoms was built. In all cases, a formal charge of −1 was set on the oxyanion atom, the catalytic His (His224) was kept neutral, and the nucleophilic nitrogen was assigned a formal charge of +1; 2) torsions were modified so that each substituent was placed in the corresponding pocket; 3) an EM of the so-created atoms was carried out; 4) the acyl chain was built atom by atom, and we used 1 ps MDS to explore the preferred location of each "new" atom in the acyl binding pocket of CALB and 5) the whole side chain of the created amino acid and 6) finally, the whole tetrahedral intermediate were energy minimised. These structures were subjected to 1 ns MDS. The analysis of the trajectories of the simulations excluded the heating period and the first 100 ps of the simulations. In particular, distances and angles of hydrogen bonds[32] and the torsions of the substrate were analysed and clustered. For the major cluster (Figures S2–S5), the structure that showed the lowest internal and interaction energy for the substrate (acyl chain, incoming nucleophile and side chain of the original Ser105) was selected as representative and used for visualization purposes after EM of the whole protein-substrate complex.

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Keywords: amides · biotransformations · enantioselectivity · hydrolases · molecular dynamics

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