Tuning Lipase Enantioselectivity in Organic Media Using Solid-State Buffers

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The enantioselectivity exhibited by *Candida antarctica* lipase B (CALB) in predominantly organic media has been studied for different enzyme protonation states. Alcoholysis of (±)-2-phenyl-4-benzyloxazol-5(4*H*)-one (**1**) using butan-1-ol as the nucleophile in low-water organic solvents was used as a model reaction. Using either organo-soluble bases or the newly introduced solid-state buffers of known pK_a, the protonation state of the lipase was altered. By choice of the appropriate solid-state buffer or organic base, the enantioselectivity could be selectively tuned. Both Et_3N and the solid-state buffer pair CAPSO/CAPSO.Na were found to increase the enantioselectivity of the reaction catalyzed by CALB and that of another lipase (*Mucor miehei).* Significant differences to both the enantioselectivity and catalytic rate were observed, especially under hydrated conditions where byproduct acid was formed.

Introduction

Much effort, over the past decade has focused on the study of the physicochemical behavior of isolated enzymes in organic solvent environments and their application to the efficient synthesis of biologically active pharmaceutical intermediates. In this sense, lipases have attracted much interest since they have proved to be powerful tools for the resolution of a wide variety of chiral compounds with potential industrial applications.[1] To this end, a range of strategies which aim to engineer the microenvironment of the lipase are currently employed to optimize lipase activity and stereoespecificity in organic solvents. Relevant factors include the degree of protein hydration,[2] nature of the organic solvent,[3] modification of substrates,[4] and ionization-state of the enzyme.[5] With the exception of ionization-state studies, most of these parameters have been studied and reported at length. There are, however, few reports concerned with the control of ionization-state in organic media.[5] It is well-known that lyophilized powders of enzymes suspended in organic media show a pH "memory effect", i.e., the activity of the enzyme can be correlated with the last aqueous pH to which the enzyme was exposed.[6] However, in many enzymatic reactions there may be unrecognized changes to the acid−base conditions due to, for example, the production of acidic side products. The result of such changes to acid/base conditions is erasure of pH memory.[7] This erasure is reflected in an altered protonation-state of either ionizable groups directly involved in catalysis or those residues elsewhere that play a key role in the overall retention of the active enzyme conformation. It has recently been reported that a convenient and practical way to avoid such problems is to use solid-state buffers[5a,e] in the reaction mixture. These buffers are able to reset and then fix the enzyme ionization-state. So far these buffers have only been extended to enhancing the catalytic activity of serine proteases in a model system.[5e,8]

Recently, we have shown that the CALB-catalyzed dynamic resolution of 4-substituted oxazol-5(4*H*)-ones was critically dependent on reaction conditions[9] and could be improved markedly on addition of a catalytic amount of a nonreactive organic base such as Et_3N to the reaction

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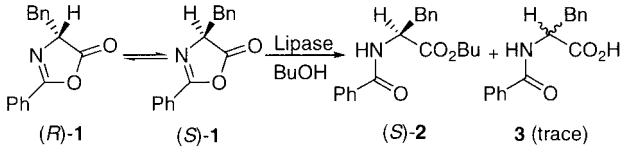
Scheme 1



medium. 5(4*H*)-Oxazalones undergo spontaneous hydrolysis and racemize with ease;[10] the role of the base is not, however, to facilitate this racemization. CALB is able to accept a wide substrate range, has proven to be very stereoselective for the resolution of secondary alcohols,[11] and thus is an ideal candidate for our studies.

In this paper, we have shown that it is possible to selectively tune both the catalytic activity and more importantly the enantioselectivity by changing the protonation-state of an enzyme in organic media. Using lipase B from *Candida antarctica* (CALB, Chirazyme L-2) and another lipase (*Mucor miehei* (Lipozyme, RM IM)), the enantioselectivity in organic media could be selectively altered and reset using solid-state organic buffers that modify the ionization state of proteins *in situ*.

Results and Discussion

The CALB-catalyzed alcoholysis of (±)-2-phenyl-4-benzyl-5(4*H*)-oxazolone (**1**) is shown in Scheme 1; the (*S*)-*N*-Benzoylphenylalanine butyl ester is obtained as the major enantiomer. This reaction is an example of a dynamic resolution (potential of 100% yield and 100% ee) and ultimately yields optically active α-amino esters that are appropriate precursors of α-amino acids.[12] Thus, a wide variety of side chains can be used in order to obtain a range of unnatural synthetic amino acids.[13]

To investigate our initial observation[9] of enhanced enantioselectivity in the presence of Et_3N in more detail, we studied systematically for the first time a range of organo-soluble bases of varying pK_a. In parallel with this we also decided to investigate the use of solid-state buffers that could buffer the ionization state of CALB in situ within a given pK_a range.

However, it was first important to establish if the inactivation and reduction to the enantiomeric ratio (*E*) that we observed during the course of a reaction was due to chiral inhibition by the acid generated. To investigate this a dry preparation of CALB in toluene ($a_w \sim 0$) that contained either trace byproduct acid (**3**) (*N*-benzoylphenylalanine) or a nonspecific acid, such as acetic acid, was assayed. From Figure 1a,b it can be seen that the presence of acetic acid significantly reduces both the catalytic activity (~50%) and enantioselectivity of CALB

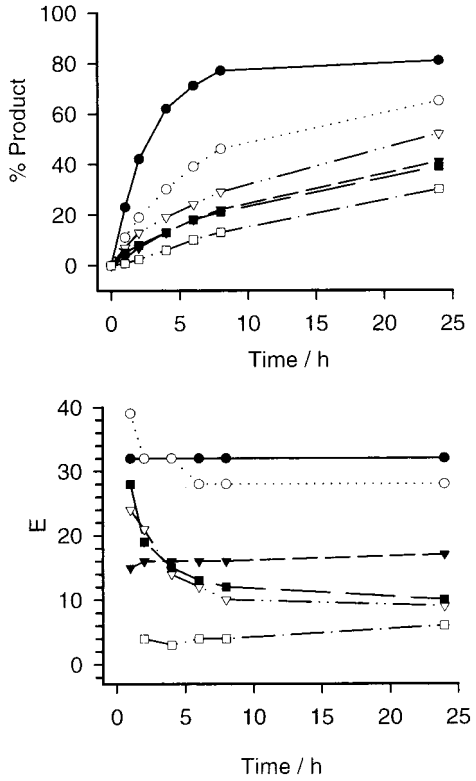


Figure 1. Effect of adding acids on (a) the catalytic activity and (b) enantioselectivity of CALB-butanolysis of (±)-2-phenyl-4-benzyl-5(4*H*)-oxazolone (**1**). The figures show the results obtained in the presence of *N*-benzoylphenylalanine (**3**) (■), acetic acid (□), Et_3N (●), Et_3N, and (**3**) (○), Et_3N, and acetic acid (▼) and additive free (▽). The amount of Et_3N added was 14 mol % and that for both acids was 24 mol %. The reactions were carried out in dry toluene ($a_w \approx 0.0$) at 37 °C.

from an initial value of $E = 24$ (92% ee) to $E = 4$ (59% ee). Since the presence of acetic acid attenuates the activity and *E*-value in a similar way to that of *N*-benzoylphenylalanine, chiral inhibition can be ruled out as a possible mechanism.

We had previously shown that Et_3N increases the enantioselectivity of CALB in organic solvents by ion-pairing byproduct acid, thereby removing acid from the enzyme microenvironment. Other researchers, too, have also shown for a number of different lipases that poor *E* can be overcome by the addition of an organic base.[14] However, there has been no systematic investigation of the effect of the base pK_a on the enantioselective behavior of lipases. We, therefore, decided to use a range of bases of differing pK_a[15] to investigate this. The bases we chose for study were *N*,*N*-dimethylaniline (DMA, pK_a = 4.38), *N*,*N*-4-(dimethylamino)pyridine (DMAP; pK_a 6.09), triethylamine (Et_3N, pK_a = 10.93), and *N*,*N*-diisopropylamine (DIPA, pK_a = 11.05). The reactions were all carried out in dry toluene ($a_w \sim 0.0$), and the organic base was added at the start of each reaction. The results obtained are shown in Figure 2a,b. Et_3N, DMAP,[16] and DIPA

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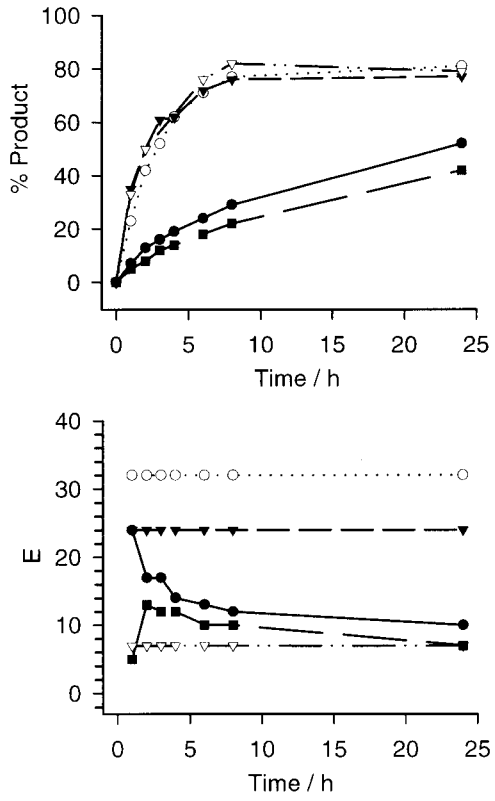

Figure 2. Effect of different organo-soluble bases on the catalytic activity (a) and enantioselectivity (b) of CALB-butanolysis of (±)-2-phenyl-4-benzyl-5(4*H*)-oxazolone. The bases added were Et_3N (○), iPr_2NH (▼), DMAP (▽), *N*,*N*-dimethylaniline (■), and in the absence of base (●). A catalytic amount (14 mol %) of the given base was added at the beginning of the reaction. The reactions were carried out in dry toluene ($a_w \approx 0.0$) at 37 °C.

accelerate the reaction rate by approximately 10-fold over that observed for the CALB-catalyzed reaction alone. The low pK_a base, *N*,*N*-dimethylaniline marginally reduces the enzyme-catalyzed reaction rate compared to additive-free CALB.

Figure 2b shows the effect of the base pK_a on the *E*-value for the product (*S*) butyl ester obtained from the CALB-catalyzed reaction. For a reaction containing no organic base, we observe an initially high *E*-value that steadily decreases with time. Remarkably, this decrease can be arrested immediately when a base of high pK_a (DIPA (11.05) or Et_3N (10.93)) is added. For these systems, control of the *E*-value is obtained and remains constant and high throughout the course of the reaction (24 h). Switching to a base of low pK_a fails to control the enantioselectivity, as observed for a reaction containing DMA (pK_a 4.38).

The highest *E* value was observed with Et_3N ($E = 32$, 94% ee). Addition of DIPA also results in enhancement and stabilization to the *E*-ratio ($E = 24$, 92% ee), as found for Et_3N. Addition of DMAP leads to the lowest *E*-value of all the bases tested, $E = 7$ (75% ee). In this case, the *E*-value is attenuated to a low but constant value. DMA reduces the *E*-value over time, to a value comparable with that found for CALB alone. It can be seen from these data that there is a correlation between CALB enantioselectivity and base pK_a: bases of high pK_a control and maintain a constant *E*-value; those of low pK_a are unable to maintain/control CALB enantioselectivity.

It is, however, difficult to quantify the effect of base pK_a on the enantioselectivity of CALB for a number of reasons. First, base solubility is solvent dependent, and this will result in differential binding of base to the enzyme surface and at or near the active-site. Also the base strength will depend on solvent polarity and protic/nonprotic nature.[17]

Potentially anomalous results associated with using organic bases can be ruled out if the enzyme protonation-state is altered *in situ* by a solid-state organic buffer. The solid-state buffers used in this study are zwitterionic biological buffers of the kind commonly used for controlling aqueous pH. As shown in eq 1, the acid and sodium salt together control the ionization-state of the enzyme's carboxylic groups. The buffer cannot bind to the protein surface/active site but can only exchange ions (Na^+/H^+), and therefore any changes to the *E*-value can be attributed to an altered enzyme protonation-state. Solid-state buffers can therefore be used in systems where reactants or products with acid−base properties are produced. These buffers have a significant advantage over the organo-soluble bases: they are present in the solvent as crystalline solids and will generate a single fixed value of exchange potential at equilibrium regardless of the solvent since they have a fixed thermodynamic activity.

$$^{+}NHR_2\sim\sim SO_3^{-} + Enz\text{-}COO^{-}Na^{+} \rightleftharpoons NR_2\sim\sim SO_3^{-}Na^{+} + Enz\text{-}COOH \quad (1)$$

With this aim in mind, we screened a range of biological buffers of differing acid−base strength: CAPSO (9.7); AMPSO (9.3); HEPPSO (7.8); HEPES (7.5); MOPS (7.2), and PIPES (6.8). The pK_a values of these buffers in aqueous solution are given in parentheses.[15] Prior to addition of the substrates, the solid-state buffer and the corresponding sodium salt were preequilibrated with the enzyme. The results obtained are shown in Figures 3a,b.

We observed (Figure 3b) that as the aqueous pK_a of the buffer increases, so too does the *E*-value, with the exception of HEPES/HEPES.Na and HEPPSO/HEPPSO.Na. Harper et al. have recently used an organo-soluble indicator[5e] to screen for appropriate buffer pairs that would be within the correct pK_a for controlling the catalytic activity of the serine protease, subtilisin Carlsberg. In the presence of these buffers, they found an anomalous indicator response using either HEPES/HEPES.Na or HEPPSO/HEPPSO.Na that may also provide an explanation for our observations.

We can also re-set a low *E*-value to a higher one, using these solid-state buffers. An initially low *E*-value, as a result of the presence of DMAP (pK_a 6.09) ($E = 7$, 75% ee) can be reset *in situ* to a higher level ($E = 10$, 81% ee) by the addition of a solid-state buffer of higher pK_a (CAPSO, pK_a 9.7). Full recovery of CALB enantioselectivity to the level observed with CALB and CAPSO/CAPSO.Na is not found, which may be as a result of residual DMAP bound to the protein.

As discussed previously, solid-state buffers will produce a fixed ionization-state regardless of the solvent, and indeed when we use *t*-BuOMe as the reaction solvent,

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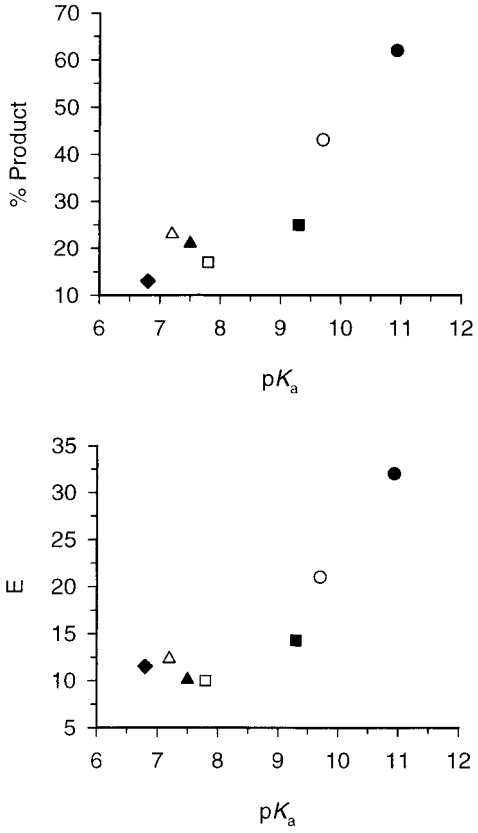

Figure 3. Effect of solid-state buffers on catalytic activity (a) and enantioselectivity (b) of CALB-butanolysis of (**1**). Figures show the results obtained after 4 h of reaction. Solid-state buffers/sodium salts were preequilibrated with the enzyme prior to the addition of substrates. The reactions were carried out in dry toluene ($a_w \approx 0.0$) at 37 °C. The solid-state buffer pairs were PIPES/PIPES.Na (◆), MOPS/MOPS.Na (△), HEPES/HEPES.Na (▲), HEPPSO/HEPPSO.Na (□), AMPSO/AMPSO.Na (■), CAPSO/CAPSO.Na (○). Also, the results obtained in the presence of Et_3N (●) are shown. Reaction catalyzed by CALB without any additive gave 19% product and an *E*-value of 14.3. Standard deviations average over three measurements for each one were between ±1.00% to ±2.50% for the % product values, and ±0.00% to ±1.15% for the *E*-values.

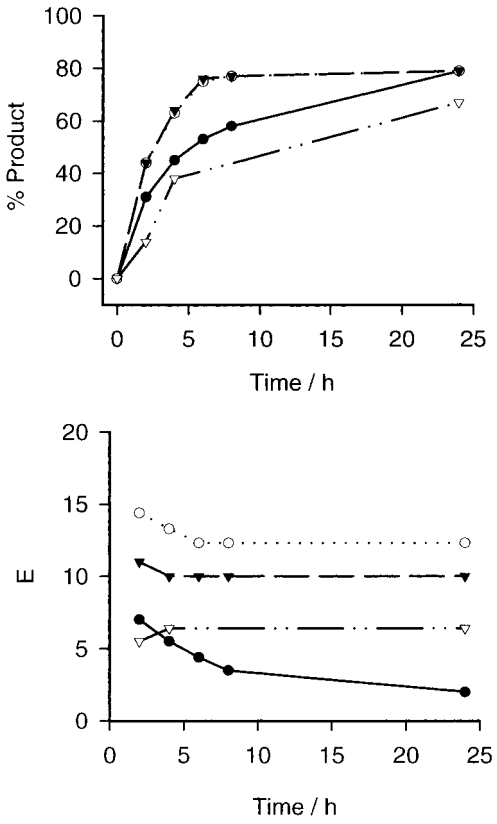

Figure 4. Catalytic performance of CALB in *t*-BuOMe. Catalytic activity (a) and enantioselctivity (b) in the presence of Et_3N (○), DMAP (▼), the biological buffer CAPSO/sodium salt (▽), and additive free (●). The reactions were carried out at 37 °C.

we find a very similar trend to that observed in toluene, thereby ruling out any specific solvent effects of toluene. Figure 4a,b shows the effect of Et_3N, DMAP, and CAPSO/CAPSO.Na on the enantioselectivity of the CALB-catalyzed (*S*)-butyl ester in *t*-BuOMe. Without the addition of any of these additives, there is a decrease to the *E*-value when monitored over 24 h from $E = 7$ (75% ee) to $E = 2$ (33% ee).

Together, with the possibility of a specific solvent effect, we also considered the posibility of interference in binding of base/buffer to the support material used in the preparation of CALB. Therefore, we used a pure preparation of CALB supplied as cross-linked enzyme crystals (CLEC−CALB). Similarly, we probed the response to changes in *E*-value of a different lipase (from *Mucor miehei*) using either Et_3N or CAPSO/CAPSO.Na, Lipase from *Mucor miehei* has been reported by several groups[14a] to be activated in the presence of Et_3N. From Table 1 it can be seen that the support has no affect on CALB enantioselectivity. Although the intrinsic selectivity of the *Mucor miehei* lipase is lower than that found for CALB, both Et_3N and CAPSO/CAPSO.Na enhance both the activity and selectivity.

Table 1. Effect of Additives on Lipase-Catalyzed Dynamic Resolution of 1

	CALB		CALB-CLEC		*M. miehei*	
additive	% P[a]	% ee[b]	% P[a]	% ee[b]	% P[a]	% ee[b]
none	19	87[c]	21	87[d]	43	48[e]
Et_3N	62	94	72	95	47	61
CAPSO	43	91	54	93	32	57

[a] Percentage of *N*-benzoyphenylalanine butyl ester after 4 h of reaction. [b] ee of (*S*)-butyl ester (**2**) after 4 h of reaction. [c] Initial value 92%. [d] Initial value 91%. [e] Initial value 59%.

Since most enzyme-catalyzed reactions in organic solvents are not practicably carried out under anhydrous conditions, we tested the catalytic performance of CALB as a function of the thermodynamic water activity (a_w).[18] Figure 5a,b shows the effect of either CAPSO/CAPSO.Na or Et_3N on the catalytic activity and *E*-value, respectively. We can see that in all cases the catalytic rate decreases with increasing hydration level. This effect is offset by Et_3N, and only under dry or low hydration conditions ($a_w \sim 0$, 0.11) by CAPSO/CAPSO.Na. In Figure 5b the *E*-value ($t = 24$ h) is plotted as a function of a_w. Whereas, the *E*-value decreases significantly as a function of a_w, remarkably both Et_3N and CAPSO maintain higher *E* values over the water activity range. Reactions were not carried out at $a_w > 0.86$ as a small amount of CAPSO.Na is likely to dissolve in the organic solvent, and this will

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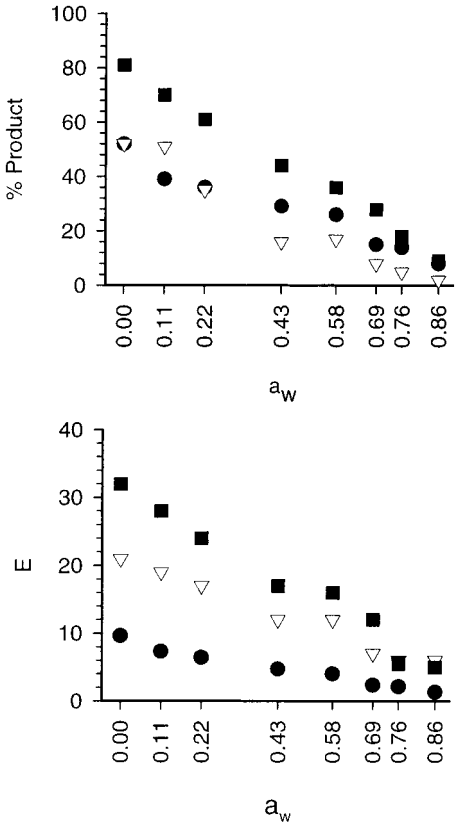

Figure 5. Catalytic performance of CALB as a function of thermodynamic water activity (a_w). Catalytic activity (a) and enantioselectivity (b) obtained after 24 h of reaction in the presence of Et_3N (■) and CAPSO/sodium salt (▽) and additive free (●) at different a_w. The reactions were carried out in dry toluene at 37 °C.

therefore adversely affect the acid−base behavior of the buffer at $a_w > 0.86$.[5e]

Despite the large number of studies[14] that have reported a qualitatively beneficial effect of Et_3N on a range lypolytic enantioselectivity, there have been no systematic studies aimed at quantifying and establishing the molecular basis for this effect. We have therefore sought to address this issue via controlled protonation-state changes to CALB. This enzyme has proven to be a potent biocatalyst for the stereoselective kinetic resolution of secondary alcohols and is also able to accept a broad substrate range.[11]

The propensity for organic bases to increase the catalytic activity of lipases has in some cases[14c,16] been attributed to the formation of an ion-pair between the base and acid byproduct. The rationale and evidence for this is that the base removes acid from the enzyme's microenvironment and restores catalytic function.[9] Even minor amounts of an acidic component present in the solvent will result in protonation and reversible loss to catalytic activity as demonstrated using a chromoionophoric indicator that is sensitive to protonation changes.[5e]

To gain an insight into the mechanism of enhancement or attenuation to the enantioselectivity of CALB, we have compared the initial rate for each of the enantiomers under a given set of conditions. We observed that when the additive to the system is either organic base or a solid-state buffer (of high pK_a, e.g., CAPSO) both the catalytic rate of the *R*- and favored *S*-butyl ester increase relative to that of CALB with no additive present. This indicates that both the solid-state buffers and the organosoluble bases (of sufficiently high pK_a) are acting via the same mechanism. A hydrated system, which results in poor enantioselectivity, leads to an increase in the rate of the *R*-enantiomer and a decrease in the catalytic rate of the *S*-enantiomer relative to that of CALB alone. When the medium is acidifed, the same effect is found.

There are a number of possible reasons for the observed effects. The ability of bases and solid-state buffers to selectively modulate the activity and enantioselectivity may be attributable to control of the protonation of the catalytic His-224. Protonation of this residue by acidic species may result in modulation of the activity and enantioselectivity. Kazlauskas and co-workers[19] have suggested a structural basis for the chiral preference of lipases. High resolution 3D structures derived from X-ray data show that the interactions between the menthyl ring of the slow-reacting enantiomer (1*S*)-menthyl hexylphosphonate and the histidine of the catalytic triad disrupt the hydrogen bond between the Nϵ2 of the imidazole ring and the menthol oxygen atom, most likely accounting for the slower reaction of the (1*S*)-enantiomer.

Uppenberg et al.[11a] have determined the three-dimensional structure of CALB cocrystallized with the detergent Tween 80 and in another structure in complex with a racemic mixture of a covalently bound phosphonate inhibitor. They observed that as the pH was raised, the short α5 helix (a potential lid forming α-helix) became disordered, as manifested by a disappearance of the electron density for this helix. Therefore, this raises the possibility that the disordered state of the helix under conditions where deprotonation occurs in organic media also results in an increase of disorder of the helix (i.e., an ionization dependence) thereby adopting a more open conformation for substrate access.

Conclusion

We have shown that a simple strategy for controlling the protonation state of a lipase, *Candida antarctica* lipase B, in organic solvents is to use solid-state buffers suspended in the reaction media or soluble organic bases of high aqueous pK_a. The enantioselectivity and the catalytic activity of CALB can be substantially improved by either addition of a base or a solid-state buffer of high pK_a (Et_3N/CAPSO.CAPSO.Na).

The method may therefore provide a reliable strategy for the prediction and control of enantioselectivity of enzymes that employ general acid−base chemistry for their catalytic processes.

Experimental Section

Reagents and Solvents. The lipases, Chirazyme L-2, c−f. C2, lyo (*Candida antarctica* lipase B) and Lipozyme, RM IM (*Mucor miehei* lipase) were received as an immobilized preparation (Boehringer Mannheim, Germany and Novo Nordisk, Denmark respectively) and were dehydrated over P_2O_5 (at room temperature) for 4 days before use. Cross-linked enzyme crystals of *Candida antarctica* lipase, CALB−CLEC were obtained from Altus Biologics Inc. (Cambridge, MA) as an aqueous suspension. The substrate, (±)-2-phenyl-4-benzyloxazol-5(4*H*)-one (**1**) was prepared from *dl*-phenylalanine as previously described.[12] *n*BuOH was purchased from Aldrich

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(HPLC grade). Inorganic salts (analytical grade) were obtained from Aldrich U.K. and used without further purification. Biological buffers: AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropanesulfonic acid), CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid), HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), HEPPSO (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-hydroxypropanesulfonic acid]), MOPS (3-[*N*-morpholino]propanesulfonic acid), PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid), and their sodium salts were purchased from Sigma. All other reagents were obtained from Aldrich, Sigma (U.K.), Fisher (U.K), or Fluka (U.K) and were of analytical grade. Solvents used in the enzyme-catalyzed reactions were of HPLC grade, dried, and stored over freshly reactivated 4 Å molecular sieves. The water content of the dry solvents was determined using a 684 Karl Fischer Coulometer coulometric titrator[20] and found to be <0.001 wt %. Hydrated solvents were also analyzed by Karl-Fischer titration.

Instruments. Chiral HPLC analyses (Chiralcel-OD column (Daicel 250 × 4.6 mm) were carried out on a Waters 2690 Separations module (Waters U.K.)

Enzyme and Solvent Hydration. Vials containing chirazyme and solvent (toluene) were placed in separate sealed jars containing a saturated salt solution for control of the thermodynamic water activity (a_w).[18] Typically, samples of enzyme were equilibrated for 4 days and solvent for 24 h, both at room temperature.

Typical Enzymatic Reaction. The substrate, (±)-2-phenyl-4-benzyloxazol-5(4*H*)-one (**1**) (0.16 mmol; 40 mg), was placed in a screw top vial together with the solvent (4 mL; either anhydrous or hydrated), butan-1-ol (0.24 mmol; 21.9 μL), and the enzyme (CALB (40 mg), *Mucor miehei* (5 mg) or CALB−CLEC (7.5 mg)). The reaction vial was shaken at 250 rpm on a rotatory shaker at 37 °C. The progress of the reaction and the ee of the product (*N*-benzoylphenylalanine butyl ester) (**2**) were monitored by HPLC using chiral chromatography (Chiralcel-OD), and the products were monitored by UV at $\lambda = 254$ nm. Aliquots (50 μL) were periodically withdrawn and dissolved with hexane/propan-2-ol (90:10 v/v) to 1 mL final volume, filtered, and then analyzed by HPLC as described. The mobile phase composition was hexane/propan-2-ol (90:10 v/v). The percentage product obtained was calculated using an external standard method. A linear calibration curve was constructed for the racemic butyl ester by preparing standard solutions containing the pure racemic butyl ester at five different known concentrations, covering that obtained for a typical reaction. Chiral HPLC analyses: (*S*)- *N*-benzoylphenylalanine butyl ester, t_R 8.95 min; (±)-*N*-benzoylphenylalanine butyl ester, t_R 5.67 and 8.69 min; $R_S = 4.25$ (hexane:propan-2-ol, 90:10 (v/v); 0.8 mL/min). The enantioselectivity values were calculated according to the literature.[21]

No product was detected when the reactions were carried out in the absence of the biocatalyst.

Enzyme Preparation of CALB−CLEC for Use in Organic Solvent. The enzyme crystals were supplied and stored in an aqueous solution. Prior to use, the aqueous CLEC suspension (150 μL; 7.5 mg) was washed with propan-1-ol (4 × 1.3 mL) and then with butan-1-ol (2 × 0.6 mL). After each wash, the enzyme suspension was centrifuged and the solvent removed. Care was taken to ensure that the enzyme pellet remained wet at all times. The enzyme was then used directly in a reaction by resuspension in the appropriate organic solvent.

Enzymatic Reaction in the Presence of Organic Bases. The substrate, (±)-2-phenyl-4-benzyloxazol-5(4*H*)-one (**1**) (0.16 mmol; 40 mg), was placed in a screw top vial together with the solvent (4 mL; either anhydrous or hydrated), butan-1-ol (0.24 mmol; 21.9 μL), enzyme (CALB (40 mg), *Mucor miehei* (5 mg) or CALB−CLEC (7.5 mg)) and the organic base (14 mol %; Et_3N, DMAP, *N*,*N*-dimethylaniline or *N*,*N*-diisopropylamine). The reaction and subsequent analyses were performed using the procedure described above.

Enzymatic Reaction in the Presence of Organic Acids. The substrate, (±)-2-phenyl-4-benzyloxazol-5(4*H*)-one (**1**) (0.16 mmol; 40 mg), was placed in a screw top vial together with the solvent (4 mL; either anhydrous or hydrated), butan-1-ol (0.24 mmol; 21.9 μL), enzyme (CALB (40 mg), *Mucor miehei* (5 mg) or CALB−CLEC (7.5 mg)) and the acid (24 mol %; *N*-benzoylphenylalanine (**3**) or acetic acid). The reactions were carried out both in the presence and absence of Et_3N (14 mol %). The reactions and subsequent analyses were performed using the procedure described above.

Enzymatic Reaction Using Solid-State Buffers. The solid-state buffers/sodium salt (25 mg of each form) were equilibrated together with the catalyst (CALB (40 mg), *Mucor miehei* (5 mg) or CALB−CLEC (7.5 mg)) for 3 h in the case of CALB and CALB−CLEC and for 5 h in that of *Mucor miehei* in dry toluene prior to the addition of the substrates and initiation of the reaction. The substrates **1** (0.16 mmol; 40 mg) and BuOH (0.24 mmol; 21.9 μL) were added to the reaction vial and shaken at 250 rpm on a rotary shaker at 37 °C. The progress of the reaction and ee of the product obtained were monitored by HPLC (Chiralcel-OD) as previously described.

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