Exploring the Active-Site of a Rationally Redesigned Lipase for Catalysis of Michael-Type Additions

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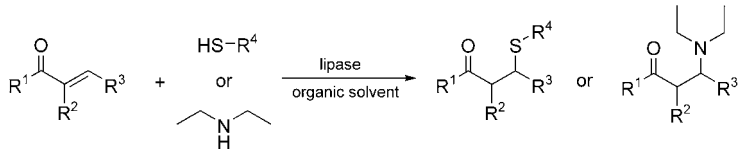
Michael-type additions of various thiols and α,β-unsaturated carbonyl compounds were performed in organic solvent catalyzed by wild-type and a rationally redesigned mutant of Candida antarctica *lipase B. The mutant lacks the nucleophilic serine 105 in the active-site; this results in a changed catalytic mechanism of the enzyme. The possibility of utilizing this mutant for Michael-type additions was initially explored by quantum-chemical calculations on the reaction between acrolein and methanethiol in a model system. The model system was constructed on the basis of docking and molecular-dynamics simulations and was designed to simulate the catalytic properties of the active site. The catalytic system was explored experimentally with a range of different substrates. The* k_{cat} *values were found to be in the range of* 10^{-3} *to 4* min^{-1}*, similar to the values obtained with aldolase antibodies. The enzyme proficiency was* 10^{7}*. Furthermore, the Michael-type reactions followed saturation kinetics and were confirmed to take place in the enzyme active site.*

Introduction

The 1,4-addition of a nucleophile to an α,β-unsaturated carbonyl compound, commonly referred to as a Michael-type addition, is a fundamental reaction in organic synthesis (Scheme 1). It is especially important for the synthesis of compounds with a thioether linkage where reactions similar to Mannich- or aldol reactions do not exist. Such hetero-Michael additions can be catalyzed by Brønsted acids[1] or variants of Lewis acids and bases.[2]



Scheme 1. *A general reaction scheme for the Michael-type addition of various thiols or an amine to α,β-unsaturated carbonyl compounds (Table 3) catalyzed by* Candida antarctica *lipase B.*

In nature, there are enzymes with Michael-type addition as a part of their natural catalytic mechanism, for instance *O*-acetylserine sulfhydrylase[3] and thymidylate synthase.[4] Despite this, there are few reports of enzyme-catalyzed Michael-type additions. However, Kitazume et al. used some hydrolytic enzymes (not *Candida antarctica* lipase B) to catalyze Michael-type additions of triflourinated α,β-unsaturated carbonyl compounds in buffer solution.[5] Recently, Lin et al. reported that alkaline protease from *Bacillus subtilis* catalyzes Michael-type additions of imidazole and pyrimidine derivates to α,β-unsaturated carbonyl esters.[6]

In this paper, we present evidence for active-site-catalyzed Michael-type additions. The results are based on experiments made by varying the pH conditions, covalent inhibition of the active-site, experiments made in the absence of enzyme and kinetic analyses. Reactions between a wide range of thiols and α,β-unsaturated carbonyl compounds could be catalyzed by the wild-type and the Ser105Ala mutant of *C. antarctica* lipase B. According to turnover numbers from kinetic studies, the mutant was more efficient than the wild-type enzyme for the catalysis of the tested substrates. In addition, the Ser105Ala mutant has previously been shown to function as catalyst for aldol additions, another unnatural reaction for the lipase.[7] The idea of targeting this particular mutant for catalysis of Michael-type additions was based on a quantum-chemical analysis that preceded the experimental work.

Results and Discussion

The reaction mechanism for the Michael-type addition between methanethiol and acrolein was explored in detail by means of quantum-chemical calculations in a model system.

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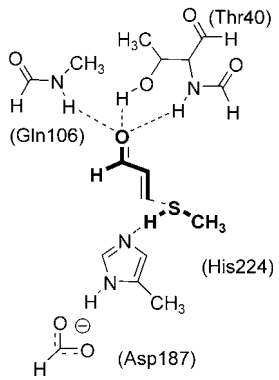
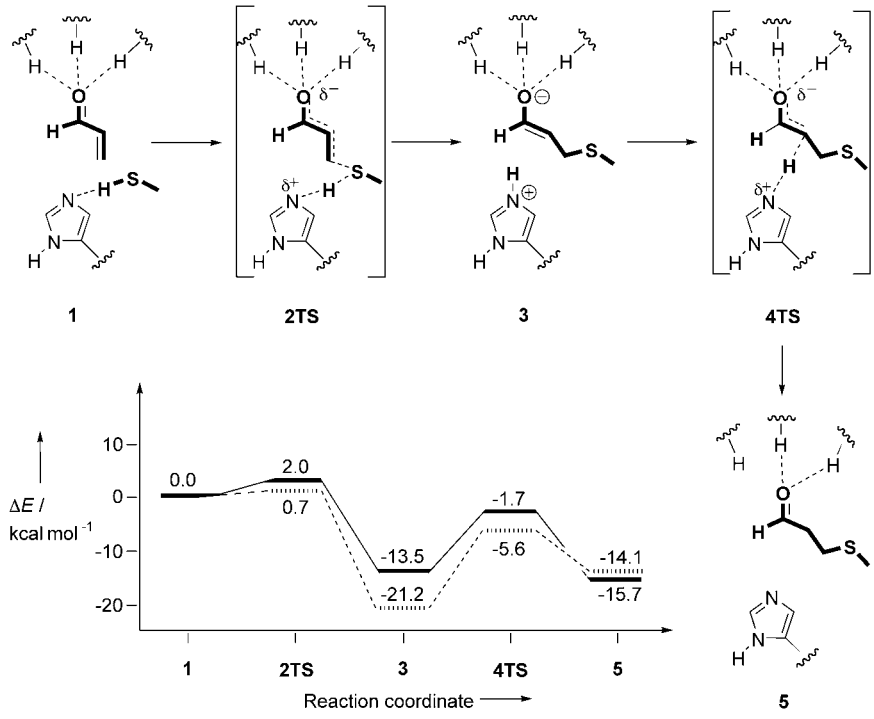
The model system incorporated all catalytically important parts of the Ser105Ala mutant's active site (Scheme 2). Similar, but smaller, model systems have been successfully used to study the catalysis of aldol addition and Baeyer–Villiger oxidation in the mutant and ester hydrolysis in the wild-type enzyme.[7–9]



Scheme 2. *Model system used in the quantum-chemical studies of the Michael-type addition of methanethiol to acrolein (bold). The catalytic machinery, the histidine–aspartate dyad, together with the oxyanion hole, is represented by molecular fragments and annotated as corresponding amino acids in the enzyme.*

Automated docking (Autodock 3.0)[12] of the two substrates in the mutant enzyme showed that the unsaturated aldehyde always bonded to the oxyanion hole, while the thiol in the majority of the dockings was in proximity to, or hydrogen bonded to, His224. Molecular dynamic simulations with the program Q[13] confirmed the docking results and showed the existence of near-attack complexes between the substrates over extended periods of time. On the basis of these results, the potential-energy surface of the reaction was explored. The quantum-chemical calculations support the two-step reaction mechanism presented in Scheme 3. Starting from the near-attack complex (**1**) with the thiol hydrogen bonded to His224, the first step is a proton transfer to His224 concerted with a nucleophilic attack of the sulfur on the β-carbon of the unsaturated aldehyde. The basicity of His224 is increased due to the negative Asp187, and the nucleophilic attack is facilitated by the oxyanion hole, since hydrogen bonding stabilizes the partial negative charge on the carbonyl oxygen in the TS (**2TS**). The combination of these two effects leads to a considerable stabilization of **2TS**; the activation energy was only 0.7 kcal mol^{-1} at the B3LYP/6-31+G* level of theory. Consideration of solvation effects lead to an increase in the activation energy of only 1.3 kcal mol^{-1}. Thus, the results indicate that this reaction step should be catalyzed very efficiently by the Ser105Ala mutant. The formation of intermediate **3** was exothermic by 21.2 or 13.5 kcal mol^{-1} before and after solvation correction, respectively. The last step, a proton transfer from His224 to the substrate (**4TS**), had an activation energy of 15.6 kcal mol^{-1} rela-

All stationary points (minima and transition states (TS)) of the reaction in the enzyme were optimized by using the B3LYP method with a specially augmented 6-31G basis set. The positions of the catalytic residues (the catalytic dyad and oxyanion hole) were determined from molecular dynamics simulations of the mutant, and a limited number (3) of constraints were used to keep the overall structure of the active site. More specifically, the distance between Asp187 and Thr40, the distance between Thr40 and Gln106 and the orientation of His224 relative to Asp187 were constrained. Thus, full flexibility were maintained within the different residue models. Single-point calculations at the B3LYP/6-31+G* level were performed to obtain more accurate energies. The Gaussian 98 suite of programs[10] was used for the gas-phase quantum-chemical calculations. In order to estimate the solvation effects of the enzyme environment on the catalytic reaction, the Poisson–Boltzmann method of Jaguar 4.1[11] was used to calculate solvation energies at the B3LYP/6-31+G* level with a dielectric constant of 4.0.



Scheme 3. *Two-step mechanism of the Michael-type addition of methanethiol to acrolein in the mutant suggested by calculations and computed potential-energy surface (PES) B3LYP/6-31+G* energies (dashed line) relative to the reaction complex (**1**). Solid line represents energies corrected for solvation effects.*

tive to **3**. The barrier height became 11.8 kcal mol^{-1} after the addition of solvation effects. This final proton transfer is clearly the rate-determining step in the reaction, and the computed activation energy is similar in magnitude to that of the natural reaction in lipases.

In normal base-catalyzed Michael-type addition of thiol to unsaturated carbonyl compounds, the nucleophilic sulfur attack is considered to be the rate-determining step. The reason for this difference is most likely the presence of the oxyanion hole in the enzyme, which has the effect of strongly stabilizing **2TS** and **3** through hydrogen bonding. It should be noted that gas-phase calculations indicate that the stabilization of **3** is so strong that the reaction might not proceed to product (**5**). However, solvation effects raise the energy of **3** relative to **5** and make the final reaction step exothermic by 2.2 kcal mol^{-1}. The most likely explanation is that the interaction between the oxyanion hole and the negative oxygen of **3** is so strong that it diminishes the interactions between the oxyanion hole and neighbouring groups in the enzyme. In agreement with this interpretation, the optimized geometries showed that the oxyanion hole formed three hydrogen bonds to the oxygen in **3** but only two in **5**.

The quantum-chemical calculations were followed by an experimental study. First, *C. antarctica* lipase B Ser105Ala was created by site-directed mutagenesis.[7] Both wild-type *C. antarctica* lipase B and the Ser105Ala variant were expressed in *Pichia pastoris*, purified, immobilized on Accurel MP1000 and equilibrated to obtain a water activity of 0.11.[14,15]

The ability of wild-type lipase and the Ser105Ala variant to catalyze the addition of pentane-2-thiol to but-2-enal was tested in cyclohexane. The pH dependence of the enzymes was studied by immobilization of the wild-type and mutant in potassium phosphate buffers at pH 7.6 or 8.6 (Figure 1). There was a tenfold increase in the apparent turnover number (k_{cat}^{app}) as the buffer was changed from pH 7.6 to 8.6 for the mutant, while the reaction rate of the wild-type was almost independent of pH (Table 1). The proposed mechanism in Scheme 3 requires the histidine 224 to be in a deprotonated state. Thus, the observed pH dependence on the reaction rate indicates that the histidine changed protonation state on going from pH 7.6 to 8.6 in the mutant, while, in the wild-type, the histidine was deprotonated at both pH values. The lower pK_a of the histidine in the wild-type is not surprising considering that the deprotonated form of the histidine can form a hydrogen bond to Ser105, an ability that the mutant obviously lacks. This pH dependence of the mutant enzyme supports the key role of histidine 224 for catalysis of the Michael-type addition reaction.

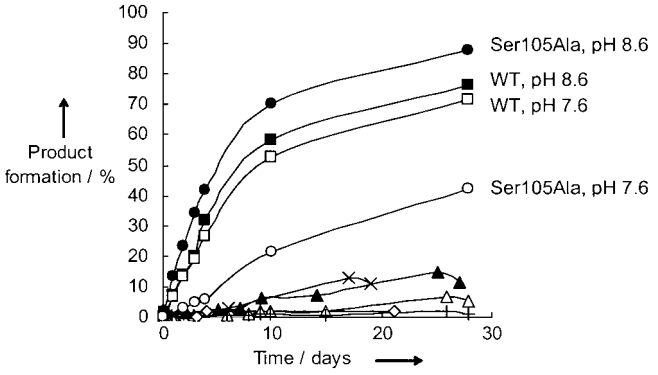


Figure 1. *Michael-type addition of pentane-2-thiol to but-2-enal in cyclohexane catalyzed by* C. antarctica *lipase B Ser105Ala mutant (●, ○) and wild-type (■, □). The bottom five curves show control experiments with imidazole (×), empty carrier (▲, △), inhibited wild-type (◇) and the background reaction with substrates only (+). Filled and open symbols denote immobilization of enzyme or prewashed carrier with potassium phosphate buffer of pH 8.6 or pH 7.6, respectively.*

Table 1. *The pH dependence of k_{cat}^{app} for the Michael-type addition of pentane-2-thiol (400 mM) to but-2-enal (300 mM) catalyzed by* C. antarctica *lipase B variants in c-hexane. The calculated turnover numbers are apparent since they are only measured at one concentration.*

Catalyst	pH[a]	k_{cat}^{app} [min^{-1}]
CALB Ser105Ala	8.6	1.03
CALB Ser105Ala	7.6	0.099
CALB WT	8.6	0.67
CALB WT	7.6	0.62
inhibited CALB WT	7.6	0.0034
imidazole (0.037 mM)		0.025[b]

[a] pH of the immobilization buffer potassium phosphate. [b] Turnover frequency.

Several control experiments were performed to prove that the reaction occurs in the active site of the enzyme. To investigate the requirement of the active-site for catalysis, immobilized wild-type *C. antarctica* lipase B was inhibited with *p*-nitrophenyl *n*-hexylphosphonate.[15] Inhibition is only possible with wild-type *C. antarctica* lipase B, since *p*-nitrophenyl *n*-hexylphosphonate reacts with serine 105 to form a covalent adduct. The inhibition blocked the catalysis (Figure 1) and the k_{cat}^{app} value decreased 180 times (Table 1) compared to that of the wild-type lipase. Further elucidation of the necessity of the protein and its oxyanion-hole for catalysis was made by the substitution of the lipase for imidazole. Imidazole was chosen as a control catalyst since the side-chain of histidine has the structure of imidazole. Free imidazole was added as catalyst to a Michael-type reaction mixture at a concentration corresponding to that of the lipase. The imidazole-catalyzed reaction was much slower than the lipase-catalyzed reactions (Figure 1 and Table 1); this suggests that the protein with the oxyanion hole is needed for efficient catalysis. Control experiments were run with empty carrier to confirm that the catalytic effect of the immobilized enzyme was not due to the carrier or the immobilization buffer. Two control experiments were run with empty carrier prewashed in an immobilization buffer of either pH 7.6 or pH 8.6 and with the same water activity as the immobilized enzyme. These two control reactions were almost as slow as the background reaction with substrates only (Figure 1). This indicates that the catalytic activity of the enzyme-catalyzed reactions is a result of the enzymes and not of the carrier or the immobilization buffers. The results of these control experiments give strong indications that the catalysis of the Michael-

type addition takes place in the active site of *Candida antarctica* lipase B.

To gain more information about the catalytic system, a kinetic study of *C. antarctica* lipase B Ser105Ala was made in which the initial rates were measured. One reaction system consisting of thiophenol and cyclohex-2-enone in toluene was chosen for evaluation. The concentration of thiophenol (30 mM) was kept constant, and the concentration of cyclohex-2-enone was varied (Table 2). Since only one of the two substrates was varied, the calculated values are apparent and underestimated. The reaction followed Michaelis–Menten kinetics when the data were treated according to pseudo-first-order kinetics. The catalytic proficiency ($(k_{cat}^{app}/K_M^{app})/k_{non}$) was estimated to be 24 million, which is comparable to natural enzymes.[16]

Table 2. The catalytic proficiency of *C. antarctica* lipase B Ser105Ala for the Michael-type addition of thiophenol to cyclohex-2-enone in toluene.

Catalyst	k_{cat}^{app} [s^{-1}]	k_{cat}^{app}/K_M^{app} [s^{-1} M^{-1}]	k_{non} [s^{-1} M^{-1}]	k_{cat}^{app}/k_{non} [M]	$(k_{cat}^{app}/K_M^{app})/k_{non}$
CALB Ser105Ala	2.2×10^{-2}	3.4			
Substrates only			1.4×10^{-7}		
Ratios				1.6×10^{5}	2.4×10^{7}

The scope of the catalytic system was explored with 13 different substrates (Table 3). Michael acceptors, like aldehydes, ketones and esters, formed Michael-type adducts with thiols. Alcohols were tested as nucleophiles, but did not react in a Michael-type addition. This can be explained by the concept of soft and hard nucleophiles. Thiols are considered soft nucleophiles and therefore preferably react at the β-carbon in a 1,4-fashion. Alcohols are hard nucleophiles and consequently react in a 1,2-fashion. Even primary and secondary amines were tested, but resulted in imine and enamine formation, respectively, when added to aldehydes. Secondary amines, however, formed Michael-type adduct when added to an α,β-unsaturated carbonyl ester, methyl acrylate. No enantioselectivity could be achieved for any of the tested substrates with either the wild-type or the Ser105Ala variant of the lipase. This may seem to be a contradictory result since these reactions were shown to take place in the enzyme active-site. The reason for this will be further investigated.

The catalytic performance of the Ser105Ala mutant was compared to the wild-type enzyme for some of the tested substrates (Table 3). As shown in Table 3, the mutant enzyme catalyzed Michael-type addition of thiols faster than the wild-type enzyme in 6 out of 9 cases. The turnover numbers of entries 2, 7 and 13 were almost the same for both enzymes. Larger differences between the two enzymes can be seen for the reactions in entries 10, 17, 21 and 22. In entries 21 and 22 thiols were added to methyl acrylate. The catalysis of these two reactions was much faster with the mutant enzyme compared to the wild-type. However, the opposite effect was shown when a secondary amine, diethyl amine, was treated with methyl acrylate. The wild-type enzyme catalyzed this reaction with a k_{cat}^{app} value of 810 min^{-1}, while the k_{cat}^{app} value was less than 15 with the mutant enzyme. The characteristic k_{cat}^{app} values for the Michael-type addition of thiols range from 10^{-3} to 4 min^{-1}, which is comparable to k_{cat} values of cross-aldol reactions catalyzed by aldolase antibodies (10^{-3} to 1 min^{-1}).[17]

Table 3. *The α,β-unsaturated carbonyl compounds (300 mM) and thiols or diethyl amine (400 mM), according to Scheme 1), used to compare the catalytic performance of* C. antarctica *lipase B Ser105Ala and wild-type* C. antarctica *lipase B. Both enzymes were immobilized in 50 mM potassium phosphate buffer at pH 8.6. The calculated turnover numbers are apparent since they are measured at only one concentration.*

No.	R^1	R^2	R^3	R^4	k_{cat}^{app} mt [min^{-1}]	k_{cat}^{app} WT [min^{-1}]	k_{cat}^{app} mt/k_{cat}^{app} WT	k_{non} [min^{-1} M^{-1}]
1	H	H	H	*t*Bu	3.5			1.5×10^{-5}
2	H	H	Me	Et	4.3	4.6	0.94	3.0×10^{-5}
3	H	H	Me	*t*Bu	0.6			3.3×10^{-6}
4	H	H	Me	2-Bu	0.18			1.1×10^{-6}
5	H	H	Me	2-pent	1.03	0.67	1.5	9.9×10^{-7}
6	H	H	Me	Bz	1.3			1.3×10^{-4}
7	H	H	Et	Et	1.03	0.93	1.1	3.8×10^{-6}
8	H	H	Et	*t*Bu	7.7×10^{-3}			3.3×10^{-6}
9	H	H	Et	2-Bu	4.4×10^{-2}			6.5×10^{-8}
10	H	H	Et	2-pent	5.6×10^{-2}	1.8×10^{-2}	3.1	5.0×10^{-6}
11	H	H	Et	Bz	1.3			3.8×10^{-5}
12	H	Me	Et	Et	0.41			2.0×10^{-7}
13	H	Me	Et	Bz	0.21	0.22	0.96	3.3×10^{-5}
14	H	H	Ph	Et	0.13			6.5×10^{-5}
15	H	H	Ph	*t*Bu	2.4×10^{-2}			2.3×10^{-8}
16	H	H	Ph	2-Bu	5.8×10^{-2}			2.0×10^{-7}
17	H	H	Ph	2-pent	0.27	9.7×10^{-2}	2.8	9.9×10^{-8}
18	H	H	Ph	Bz	1.2			1.0×10^{-6}
19	[a]	[a]	[a]	2-pent	0.27	0.48	0.56	1.9×10^{-5}
20	[a]	[a]	[a]	Ph	1.4			1.1×10^{-7}
21	OMe	H	H	Et	6.4×10^{-2}	$<1.0\times10^{-2}$	>6.4	2.0×10^{-7}
22	OMe	H	H	2-pent	2.8×10^{-2}	$<1.7\times10^{-5}$	>1600	2.3×10^{-8}
23	OMe	H	H	[b]	<15	810	$<1.9\times10^{-2}$	2.4×10^{-3}

[a] Cyclohex-2-enone. [b] Diethyl amine.

Similar reaction rates for both enzymes were obtained in some of the reactions. This indicates that the serine in the wild-type does not significantly influence the catalysis by its presence. The reason might be that serine 105 can only interfere with the first step of the mechanism (Scheme 3). Since the second step of the reaction mechanism, the transfer of the proton from histidine 224 to the α-position of the substrate, was found to be rate limiting (Scheme 3), the presence of the serine 105 cannot be expected to significantly alter the reaction rate.

It can be noted that the measured reaction rates are not as high as indicated by the quantum-chemical calculations. How-

ever, the average error in calculations of reaction energetics with the computational method (B3LYP/6-31+G*) used in this work is around 4–5 kcal mol^{-1}.[18,19] In addition, the calculations were performed in a limited model system without consideration of enzyme dynamics. The frequency of formation of near-attack complexes (NACs) as well as the occurrence of promoting and demoting motions can have a significant impact on the reaction rates.[20–24] Thus, considering the importance of such effects and the accuracy of the quantum-chemical calculations, the discrepancy between theory and experiments is not surprising.

In conclusion, the quantum-chemical calculations indicate that the Michael-type addition of thiols to α,β-unsaturated carbonyl compounds should be catalyzed in the active-site of the Ser105Ala mutant of *C. antarctica* lipase B. This was confirmed by experimental data. Inhibition of the wild-type lipase blocked the catalysis; this proves that the Michael-type addition reaction is catalyzed in the active-site of the enzyme. The pH dependence of the immobilized *C. antarctica* lipase B Ser105Ala supports the key role of histidine 224 in the catalysis. This was also confirmed by using imidazole as catalyst. The kinetic study showed saturation (Michaelis–Menten) kinetics, and the catalytic proficiency of the Ser105Ala mutant to catalyze the Michael-type addition was in the order of 10^7. This value is in the same range as that of natural enzymes. The turnover numbers for *C. antarctica* lipase B towards Michael-type additions were improved in many cases by the mutation of serine 105 to alanine for the tested substrates.

Experimental Section

Protein production: *C. antarctica* lipase B Ser105Ala and wild-type *C. antarctica* lipase B were produced as previously described.[7,14]

Immobilisation: The Ser105Ala and wild-type *C. antarctica* lipases B were immobilized on the polypropylene carrier Accurel MP1000 (< 1500 μm) at 2.31% (*w*/*w*) and 1.78% (*w*/*w*), respectively, in potassium phosphate buffers (50 mM) at pH 7.6 or 8.6 and 20 °C for 24 h in an end-over-end rotator. The enzymes were then filtered and equilibrated against a saturated aqueous solution of LiCl, to obtain a water activity of 0.11.[15]

Irreversible inhibition: Irreversible inhibition of immobilized wild-type *C. antarctica* lipase B was carried out with methyl *p*-nitrophenyl *n*-hexylphosphonate as described by Rotticci et al.[15]

Michael-type reactions: In a typical reaction, immobilized lipase (42.9 mg mL^{-1}) was added to a solution of α,β-unsaturated carbonyl compound (300 mM), thiol/diethyl amine (400 mM) and internal standard (*n*-decane), dissolved in *c*-hexane. Reaction mixtures containing methyl acrylate were dissolved in diethyl ether. For the Michael-type addition of thiophenol to cyclohex-2-enone, the mixture was dissolved in toluene. The mixtures were incubated at 20 °C in an end-over-end rotator. Samples were taken for GC-FID (flame-ionization detection) and GC-MS analyses on capillary columns (J & W CycloSil-B, 30 m × 0.32 mm i.d., thickness of stationary phase $d_f = 0.25$ μm and Chrompack CP-Sil 5 CB, 25 m × 0.32 mm i.d., $d_f = 1.2$ μm). The reactions were run on a 1.75 mL scale; some of them also on a larger scale (8 mL). All products were confirmed by GC-MS, and some products also by NMR spectroscopy.

Michaelis–Menten kinetics: The apparent turnover numbers (k_{cat}^{app}) were calculated at low conversion by dividing the number of millimoles of product by the reaction time and number of millimoles of enzyme. The turnover numbers are apparent since they were only measured at one concentration (300 mM). The lipase concentration was determined spectrophotometrically before and after immobilization, and the amount of active enzyme was determined by active-site titration.[15] Both wild-type and mutant enzymes contained the same amount of correctly folded enzyme before immobilization according to CD measurements. The k_{non} values were calculated from the reaction rates, according to $v = k_{non}[S]_1[S]_2$, where $[S]_1$ and $[S]_2$ are the concentrations of substrates 1 and 2, respectively, at 300 mM α,β-unsaturated carbonyl compound and 400 mM thiol/diethyl amine for all reactions except the enzyme proficiency measurement experiment. For the enzyme-proficiency experiment, thiophenol (30 mM) and cyclohex-2-enone (300 mM) were used for calculations of k_{non} and k_{cat}^{app} values. The k_{cat}^{app}/K_M^{app} value was calculated from the initial rate of the Michaelis–Menten curve as cyclohex-2-enone was varied and the thiophenol was kept constant.

Note added in proof

During the production of this paper, Gotor and co-workers published the Michael addition of secondary amines to acrylonitrile catalyzed by wild-type *Candida antarctica* lipase B. Their results are in agreement with our result (Table 3, entry 23) since we, in this case, found that the wild-type were faster than the mutant by a factor of more than 50: O. Torre, I. Alfonso, V. Gotor, *Chem. Commun.* **2004**, 1724–1725.

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Keywords: enzyme catalysis · hydrolases · Michael addition · protein design · quantum chemistry

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