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Fast Carbon−Carbon Bond Formation by a Promiscuous Lipase

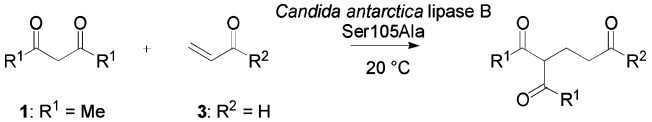
Maria Svedendahl, Karl Hult, and Per Berglund*

Department of Biochemistry, School of Biotechnology, Royal Institute of Technology (KTH), AlbaNova University Center, SE-106 91 Stockholm, Sweden

Received September 28, 2005; E-mail: per.berglund@biotech.kth.se

Enzymes can be used in organic synthesis, where they are efficient catalysts.[1] We have used lipase B from *Candida antarctica* as a scaffold and increased its reaction specificity for Michael additions by the substitution of one amino acid (Ser105Ala) in the active site through rational design. This mutant, *C. antarctica* lipase B Ser105Ala, catalyzes carbon−carbon bond formation between 1,3-dicarbonyls and α,β-unsaturated carbonyl compounds faster than the wild-type enzyme. This Michael addition is one of several possible promiscuous reactions[2] for *C. antarctica* lipase B. The common feature of both the natural hydrolytic reaction and the promiscuous reactions is the activation of a carbonyl functionality of the substrate by the oxyanion hole of the enzyme.[3] 1,4-Addition

Scheme 1. Michael Addition of 1,3-Dicarbonyl Compounds to α,β-Unsaturated Carbonyl Compounds Catalyzed by a *C. antarctica* Lipase B Mutant



1: R^1 = Me
2: R^1 = OMe

3: R^2 = H
4: R^2 = Me
5: R^2 = OMe

of a carbon nucleophile to an α,β-unsaturated carbonyl compound is called Michael addition and is a useful and efficient method to create carbon−carbon bonds in organic synthesis. Michael additions are traditionally catalyzed by strong bases, which can cause unwanted side reactions of the starting materials and subsequent reactions of the products. To avoid these problems, various types of catalysts have been developed. The most common catalysts reported for Michael additions are transition metals or lanthanide catalysts.[4] An alternative to these catalysts is enzymes. Enzyme-catalyzed conjugate additions, however, are rarely reported. Kitazume et al. used hydrolytic enzymes (not lipase from *Candida antarctica*) to catalyze the Michael-type addition of thiols and amines to triflourinated α,β-unsaturated carbonyl compounds in the 1980s.[5] Wild-type *C. antarctica* lipase B has been reported to catalyze Michael-type addition of secondary amines to acrylonitrile and primary amines to methyl acrylate.[6] Lin and co-workers used alkaline protease from *Bacillus subtilis* to catalyze Michael-type addition of pyrimidine derivatives and imidazole to acrylates in organic solvent.[7] Recently, we reported that wild-type and the Ser105Ala mutant of *C. antarctica* lipase B catalyzed the Michael-type addition of thiol and amine nucleophiles to a range of α,β-unsaturated carbonyl compounds.[3c] This led us to believe that *C. antarctica* lipase B Ser105Ala could even catalyze Michael additions of carbon nucleophiles, if their pK_a values were sufficiently low.

A hypothetical model of the catalytic addition of acetylacetone to acrolein in *C. antarctica* lipase B Ser105Ala is shown in Figure 1. This model is based on previously published QM calculations.[3] α,β-Unsaturated carbonyl compounds will bind to the oxyanion

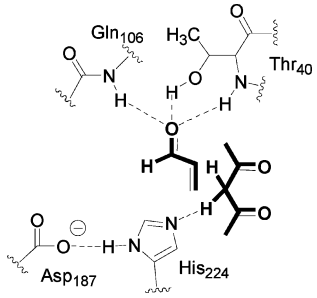


Figure 1. The hypothetical catalytic addition of a carbon nucleophile (acetylacetone) to an α,β-unsaturated carbonyl compound (acrolein) in the active site of *C. antarctica* lipase B Ser105Ala. The carbonyl oxygen of the α,β-unsaturated carbonyl compound is bound to the oxyanion hole formed by Gln_{106} and Thr_{40}, and the nucleophile is activated by His_{224}.

hole and be activated for a nucleophilic attack by a second substrate. The substitution of Ser_{105} to Ala facilitates this attack by opening up extra space and avoiding unproductive hemiacetal formation. His_{224} may act as a general base to activate the carbon nucleophile by removing one of its α-protons. The two substrates are then close to each other in the active site, which can result in a nucleophilic attack of the carbon nucleophile on the β-carbon of the α,β-unsaturated carbonyl compound. Gln_{106} and Thr_{40} could then stabilize the formation of an enolate by three hydrogen bonds to the carbonyl oxygen of the α,β-unsaturated carbonyl compound. The product can then be released from the enzyme active site.

The Ser105Ala variant of *C. antarctica* lipase B was created by site-directed mutagenesis.[3] Both wild-type and mutant enzyme were expressed in *Pichia pastoris*, purified, immobilized on Accurel MP1000, <1500 μm, and equilibrated to a water activity of 0.11.[8,9] The amount of active wild-type enzyme was determined by active-site titration using *p*-nitrophenyl *n*-hexylphosphonate.[9] The amount of protein adsorbed on the carrier was determined spectrophotometrically before and after immobilization.[3c] In a typical experiment, 10 mg of immobilized enzyme (containing 1.8% w/w wild-type lipase or 2.3% w/w mutant lipase), 8.4 mmol of α,β-unsaturated carbonyl compound, 6 mmol of 1,3-dicarbonyl compound, and internal standard (dodecane) were mixed without solvent and incubated at 20 °C in an end-over-end rotator. The reactions were followed by capillary column GC-FID (Chrompack Chirasil-dex, 25 m × 0.32 mm i.d., d_f 0.25 μm). The products were identified by GC−MS (J&W CycloSil-B, 30 m × 0.32 mm i.d., d_f 0.25 μm). The product of **2** + **4** showed identical MS spectra as was earlier published by Picquet et al.[10] The product of **1** + **5** showed MS spectra identical to that of the commercial methyl-4-acetyl-5-oxohexanoate from Sigma Aldrich (Product No. 296171). The specific rates (v) were calculated from initial rates as mole product per mole enzyme and second. The k_{non} values were calculated from the reaction rates, $v = k_{non}[S]_1[S]_2$, where $[S]_1$ and $[S]_2$ are the concentrations of the two substrates.

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Table 1. Specific Rates (v) for the Solvent-Free Michael Addition of the Substrates Shown in Scheme 1[a]

exp.	v_{mt} (s^{-1})	v_{wt} (s^{-1})	k_{non} (s^{-1} M^{-1})	v_{mt}/k_{non} (M^{-1})
1 + **3**	4.0×10^{3}	1.1×10^{2}	2.6×10^{-5}	1.5×10^{8}
1 + **4**	1.2×10^{0}	9.0×10^{-1}	4.8×10^{-9}	2.4×10^{8}
1 + **5**	8.1×10^{-2}	2.4×10^{-3}	2.6×10^{-8}	3.1×10^{6}
2 + **3**	$<10^{-7}$	$<10^{-7}$	$<10^{-10}$	
2 + **4**	3.2×10^{-1}	5.8×10^{-3}	4.4×10^{-7}	7.2×10^{5}
2 + **5**	9.0×10^{-3}	1.1×10^{-5}	1.5×10^{-11}	5.9×10^{8}

[a] Specific rates (v) were calculated from initial rates as mole product per mole enzyme and second. The k_{non} values were calculated from the reaction rates, $v = k_{non}[S]_1[S]_2$, where $[S]_1$ and $[S]_2$ are the concentrations of the two substrates.

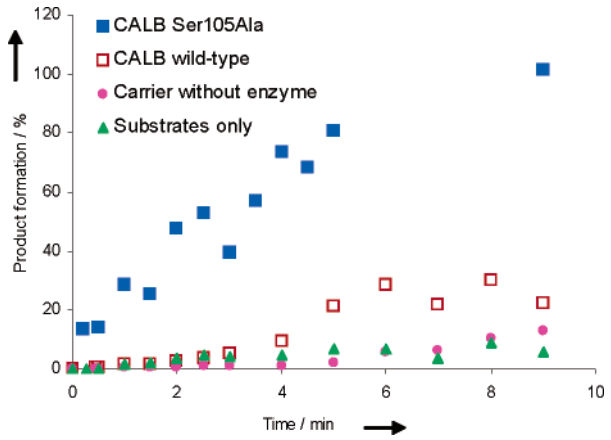

Figure 2. The progress curve of the solvent-free Michael addition of acetylacetone to acrolein **1** + **3** in Table 1. CALB = *C. antarctica* lipase B.

The ability of wild-type and Ser105Ala mutant of *C. antarctica* lipase B to catalyze the Michael addition of 1,3-dicarbonyl compounds to α,β-unsaturated carbonyl compounds was tested under solvent-free conditions (Table 1). All experiments proceeded faster with the mutant than with the wild-type enzyme (1.3−830 times). The reaction of acetylacetone with acrolein **1** + **3** (Table 1) proceeded extremely fast with the mutant reaching 100% conversion in less than 10 min (Figure 2). The specific rate (4000 s^{-1}) was found to be 36 times higher than the specific rate for the wild-type lipase. This reaction has previously been catalyzed by $CeCl_3 \cdot 7H_2O$/NaI without solvent, which resulted in 86% yield after 8 h.[11] On the basis of mole catalyst, the enzymatic process is 10^7 more efficient. Methyl acrylate **5** showed the lowest specific rates of the three α,β-unsaturated carbonyl compounds tested. Dimethyl malonate **2** reacted slower than acetylacetone **1** in all reactions (Table 1). This can be due to the more acidic α-protons of acetylacetone or steric hindrance. The rate enhancements (v_{mt}/k_{non}) are high and are in the same order as those observed for enzymes with native substrates.[12]

The **1** + **3** reaction is extremely fast. If the amount of enzyme is increased, the reaction will start to boil. The progress curve of the Michael addition of acetylacetone to acrolein is shown in Figure 2. This curve shows the progression of the reaction catalyzed by the mutant enzyme, wild-type enzyme, and two uncatalyzed reactions. The two uncatalyzed reactions (one with carrier without enzyme and one with substrates only) showed low reaction rates. This indicates that the Michael addition is catalyzed by the enzyme.

Table 2. Kinetic Constants for the Michael Addition of Acetylacetone to Methyl Vinyl Ketone (**1** + **4**) Catalyzed by *C. antarctica* Lipase B Ser105Ala in Cyclohexane at Various Acetylacetone Concentrations

concentration (**1**)	0.10 M	0.50 M	1.0 M
k_{cat}^{app}/K_M^{app} (s^{-1} M^{-1})	1.2	1.0	0.66
k_{non} (s^{-1} M^{-1})	4.8×10^{-9}	4.8×10^{-9}	4.8×10^{-9}
$(k_{cat}^{app}/K_M^{app})/k_{non}$	2.4×10^{8}	2.2×10^{8}	1.4×10^{8}

Kinetic constants for the Michael addition catalyzed by the Ser105Ala mutant of *C. antarctica* lipase B were determined for methyl vinyl ketone **4** under pseudo-one substrate conditions, keeping the concentration of acetylacetone constant (Table 2). The values of k_{cat}^{app}/K_M^{app} (~1 s^{-1} M^{-1}) were calculated from initial rate determinations at a low concentration of methyl vinyl ketone **4** (0.04 M). The experiment was repeated at three concentrations of acetylacetone **1** (0.1, 0.5, and 1.0 M). The decrease in k_{cat}^{app}/K_M^{app} with increased concentration of acetylacetone is probably caused by binding of that substrate to the oxyanion hole, competing with methyl vinyl ketone. The catalytic proficiency ($(k_{cat}^{app}/K_M^{app})/k_{non}$) was over 10^8, which is in the same order as the value observed for enzymes with native substrates.[12]

In conclusion, we have increased the reaction specificity of *C. antarctica* lipase B Ser105Ala toward carbon−carbon bond forming Michael additions. The specific rates for the tested substrates were high and even extremely high for the Michael addition of acetylacetone to acrolein. Data from Michael addition reactions catalyzed by *C. antarctica* lipase B Ser105Ala showed saturation kinetics with substrate inhibition of both substrates. The rate enhancement (10^5−10^8) and the catalytic proficiency (>10^8) were in the same range as the values observed for enzymes with native substrates.

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