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Transition-State Model for Subtilisin-Catalyzed Transesterifications of Secondary Alcohols

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Abstract: In the subtilisin-catalyzed transesterifications of a variety of secondary alcohols, the (*S*)-enantiomers were acylated faster. Kinetic measurements indicated that the enantioselectivity originates from chiral discrimination in the transition state. A transition-state model capable of explaining the *S*-preference of subtilisin toward secondary alcohols has been proposed. © 1999 Elsevier Science Ltd. All rights reserved.

An accurate understanding of the principles of the stereoselectivity of enzymes is essential for the distinct advancement of the chemoenzymatic organic synthesis, and hence much effort has been devoted to this subject.[1–4] We have recently reported a new proposal on the mechanism of the enantioselectivity in the lipase-catalyzed kinetic resolution of secondary alcohols.[3,4] The main points are as follows: (1) Chiral discrimination operates in the transition state, but not in the substrate-binding step. (2) The enantioselectivity results from the reduced reactivity of the slower-reacting enantiomers due to steric repulsion, but not from the enhanced reactivity of the faster-reacting enantiomers due to attractive interactions. (3) The transition-state model, which consists of substrate/product and several amino acid residues at the active site, is consistent with the empirical rule (*R*-preference of lipases toward secondary alcohols[2a]). In our transition-state model, the enantioselectivity is accounted for by the conformational requirements of the local transition structure, and the concept of molecular recognition is not used except steric repulsion. Therefore, high enantioselectivity and broad substrate specificity can be achieved simultaneously, in agreement with the experimental observation.

Whether or not the above concept can be applied to other enzymes is a significant and interesting subject. We directed our attention to the serine protease subtilisin because of its intriguing features and commercial availability.[5] Here we employed the cross-linked enzyme crystals of subtilisin Carlsberg (CLEC-subtilisin)[5] as a catalyst and a variety of secondary alcohols **1–8** shown in Figure 1. In this paper, we propose a transition-state model for the subtilisin-catalyzed transesterifications of secondary alcohols on the basis of kinetic resolutions, kinetic analysis, and crystal structures of subtilisins available from the Protein Data Bank.

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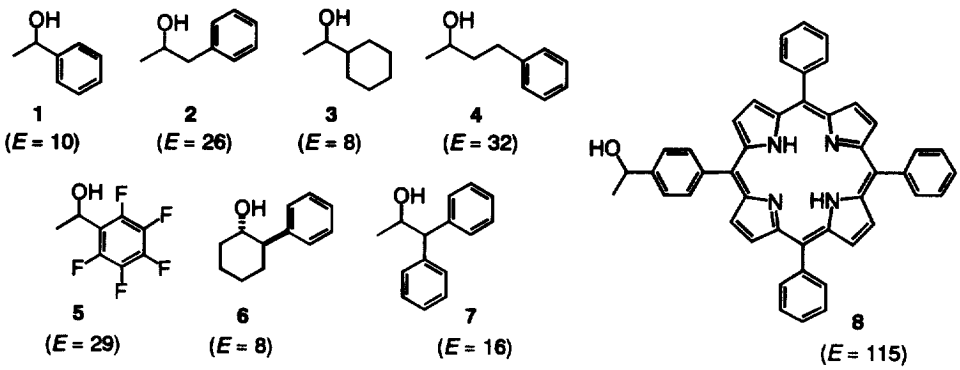


Figure 1. Structures of secondary alcohols and the *E* values for the subtilisin-catalyzed kinetic resolutions. The *E* values were calculated according to ref 6. The (*S*)-enantiomers were acylated preferentially in all cases. Typical reaction conditions: CLEC-subtilisin (40 mg), alcohol (0.82 mmol), vinyl acetate (1.6 mmol), molecular sieves 4A (400 mg), dry *i*-Pr_2O (5 mL), 30 °C.

Table 1. Kinetic Parameters for Subtilisin-Catalyzed Transesterifications.[a]

	V_{max} (M min^{-1} mg(enzyme)$^{-1}$)		K_m (M)	
Alcohol	(*S*)	(*R*)	(*S*)	(*R*)
1	$(1.1 \pm 0.2) \times 10^{-4}$	$(1.1 \pm 0.2) \times 10^{-5}$	$(2.0 \pm 0.7) \times 10^{-2}$	$(2.2 \pm 0.8) \times 10^{-2}$
2	$(6.1 \pm 0.3) \times 10^{-5}$	$(5.3 \pm 0.2) \times 10^{-7}$	$(1.7 \pm 0.2) \times 10^{-2}$	$(1.7 \pm 0.1) \times 10^{-2}$
3	$(3.6 \pm 0.4) \times 10^{-5}$	$(2.2 \pm 0.1) \times 10^{-6}$	$(2.2 \pm 0.5) \times 10^{-1}$	$(1.6 \pm 0.2) \times 10^{-1}$
cyclopentanol	$(2.2 \pm 0.1) \times 10^{-4}$		$(1.5 \pm 0.2) \times 10^{-1}$	

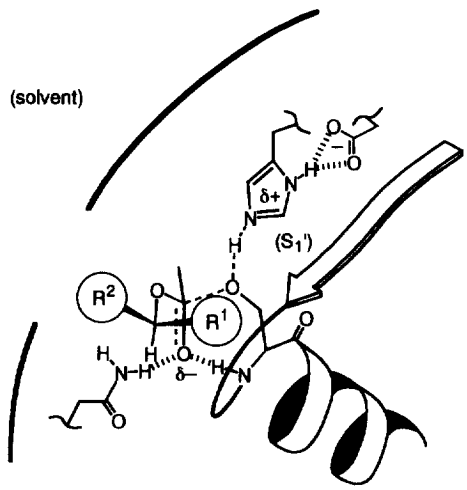
[a] Conditions for the kinetic measurements: CLEC-subtilisin (typically, 3–10 mg and 3–30 mg for (*S*)- and (*R*)-enantiomers, respectively), (optically pure) alcohol (arbitrary concentration range across the K_m value), vinyl acetate (0.5 M), molecular sieves 4A (80 mg), dry *i*-Pr_2O (4 mL), 30 °C. Because of the heterogeneous reaction, the nonlinear least-squares method was applied to the Michaelis-Menten type of equation: $v_0 = V_{max}(E)_{mg}[S]_0/(K_m + [S]_0)$, where V_{max} is normalized by the weight of CLEC-subtilisin $(E)_{mg}$.

The subtilisin-catalyzed kinetic resolutions of racemic secondary alcohols **1–8** were carried out with vinyl acetate in dry diisopropyl ether at 30 °C. The results are shown in Figure 1, where the enantiomeric ratios (*E* values) are calculated according to the literature.[6] Subtilisin showed good to high enantioselectivities for **1–8**. Interestingly, subtilisin showed the *S*-preference for all the secondary alcohols examined,[7] which is opposite to the well-known empirical rule for lipases (*R*-preference).[2a] Moreover, the more unbalanced the bulkiness of the two substituents attached to the stereocenter, the higher the enantioselectivity,[8] which is often observed in the lipase-catalyzed kinetic resolutions.[3]

Next, in order to specify the enantiomer-discriminating step, we determined the kinetic parameters (V_{max} and K_m values) of subtilisin for **1–3** and cyclopentanol according to the reported procedure.[3] The obtained constants are listed in Table 1. Cyclopentanol was used as a reference alcohol to investigate the effect of the larger substituent of chiral secondary alcohols. Table 1 clearly shows that the difference in the V_{max} values between the enantiomers of **1–3** is larger than that in the K_m values. This indicates that chiral discrimination originates from the transition state, but not from the substrate-binding step. Furthermore, comparison of the V_{max} values for cyclopentanol with those for chiral alcohols **1–3** reveals that the enantioselectivity results

from the reduced activity of the enzyme for the slower-reacting enantiomers, but not from the enhanced activity for the faster-reacting enantiomers. These trends are the same as those observed in the lipase-catalyzed transesterifications.[3] Although the K_m values reported here are smaller than those for the lipase-catalyzed transesterifications,[3] it is evident that chiral recognition based on specific binding is unimportant.

In view of the dominant contribution from the transition state, we derived the transition-state model for the subtilisin-catalyzed transesterifications of secondary alcohols (Figure 2) applying the protocol for lipases[3] to subtilisin Carlsberg[9]. Importantly, this simple transition-state model can rationalize the experimentally observed *S*-preference of subtilisin for secondary alcohols. It is known that the approximate mirror-image relationship exists between the catalytic residues (the catalytic triad and the oxyanion hole) of the serine proteases and those of lipases.[10] It is also clear from high-resolution crystal structures that the size and shape of the binding pockets of subtilisin are very different from those of lipases. Nevertheless, the opposite enantiopreferences of subtilisin (*S*-preference) and lipases (*R*-preference) are reasonable, if the enantioselectivity of these hydrolases results predominantly from the conformational requirements of the local transition structure and from repulsive interactions (not from attractive interactions at binding pockets).



$R^1 < R^2$: faster-reacting enantiomer
$R^1 > R^2$: slower-reacting enantiomer

Figure 2. Transition-state model to rationalize the enantioselectivity of subtilisins toward secondary alcohols in organic solvents.

(i) The enantiomer-differentiating transition state in the rate-determining step is shown.

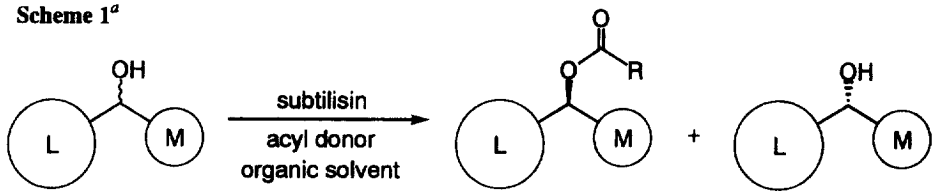
(ii) The absolute configuration of the carbonyl carbon atom where the oxygen atom of the serine of the catalytic triad is leaving is determined by the spatial arrangement of the catalytic residues.

(iii) The C–O bond of the substrate (secondary alcohol) takes the *gauche* conformation with respect to the breaking C–O bond, which is due to the stereoelectronic effect.

(iv) The hydrogen atom attached to the stereocenter in the substrate is *syn*-oriented toward the carbonyl oxygen atom.

For details of these requirements, see ref 3. Compare the present transition-state model with that reported for lipases.[3,4]

Scheme 1[a]



[a] A rule for the subtilisin-catalyzed kinetic resolutions of secondary alcohols in organic solvents. L and M represent the substituents of large and medium sizes, respectively.

Previously, we have used **8** in order to demonstrate that generally, the accommodation of the larger substituent of secondary alcohols in a binding pocket of lipases is not necessary for high enantioselectivity.[4] Obviously, the tetraphenylporphyrin moiety of **8** is so large that it cannot be accommodated by any pocket of subtilisin. The transition-state model (Figure 2) suggests that severe steric repulsion occurs between the tetraphenylporphyrin moiety of (*R*)-**8** and the protein moiety, leading to the highest *E* value.

There is a clear tendency that subtilisin shows lower enantioselectivity for secondary alcohols than lipases. The diminished enantioselectivity of subtilisin is probably due to the lack of the protein wall corresponding to the "triangular wall" of lipases, which we have proposed to be important for high enantioselectivity.[3] Instead of such a wall, subtilisin has a shallow depression (S_1'), which is made up of the β-strand, the α-helical turn, and the histidine imidazole of the catalytic triad, and which is open to external solvent (Figure 2). Therefore, subtilisin has poor ability to discriminate the chirality of relatively small alcohols such as **1** and **3**, and shows high but incomplete enantioselectivity toward even the very large alcohol **8**.[11]

Although the number of the secondary alcohols so far resolved by subtilisins is not large, our transition-state model predicts or validates a rule that subtilisins preferentially acylate the (typically, (*S*)-) enantiomer shown in Scheme 1. Further work is under way to investigate the validity and usefulness of the present transition-state model.

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7. The *S*-preference of subtilisins for secondary alcohols has also been reported elsewhere. (a) Fitzpatrick, P. A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1991**, *113*, 3166–3171. (b) Kazlauskas, R. J.; Weissfloch, A. N. E. *J. Mol. Cat. B: Enz.* **1997**, *3*, 65–72. (c) Lloyd, R. C.; Dickman, M.; Jones, J. B. *Tetrahedron: Asymmetry* **1998**, *9*, 551–561. See also ref 5.
8. Alcohols **6** and **7** are the exceptions. This is probably because even the (*S*)-enantiomers of **6** and **7** undergo steric hindrance, judging from the low reactivity and the low *E* values of **6** and **7**.
9. For example: McPhalen, C. A.; James, M. N. G. *Biochemistry* **1988**, *27*, 6582–6598.
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11. The enantioselectivity in the lipase-catalyzed kinetic resolutions of **8** was complete, because the slower-reacting (*S*)-enantiomer of **8** did not undergo the acylation at all.