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Reduction of β-Keto Esters with a Reductase: Construction of Plural Stereocenters Remote from the Reaction Center

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The reduction of *sec*-alkyl 2-methyl-3-oxobutyrate with a keto ester reductase from bakers' yeast (YKER-I) is accompanied by simultaneous dynamic and static resolution of chiral centers affording the corresponding (2*R*,3*S*,1′*R*)-hydroxy esters preferentially. Thus, the enzyme discriminates three chiral centers simultaneously in high stereoselectivity producing useful chiral building blocks. To study the effect of the alcohol moiety which is located at a remote position from the reaction center, upon the interaction between the enzyme and a substrate, steady-state kinetic parameters, K_m and k_{cat}, of YKER-I for each (1′*R*)- and (1′*S*)-substrate have been determined. The results reveal that the stereochemistry at the alcohol moiety affects K_m rather than k_{cat}.

INTRODUCTION

A number of enzymes are now commercially available and are used for asymmetric syntheses extensively (*1*). Most of the enzymes, however, discriminate the stereochemistry at the reaction center or its vicinity only. Generally, the further the chiral center is kept away from the reaction center, the less the ability of enzyme to discriminate the chirality. Although several examples of enzymatic discrimination of a chiral center three or more bonds apart from the reaction center have been reported, most examples are seen in hydrolytic reactions (*2–6*). Although a few alcohol dehydrogenases that discriminate such chiral centers have been reported, all the examples available are those that operate on cyclic compounds with a rigid skeleton (*7*, *8*). No alcohol dehydrogenase has been reported on the discrimination of remote and multichiral centers in acyclic compounds that have flexible skeletons.

Hudlicky *et al*. reported the reduction of *sec*-alkyl acetoacetate with bakers' yeast (*9*). It is noteworthy that bakers' yeast discriminates stereochemistry not only at the reaction center but also at the alcohol moiety which is four bonds apart from the reaction center, although the stereoselectivity of this reaction is not satisfactory. The fact suggests that bakers' yeast contains at least one enzyme which discriminates the chiral center in the alcohol moiety in the reduction of β-keto esters.

Keto ester reductases have been isolated from bakers' yeast (*10–12*). YKER-I

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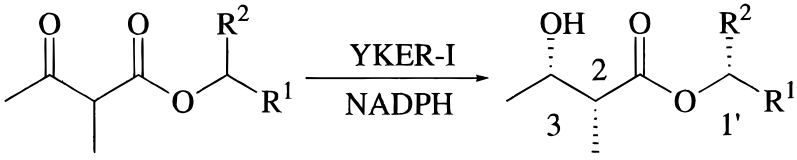
(*y*east *k*eto *e*ster *r*eductase - *I*), an NADPH-dependent enzyme, is one of those enzymes isolated and reported previously as L-enzyme-1 (*11*). The enzyme has wide specificity in substrates with extremely high enantioselectivity (*13*). Although the enzyme reduces various keto esters, its reaction rate highly depends on the structure of alcohol moiety in the keto esters. For example, in the reduction of alkyl 2-methyl-3-oxobutyrate, the reaction rate of the neopentyl ester is about 60 times larger than that of the t-butyl ester. Kinetic parameters of YKER-I for these esters have been studied and it was found that K_m for the t-butyl ester is 20 times larger than that for the neopentyl ester, whereas k_{cat} for the t-butyl ester is only one-third of that for the neopentyl ester (*14*). Thus, the contribution of K_m is larger than that of k_{cat} in the sensitivity toward the change in structure of the alcohol moiety: the variation in substrate structure does not affect k_{cat} appreciably. The finding suggests that anchoring of a substrate into the pocket of YKER-I is largely affected by the structure in the alcohol moiety, or YKER-I might have the ability to discriminate the stereochemistry in the alcohol moiety, and the reduction of α-alkyl β-keto ester of a secondary alcohol will construct plural asymmetric centers in the product simultaneously (Eq. [1]) (*15*).



[1]

1a - n (2*R*,3*S*,1'*R*)-**2a - n**

a : R^1 = Hex , R^2 = Me
b : R^1 = c-Hex , R^2 = Me
c : R^1 = Ph , R^2 = Me
d : R^1 = Ph , R^2 = Et
e : R^1 = 2-Cl-Ph , R^2 = Me
f : R^1 = 4-Cl-Ph , R^2 = Me
g : R^1 = 2-Me-Ph , R^2 = Me
h : R^1 = 4-Me-Ph , R^2 = Me
i : R^1 = 4-NO_2-Ph , R^2 = Me
j : R^1 = 2-Py , R^2 = Me
k : R^1 = 3-Py , R^2 = Me
m : R^1 = 4-Py , R^2 = Me
n : R^1 = 1,3-Dithianyl , R^2 = Me

RESULTS AND DISCUSSIONS

Asymmetric Reduction of α-Methyl β-Keto Esters with YKER-I

α-Methyl β-keto esters, **1**, were prepared by allowing the corresponding alcohols to react with diketene and succeeding methylation at the β-position (*16*). The results

from the reductions of **1** with YKER-I are listed in Table 1, in which the stereoselectivity in the carboxylic acid moiety is evaluated on the basis of enantiomeric and diastereomeric excesses and that in the alcohol moiety in the product is evaluated from *E* values (*17*). β-Hydroxy esters, **2**, obtained as the products have three chiral centers in the molecules and, therefore, have eight stereoisomers. After the reduction, the unreacted substrate and the products formed were separated by column chromatography on silica gel, and then each compound was hydrolyzed. The free alcohols were converted into the corresponding acetyl (*18*) or α-methoxy-α-trifluoromethylphenylacetyl (MTPA) (*19*) esters, and enantiomeric excesses in the esters were measured by GC or HPLC analysis. The enantiomeric and diastereomeric excesses in the β-hydroxybutyric acid were determined by GC analysis of the corresponding ethyl ester (Scheme 1).

In the reduction of *sec*-alkyl 2-methyl-3-oxobutyrate, the chiral carbon at the 2-position remains racemic throughout the reaction due to enolization (dynamic resolution) (*20*), and only (2*R*)-**1** is reduced with YKER-I to the corresponding (2*R*)-**2** in nearly 100% chemical and enantiomeric yields. On the other hand, the chiral center at the 1′ position does not racemize throughout the reaction (static resolution). Therefore, if YKER-I discriminates the chiral center at the 1′ position, the reaction must cease at 50% conversion of the substrate. To regulate the conversion of the substrate, a coupling system with glucose-6-phosphate (G6P) − glucose-6-phosphate dehydrogenase (G6PDH) was employed to regenerate the appropriate amount of coenzyme

TABLE 1

Asymmetric Reduction of α-Methyl β-Keto Esters with YKER-I

	R^1	R^2	Recovered (%)	ee[a] (%)	Yield (%)	ee[b] (%)	de[c] (%)	ee[d] (%)	*E*[e]
a	Hex (0.73)[f]	Me	64	32	33	>98	>98	66	6.5
b	*c*-Hexyl (0.87)[f]	Me	63	40	35	>98	91	78	12.2
c	Ph (1.66)[f]	Me (0.52)[f]	52	68	48	>98	>98	74	13.3
d	Ph	Et (0.56)[f]	61	36	40	>98	>98	54	4.6
e	2-Cl-Ph	Me	59	39	35	>98	>98	70	8.2
f	4-Cl-Ph	Me	64	36	29	>98	>98	77	10.7
g	2-Me-Ph	Me	46	72	49	>98	93	75	14.6
h	4-Me-Ph	Me	58	58	40	>98	>98	75	12.2
i	4-NO_2-Ph	Me	56	55	36	>98	>98	80	15.9
j	2-Py	Me	58	59	38	>98	98	80	15.9
k	3-Py	Me	56	71	43	>98	>98	86	29.0
m	4-Py	Me	62	50	36	>98	87	91	35.8
n	1,3-Dithianyl	Me	57	68	42	>98	98	91	41.4

[a] ee at the 1′ position of **1** recovered.

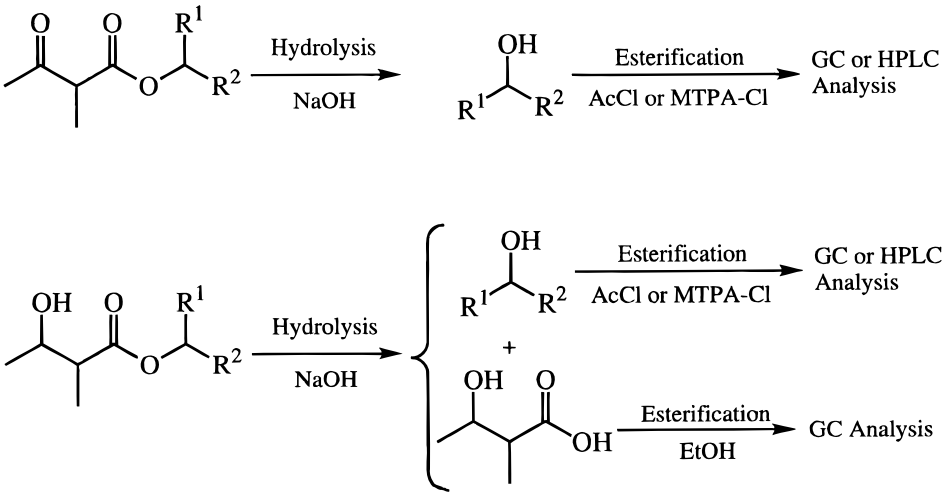
[b] Excess of (2*R*,3*S*)-**2** over (2*S*,3*R*)-**2**.

[c] Excess of (2*R*,3*S*) over other diastereomers of **2**.

[d] ee at the 1′ position of **2**.

[e] *E* value was calculated from enantiomeric excesses at the 1′ positions in the substrate recovered unreacted and the product (*17*).

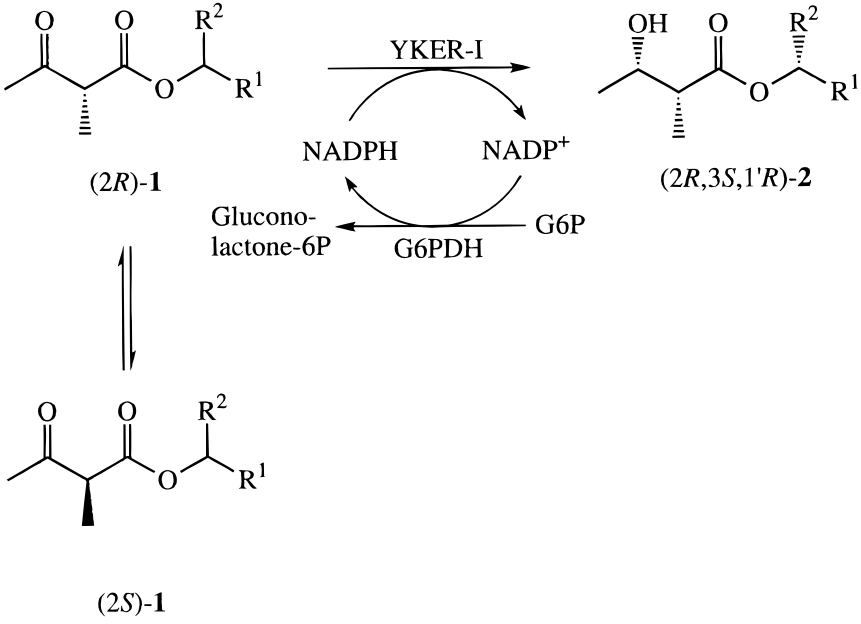
[f] Steric parameter, *v*, of a substituent is defined as its van der Waals radius (*22*).



SCHEME 1

(Scheme 2) (*21*), and the procedure was found to be effective to obtain the product, (2*R*,3*S*,1′*R*)-**2**, in moderate yield.

The majority of the materials isolated from the reaction mixture were (2*R*,3*S*,1′*R*)-**2** and (1′*S*)-**1** which remained unreacted. The fact reveals that YKER-I discriminates



SCHEME 2

the stereochemistry at the 1′-position as well as those at the reaction center and the 2-position. It is noteworthy that YKER-I is quite unique in the sense that it discriminates a chiral center four bonds apart from the reaction center.

Stereoselectivity in the acid moiety is very high (>98% ee and ≥98% de) in most of the products except for **2b** (91% de), **2g** (93% de), and **2m** (87% de). However, the stereoselectivity in the alcohol moiety changes depending on the property of the substituent. For instance, in the series of **1a**–**1c**, steric bulk of R^1 is larger in **1b** and **1c** than that in **1a** on the basis of the steric parameter, *v* (*22*), and the stereoselectivity at the 1′-position in the enzymatic reduction of **1b** and **1c** is higher than that of **1a**, suggesting that steric bulk is one of the important factors for controlling the stereoselectivity in the alcohol moiety. Particularly, the reduction of **1n** affords (2*R*, 3*S*, 1′*R*)-**2n** in more than 94% purity out of a possible eight stereoisomers. Although the steric bulk of R^2 also affects stereoselectivity at the 1′ position, its direction is opposite to that of R^1 (**2c** and **2d**); when R^2 is bulkier than the methyl group, a decrease in stereoselectivity is observed. The effect of the substituent on the phenyl group, however, is not remarkable (**2c**, **2e**–**2i**); *E* value does not depend on the position and electronic property of the substituent appreciably. Remarkable improvement of the stereoselectivity at the 1′ position is observed when R^1 has an electronegative heteroatom as in the 3-pyridyl, 4-pyridyl group (**2k**–**2m**). Usually, an electronegative heteroatom acts as an electron donor and interacts with an electron acceptor such as a proton. Therefore, the fact suggests that YKER-I has an electron acceptor at the binding site and a heteroatom in R^1 interacts with this electron acceptor to stabilize the enzyme–substrate complex affording the product in high stereoselectivity (Fig 1).

Substituent Effect on Kinetic Parameters

The mechanism of discrimination of a chiral center by an enzyme has often been discussed on the basis of active-site models such as diamond lattice (*23*), cubic section (*7*,*24*), and so on (*25*–*30*). The models have been proposed on the basis of size and shape of the substrate, which would be accommodated by the active site of the

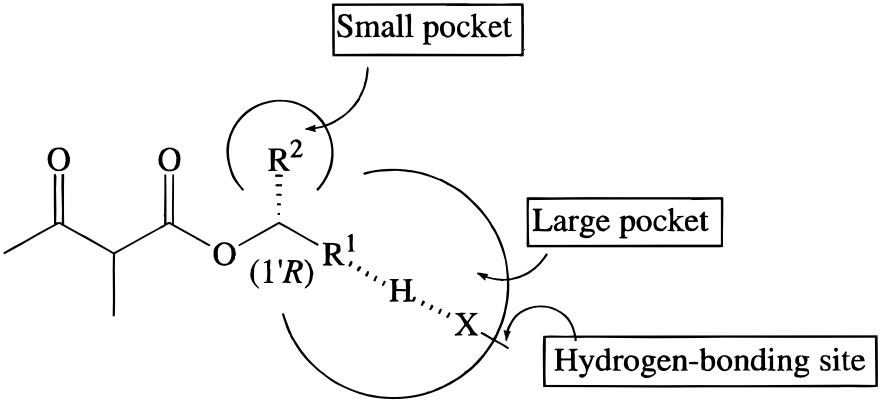


FIG. 1. Schematic model for the active site of YKER-I.

enzyme. The relative location of catalytic residues of the enzyme from the reaction center of the substrate is the crucial factor for controlling the reactivity. Although examples are few, the mechanism of enantiomer discrimination has been explored by means of kinetic parameters, K_m and k_{cat} (*8,31–36*). For instance, in the optical resolution of secondary alcohols by a lipase, the discrimination was explained to stem from the dominant contribution of k_{cat} over K_m (*31–35*), whereas, in the oxidation of some alcohols by horse liver alcohol dehydrogenase, the discrimination is due to the dominancy of K_m (*36*). Thus, kinetic studies may provide important information for the discussion on the mechanism of enantiomer discrimination.

Lee *et al.* demonstrated, using chiral inhibitors, that serine proteases have the ability to discriminate remote chiral centers (*37*). (*R*)- and (*S*)-3-phenylbutanols, chiral transition-state-analog inhibitors of α-chymotrypsin, have an 88-fold difference in K_i value in favor of the (*R*)-enantiomer. Thus, kinetic study has elucidated a potential of α-chymotrypsin quantitatively for the discrimination of a chiral center two bonds apart from the reaction center.

As noted above, YKER-I discriminates the stereochemistry of *sec*-alkyl 2-methyl-3-oxobutyrate at the 2 and 1′ positions as well as the stereochemistry at the reaction center yielding (2*R*,3*S*,1′*R*)-hydroxy esters preferentially. YKER-I is the first reductase, to the authors' best knowledge, that has been found to discriminate three chiral centers of acyclic compounds simultaneously.

When attention is focused on *E* values listed in Table 1, it appears that a 3- or 4-pyridyl substituent in the alcohol moiety of the ester exerts much higher selectivity than the ester with a 2-pyridyl substituent, despite the fact that their structures are quite similar. To elucidate the origin of this difference, we investigated the kinetics of the reduction with these substrates. Optically pure secondary alcohols with an appropriate pyridyl substituent were prepared by kinetic resolution of the corresponding racemic alcohols by means of a lipase and employed for the preparation of esters with a chiral alcohol moiety, (1′*R*)- and (1′*S*)-**1j**, (1′*R*)- and (1′*S*)-**1k**, and (1′*R*)- and (1′*S*)-**1m**. Steady-state kinetic parameters, K_m and k_{cat}, of YKER-I for each substrate were determined from [S] − [S]/*v* plots and the results are listed in Table 2.

When $\delta\Delta G^{\neq}$ is defined as the difference between the free energy of activation in the reaction of the (1′*R*)-isomer ($\Delta G_R^{\neq}$) and that of the (1′*S*)-isomer ($\Delta G_S^{\neq}$), the quantity is given by

TABLE 2

Kinetic Parameters of YKER-I for 1′-Optically Pure Esters

Substrate	R^1	R^2	K_m, mM	k_{cat}, s^{-1}
(1′*R*)-**1j**	2-Py	Me	1.4 ± 0.1	3.5 ± 0.5
(1′*S*)-**1j**	Me	2-Py	10.3 ± 0.8	1.3 ± 0.04
(1′*R*)-**1k**	3-Py	Me	0.89 ± 0.1	3.7 ± 0.2
(1′*S*)-**1k**	Me	3-Py	9.6 ± 0.9	1.3 ± 0.1
(1′*R*)-**1m**	4-Py	Me	0.56 ± 0.1	3.8 ± 0.1
(1′*S*)-**1m**	Me	4-Py	8.8 ± 1.1	1.3 ± 0.09

TABLE 3

The Difference in Activation Energy between the Reactions of (*R*)- and (*S*)-Isomers

Substrate	$-RT \ln k_{catR}/k_{catS}$ (kcal/mol)	$-RT \ln K_{mS}/K_{mR}$ (kcal/mol)	$-\delta\Delta G^{\neq}_{kin}$ [a] (kcal/mol)	$-\delta\Delta G^{\neq}_{E}$ [b] (kcal/mol)
1c	—	—	—	1.56
1j	0.60	1.20	1.80	1.67
1k	0.62	1.43	2.05	2.03
1m	0.64	1.66	2.30	2.15

[a] Calculated from kinetic parameters.
[b] Calculated from *E* value.

$$\begin{aligned}\delta\Delta G^{\neq} &= -RT \ln E \\ &= -RT \ln(k_{cat}/K_m)_R/(k_{cat}/K_m)_S \\ &= -RT \ln(k_{cat\,R}/k_{cat\,S}) - RT \ln(K_{mS}/K_{mR}),\end{aligned} \quad [2]$$

where *R*, *T*, and *E* are gas constant, reaction temperature in K, and *E* value, respectively. Suffixes *R* and *S* represent (1′*R*)- and (1′*S*)-isomers.

The first and second terms in the right-hand side of the last equality in Eq. [2] are summarized in Table 3. The differences in free energy of activation between the reactions of (1′*R*)- and (1′*S*)-isomers were calculated from the *E* value which was determined from enantiomeric excesses in the product and the reactant that remained unreacted ($\delta\Delta G^{\neq}_{E}$), and those calculated from kinetic parameters ($\delta\Delta G^{\neq}_{kin}$) agree with each other quite well within a limit of experimental error. The fact guarantees that all the experimentally observed quantities are reasonable.

Table 3 also reveals that the contribution of k_{cat} for $\delta\Delta G^{\neq}$ is smaller than the contribution of K_m. Thus, it is apparent that the contribution of K_m dominates the contribution of k_{cat} in discrimination of the chiral center at the alcohol moiety. In other words, the active site of YKER-I accommodates the (1′*R*)-isomer better than the (1′*S*)-counterpart. However, after the substrate is accommodated in the enzyme pocket, the chemical reaction between the enzyme and the substrate proceeds with similar rates for the (1′*R*)- and (1′*S*)-isomers.

E value has a wide distribution depending on the structure of R^1, and here a question

TABLE 4

The Difference in Activation Energy between the Reactions with **1j** and **1k** or **1m**

	$-RT \ln k_{catB}/k_{catA}$ (kcal/mol)		$-RT \ln K_{mA}/K_{mB}$ (kcal/mol)	
Substrates	1′*R*	1′*S*	1′*R*	1′*S*
1k–1j	0.03	0.00	0.27	−0.04
1m–1j	0.05	−0.00	0.55	−0.09

arises: Which of the (1′*R*)- and (1′*S*)-isomers is responsible for exerting different sensitivities for different compounds? To answer this question, we selected **1j** as a representative low selective compound ($E = 16$), and **1k** ($E = 29$) and **1m** ($E = 36$) as candidates for highly selective compounds. Because these compounds are similar in structure, it is safe to take into account the position of the nitrogen atom only as the difference in substrate structure.

The relationship shown in Eq. [2] also holds for two different substrates, A and B, of the same configuration, giving

$$\begin{aligned}\delta\Delta G^{\neq} &= \Delta G_B^{\neq} - \Delta G_A^{\neq} \\ &= -RT \ln(k_{catB}/k_{catA}) - RT \ln(K_{mA}/K_{mB}),\end{aligned} \quad [3]$$

where suffixes A and B represent the substrates A and B. The results are listed in Table 4.

The difference in energetic contribution of K_m between the reactions of (1′*R*)-**1j** and (1′*R*)-**1k** or (1′*R*)-**1j** and (1′*R*)-**1m** is 0.27 or 0.55 kcal/mol, respectively, whereas the value is one order of magnitude smaller in the corresponding series of the (1′*S*)-counterparts (−0.04 and −0.09, respectively). On the other hand, the difference in k_{cat} remains almost unchanged for the two isomers.

It is worth emphasizing that **1c** exerts $\delta\Delta G_E^{\neq}$ of 1.56 cal/mol, a value similar to that of **1j** (1.67 kcal/mol), which suggests that the nitrogen in the 2-pyridyl group exerts no role in binding the substrate in the large pocket; the 2-pyridyl and phenyl groups behave similarly as substituents. The observation may be interpreted by means of the ability of a substrate to act as a hydrogen-bond acceptor. The active site of YKER-I has two pockets, a large and a small which accommodate the alcohol moiety. The large pocket may have enough volume to accommodate R^1 (large) of both (*R*)- and (*S*)-configurations of a substrate without causing appreciable steric stress for the enzyme. The stress-free enzyme is expected from the constancy of k_{cat} terms for the (1′*R*)- and (1′*S*)-isomers. However, of course, the position of R^1 in the (1′*R*)-isomer in the pocket is different from the position of R^1 in the (1′*S*)-isomer, provided the volume of the small pocket is nearly the same as that of the small substituent, R^2 (*38*).

If one assumes a hydrogen-bonding site at the end of the large pocket that can form a hydrogen bond with the nitrogen in the 3- or 4-pyridyl group, but not with the 2-pyridyl group, the large stability of the ES complex with **1k** or **1m** compared with the stability of **1c** or **1j** is understood. The strength of hydrogen-bond in a protein has been reported to be about 0.5–1.5 kcal/mol (*39*) and the difference in binding energy observed may be accounted for on the basis of a hydrogen bond.

Thus, we propose that the active site of YKER-I has a large and a small pocket and there exists a hydrogen-bonding residue at the end of the large pocket, although the pockets are essentially hydrophobic.

EXPERIMENTAL

Instruments

^{1}H NMR spectra were recorded on a Varian VXR-200 spectrometer in $CDCl_3$. Capillary gas chromatograms (GC) were recorded on a Shimadzu GC-9A or GC-14B