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Stereochemical Control in Microbial Reduction. 18. Mechanism of Stereochemical Control in the Diastereoselective Reduction with Bakers' Yeast

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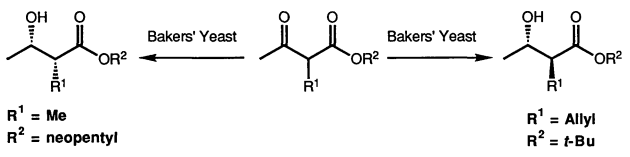
Reduction of a variety of 2-alkyl-3-oxobutanoates with bakers' yeast yields the corresponding L-(3*S*)-hydroxy esters with exclusive enantioselectivity, while the diastereoselectivity, syn/anti ratio, varies depending on the structure of the alkoxyl moiety. In order to elucidate the mechanism for this stereochemical control, oxidoreductases that reduce 2-alkyl-3-oxobutanoate have been isolated from the cells of bakers' yeast and purified. Two dominant competing 2-alkyl-3-hydroxybutanoate oxidoreductases have been obtained: one of them reduces 2-alkyl-3-oxobutanoates stereoselectively yielding the corresponding L-*syn*-hydroxy esters, whereas the other affords a mixture of L-*syn*- and L-*anti*-hydroxy esters. The experimental results on stereoselectivity have nicely been simulated using kinetic parameters elucidated.

Optically active 2-alkyl-3-hydroxyalkanoates are versatile chiral building blocks in organic synthesis[1] because they have two chiral centers necessary for asymmetric synthesis as well as two functional groups that are readily convertible into many other functions. A number of researchers have made efforts to obtain the compounds of this class.[2-4] Recently, we developed the methods to synthesize optically active 2-alkyl-3-hydroxy esters under the catalysis of biological systems:[5-10] bakers' yeast reduces various 2-alkyl-3-oxobutanoates affording the corresponding L-(3*S*)-hydroxy esters with exclusive enantioselectivity, while the diastereoselectivity varies depending on the structure of the alkoxyl group. The presence or absence of a substituent at the carbon adjacent to the alkoxyl oxygen is crucial to control the reduction course leading to the *syn*- or *anti*- product.[11] For example, the reduction of neopentyl 2-methyl-3-oxobutanoate (**1c**) affords the corresponding (2*R*,3*S*)-*syn*-hydroxy ester with 92% diastereomer excess (d.e.), whereas the reduction of *t*-butyl 2-allyl-3-oxobutanoate (**2b**) yields the corresponding (2*S*,3*S*)-*anti*-hydroxy ester with 88% d.e. (Scheme 1).

In order to clarify the mechanism for stereochemical control in microbial reduction, we isolated certain β-hydroxy ester oxidoreductases effective to the reduction of 2-alkyl-3-oxobutanoates from the cells of bakers' yeast and studied the kinetics as well as stereochemical properties of the enzymes. In the previous paper of this series, we reported that the studies at the enzyme level is successful to explain stereochemical behavior of their host microbe under various reaction conditions at least qualitatively.[12]



Scheme 1.

Experimental

Instruments. ^{1}H NMR spectra were recorded on a Varian VXR-200 spectrometer in $CDCl_3$ with Me_4Si as an internal standard. Gas chromatograms were recorded on a Yanaco G-2800 (PEG-20M, 1.5 m) and a Shimadzu GC-14A (PEG-20M Bonded, 25 m) gas chromatographs. Preparative gas chromatography was done on a Varian Aerograph Model 920 gas chromatograph (PEG-20M, 1.5 m, 150 °C). UV spectra were obtained on a Hitachi U-3210 spectrophotometer. Optical rotation was measured with a Perkin-Elmer 241 polarimeter.

Materials. Organic reagents and solvents were purchased from Nacalai Tesque Co., Tokyo Kasei Co., and Aldrich Chemical Co. unless otherwise indicated. Bakers' yeast was purchased from Oriental Yeast Co. and stored in a refrigerator. The substrates (**1a—c**) and (**2a—c**) were prepared according to the method described in our previous paper.[7] The detailed procedure for purification of the enzymes was described in the previous paper.[12]

Enzyme Assay. A 50 μl aliquot of a fraction of chromatography was added to a 3.10 ml solution (0.10 M potassium phosphate buffer at pH 7.0, 1M=1 mol dm^{-3}) containing ethyl 2-allyl-3-oxobutanoate (**2a**) (9.32×10^{-4} M) and NADPH (9.00×10^{-5} M). The kinetics was followed spectrophotometrically at 30 °C following the decreases in absorbance of NADPH at 340 nm. One unit of the enzyme activity was defined as the amount of an enzyme which catalyzes the oxidation of 1 μmol NADPH per minute at 30 °C under the assay conditions.

Reduction of Alkyl 2-Alkyl-3-oxobutanoate with Enzymes. In a 10 ml round-bottomed flask equipped with magnetic stirrer and silicone stopper, were placed a concentrated solution of an enzyme (1 unit), NADPH (8 mg), a substrate (5 mg), and 0.1 M phosphate buffer (pH 7.0, 4 ml). The flask

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was protected from light and the mixture was stirred for 24 h at 30 °C. The mixture was extracted with ether, washed with water and brine, and dried over anhydrous sodium sulfate. The diastereomer excess (% d.e.) in the hydroxy ester was determined by gas chromatographic analyses (PEG-20M Bonded, 25 m, 90 °C for 2-methyl-3-hydroxy esters and 150 °C for 2-allyl-3-hydroxy esters).[13]

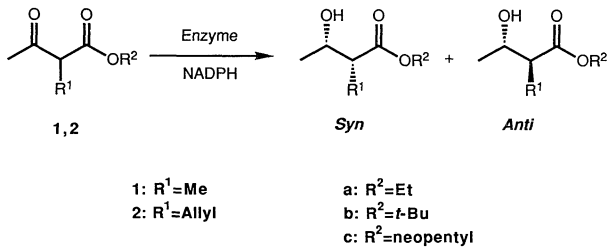
Results

Purification of 2-Alkyl-3-hydroxybutanoate Oxidoreductases. 2-Alkyl-3-hydroxy ester oxidoreductases were separated from the cell-free solution of bakers' yeast by column chromatography on DEAE-Toyopearl anion exchange resin, and two enzymes were found to exert the desired activity. One of them (E1) was not adsorbed on the anion exchange resin, whereas the other (E2) was adsorbed on it at pH 7.0 (10 mM phosphate buffer). Both enzymes were further purified with chromatographic techniques including ion-exchange, hydrophobic, and gel filtration chromatographies. The activity was determined based on the velocity of the reduction of ethyl 2-allyl-3-oxobutanoate (**2a**). Both enzymes utilize NADPH preferentially as the coenzyme. Total activities of these enzymes measured under the assay and preparative conditions are summarized in Table 1.

In the previous paper, we reported that bakers' yeast has four β-hydroxy ester oxidoreductases. Two of them (L-enzymes) afford the corresponding L-hydroxy esters, whereas the other two (D-enzymes) give the D-hydroxy esters.[12] Since the yeast reduction of 2-alkyl-3-oxobutanoates affords the corresponding L-(3*S*)-hydroxy esters exclusively, it is probable that these two 2-alkyl-3-hydroxy ester oxidoreductases are identical with the two L-enzymes reported previously. In order to confirm the identity, the substrate specificities of E1 and E2 were kinetically studied for the reductions of ethyl 4-chloro-3-oxobutanoate and found, together with the chromatographic evidence, that non-adsorbed enzyme (E1) and adsorbed enzyme (E2) are identical with the previously reported L-enzyme-1 and L-enzyme-2, respectively. Detailed descriptions of the purification methods and enzyme properties are given in the previous paper.[12]

The diastereoselectivity in the reduction of various esters of 2-methyl-3-oxobutanoates (**1**) or 2-allyl-3-oxobutanoates (**2**) with these two enzymes were determined (Scheme 2) and the results are summarized in Table 2.

In order to characterize the enzymes, the kinetic parameters, K_M and V_{max}, for the present particular substrates were elucidated from the Lineweaver–Burk plot[14] and the results are listed in Table 3.



Scheme 2.

Table 1. Total Activities of Enzymes for Ethyl 2-allyl-3-oxobutanoate (**2a**) as Substrate

Enzyme	Total activity (unit)	
	[S]=1 mM[a]	[S]=40 mM[b]
E1	8.8[c]	102
E2	93	273

a) Substrate concentration under the assay conditions. b) Substrate concentration under the preparative reaction conditions. c) Note that [S]≪K_M under the assay conditions.

Table 2. Reduction of Alkyl 2-Methyl- and 2-Allyl-3-oxobutanoates (**1**) and (**2**) with Isolated Enzymes

Substrate	*syn*:*anti* Ratio in the product	
	E1	E2
1a	>99:1	82:18
1b	97:3	51:49
1c	>99:1	96:4
2a	>99:1	6:94
2b	>99:1	3:97
2c	>99:1	19:81

Table 3. Kinetic Parameters for the Enzymes[a]

Substrate	K_M/mM		k_{cat}/s^{-1}		$10^6 V_{max}$/M s^{-1} mg^{-1} protein		$10\,k_{cat}/K_M$/s^{-1} mM^{-1}	
	E1	E2	E1	E2	E1	E2	E1	E2
1a	6.4	3.1	4.06	0.179	41.0	1.80	6.35	0.577
1b	15	2.3	2.87	0.070	28.9	0.701	1.91	0.302
1c	0.72	2.0	8.55	0.128	86.1	1.29	119	0.642
2a	15	2.1	3.48	0.452	35.1	4.56	2.32	2.15
2b	30[b]	2.1	8.32	1.22	83.8	12.3	2.77	5.80
2c	1.1	1.0	1.90	0.403	19.1	4.06	17.3	4.03

a) Errors in K_M and k_{cat} are estimated to be less than ±10%. b) The error in this value is about ±20% because the solubility of this substrate is only 3 mM.

Discussion

Several hydroxy ester oxidoreductases have been isolated from bakers' yeast. Shieh et al. isolated three kinds of β-hydroxy ester oxidoreductases, which reduced ethyl 4-chloro-3-oxobutanoate.[15] One of them possesses similar properties to fatty acid synthetase (FAS) and reduces β-keto esters to afford D-hydroxy ester. One of the other enzymes also gives D-hydroxy ester (D-enzyme), whereas the last one yields L-hydroxy ester (L-enzyme). Their L-enzyme is distinct from our both L-enzymes since there have different molecular weight and kinetic constants. Furuich et al. isolated a β-hydroxy ester oxidoreductase from bakers' yeast.[16] It reduces benzyl 2-methyl-3-oxobutanoate affording the corresponding L-hydroxy ester (L-enzyme). This enzyme differs from our enzymes, because the latter enzymes are able to reduce ethyl 2-methyl-3-oxobutanoate (**1a**), whereas the former inactive toward this substrate. Although they reported the isolation of another oxidoreductase from *Saccharomyces fermentati*, this enzyme also differs from our enzymes both in molecular weight and substrate specificity.[17]

As listed in Table 2, E1 is distinct from E2 in diastereoselectivity of the reduction. The former affords the L-*syn*-hydroxy ester with exclusive stereoselectivity for all the substrates. On the other hand, the diastereoselectivity of the latter enzyme depends on the structure of the substrate; not only on the structure of the alkoxyl group but also on the structure of the substituent at the C_2-position. The substrates (**1**) that have a methyl group at the C_2-position tend to afford the corresponding *syn*-hydroxy esters under the catalysis of E2, whereas the substrates (**2**) having an allyl group at the same position prefer to afford the corresponding *anti*-hydroxy esters under the same reaction conditions.

In the reaction with E2, the values of K_M remains almost constant regardless of the structure through all of the substrates employed. However, this tendency is not held in the reaction with E1: the K_M for the neopentyl ester is small but that for the *t*-butyl ester is large in both of 2-methyl and 2-allyl derivatives. That is, it has been elucidated that the active site of E1 is adopted to the neopentyl derivative more effectively than to the ethyl derivative. The *t*-butyl derivative is the least effective substrate.

In addition, it is interesting to compare the substrate selectivity of E1 with that of E2 from the viewpoint of the stereochemistry at the C_2-position. Only the (2*R*)-isomer can be recognized by E1 as the substrate affording the (2*R*)-*syn*-product exclusively. The (2*S*)-isomer is not reduced until it is converted into the (2*R*)-isomer by isomerization equilibrium. This characteristics of the reaction is independent of the structure of the substituent at the C_2-position. On the contrary, E2 has a rather wide selectivity on stereochemistry at the C_2-position: although a substrate which has the 2-methyl substituent exerts a tendency to react as the (2*R*)-isomer, the selectivity at the C_2-position depends largely on the structure of the alkoxyl moiety. Namely, the neopentyl ester (**1c**) is reduced almost exclusively through the (2*R*)-isomer (2*R*:2*S*=96:4), whereas there is no enantiomeric discrimination between the (2*S*)- and (2*R*)-configurations in the *t*-butyl ester (**1b**) (2*R*:2*S*= 51:49).

In the reduction with E2, the substrate with the 2-allyl substituent reacts mainly through the (2*S*)-isomer. Among three substrates studied, the *t*-butyl ester (**2b**) exerts the largest (2*S*)-selectivity, which is a distinct contrary to the selectivity on the 2-methyl derivative mentioned above. In other words, it may be concluded that the *t*-butoxyl group prefers to afford the (2*S*)-*anti*-product, but the neopentyloxyl group suits to yield the (2*R*)-*syn*-product.

Knowing relative activities of the enzymes in bakers' yeast and the concentration of the substrate, we can simulate the stereoselectivity of the microbial reduction theoretically. The substrate concentration employed for the present preparative reactions is 40 mM.[7] Although **1a** and **2a** have enough solubility to maintain this stoichiometric concentration during the reaction, **1b**, **1c**, **2b**, and **2c** are less soluble to water and their concentrations in saturated aqueous solutions are about 12, 3, 3, and 1 mM, respectively. That is, the effective concentrations of the substrates in the latter group are much smaller than 40 mM in the preparative reactions. Therefore, the diastereoselectivities of the reduction can be evaluated based on the effective concentrations of the substrates, the relative activities: velocity (listed in Table 3) × total unit (listed in Table 1), and the syn/anti selectivities of these two competing enzymes. The results are summarized in Table 4. The diastereoselectivities experimentally observed for the reduction with bakers' yeast[7] are also listed in Table 4 for comparison. It is apparent that the calculated selectivities are in good agreement with the experimental values.

The mechanism for stereochemical control in the reduction with bakers' yeast by modification of the alkoxyl group is thus explicated by kinetic parameters

Table 4. Calculated Diastereoselectivity of the Reduction Based on Kinetic Parameters

Substrate	*syn*:*anti* Ratio in the product	
	Bakers' yeast[a]	Calcd
1a	87:13	92:8
1b	72:28	78:22
1c	96:4	99:1
2a	35:65	31:69
2b	6:94	9:91
2c	56:44	37:63

a) Ref. 8.

for the combinations of various substrates and enzymes. The K_M values for E2 do not change appreciably upon the modification in structure of the alkoxyl group, whereas those for E1 change so that they increase when the alkoxyl oxygen is bound directly to a tertiary carbon such as in the *t*-butyl group, and decrease when a methylene group is inserted between the alkoxyl oxygen and a bulky alkyl group such as in the neopentyl group.

It is conceivable that the binding site of E1 has a shape such that there is no enough room to set a bulky tertiary carbon in the vicinity of the alkoxyl oxygen. The steric tension in the pocket can be released, however, by the insertion of a methylene group between the alkoxyl oxygen and a bulky alkyl group because the molecule now procures an ability to fracture the bulky substituent to assume the least volume.[7] Consequently, the presence or absence of a methylene group adjacent to the alkoxyl oxygen is crucial to control the diastereoselectivity of the reduction.

The pocket of E2, on the other hand, is so vague that not only the structure of the alkoxyl group but also the stereochemistry at the C_2-position are not discriminated strictly by this enzyme. It is assumed that a substrate can be contained in the pocket of E2 setting either of its 2*S*- and 2*R*-isomer against the reacting function of the enzyme without causing serious steric strain. The dominant effect to choose the isomer is the force to set the substrate in the stable form in the pocket of the enzyme. The *syn*- and *anti*-products are, therefore, afforded in various ratios under the catalysis of E2 depending on the structure of substituent at the C_2-position.

The enzymatic studies of bakers' yeast for other substrates are now in progress in our laboratory and will be reported elsewhere.

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