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Mechanism for improving stereoselectivity for asymmetric reduction using acetone powder of microorganism

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Abstract

The mechanism of a dramatic improvement in stereoselectivity for the reduction of ketones using acetone powder of *Geotrichum candidum* is elucidated by examining stereoselectivity of the cell-free system. The improvement in stereoselectivity by the present method was also applicable to the reactions with enzymes from other kinds of microorganisms.

Keywords: enantiocontrol; enzymes and enzyme reactions; coenzymes; reduction.

Microbial reduction has been widely used for the synthesis of chiral alcohols.[1–7] However, microbial reduction does not afford an alcohol of satisfactory optical purity[1–7] when enzymes of different stereoselectivities are present in the cell. Therefore, inhibitors,[8,9] additives such as reducing agents[10–12] and organic solvents[13–16] have been used to improve the enantioselectivity. Recently, we reported that the stereoselectivity of the reduction of aromatic ketones, β-keto esters, and simple aliphatic ketones can be improved significantly by treating resting cells of *Geotrichum candidum* IFO 4597 with acetone to prepare acetone powder (APG4) and using this powder with a coenzyme and a secondary alcohol.[11,12] The stereoselectivity of the APG4 reduction system is shown to be extremely high (>99% ee) and the substrate specificity of the system is very wide.[11,12] However, it has not yet been clarified how the high stereoselectivity is achieved just by treating the cells with acetone. In this report, the mechanism of a dramatic improvement in stereoselectivity for the reduction of ketones using the acetone powder of *G. candidum* is elucidated by examining stereoselectivity of the cell-free, enzymatic reaction in the homogeneous system.

As reported previously, the stereoselectivity for the reduction of acetophenone **1** by the resting cell is poor (>52% ee), and reaction does not proceed with only APG4 and coenzyme due to the loss of

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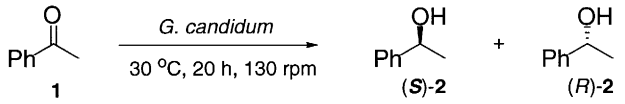
reducing agents. When secondary alcohols such as cyclopentanol are added to the reaction with APG4 and coenzyme, the (*S*)-phenylethanol (*S*)-**2** is obtained with >99% ee (Table 1).[12]

Table 1
Reduction of acetophenone **1** by APG4,[12] cell-free extract and separated enzymes from *Geotrichum candidum*



Entry	Catalyst	Coenzyme	Cyclopentanol	Yield(%)	Ee(%)	Config.
1	Resting Cell[12]	-	-	52	28	*R*
2	APG4[12]	$NADP^+$	-	0	-	-
3	APG4[12]	$NADP^+$	+	86	>99	*S*
4	Cell-free Extract	NADPH	-	59	80	*S*
5	Cell-free Extract	$NADP^+$	+	77	>99	*S*
6	S-enzyme	NADPH	-	5	>99	*S*
7	S-enzyme	$NADP^+$	+	60	>99	*S*
8	R-enzyme	NADPH	-	69	92	*R*
9	R-enzyme	$NADP^+$	+	12	92	*S*
10	S-enzyme + R-enzyme	NADPH	-	64	22	*R*
11	S-enzyme + R-enzyme	$NADP^+$	+	59	>99	*S*

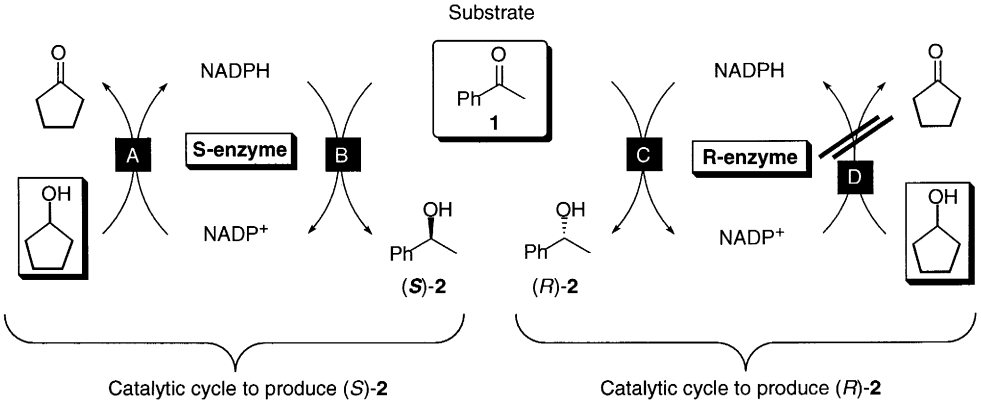
S-enzyme and R-enzyme isolated by FPLC (MonoQ-HR) and rechromatographed by FPLC (MonoQ-HR) were used for the reactions. Yield, ee and absolute configurations of the reduction of **1** (2.5 mM) with NADPH (13 mM) or $NADP^+$ (1.5 mM) and cyclopentanol (5.0 mM) were determined by GC analysis (Chirasil-DEX CB; 25 m; He 2 mL/ min).

Obviously, poor stereoselectivity with resting cells suggests that the resting cell contains both S-enzyme(s) and R-enzyme(s). The question is whether the acetone treatment deactivates R-enzyme(s) selectively and APG4 contains only S-enzyme(s) resulting in a high ee, or both enzymes remain present and it still shows a high ee. A cell-free extract prepared from APG4 was charged on an anion exchange column and enzymes in APG4 were separated and both S-enzymes and an R-enzyme were found.[17] The stereoselectivity of the reduction was examined using either an excess amount of NADPH or a small amount of $NADP^+$ with cyclopentanol. The result is shown in Table 1. The stereoselectivity of the reduction of **1** by the cell-free extract with NADPH was 80% ee (Table 1, Entry 4). This stereoselectivity (80% ee) does not explain the high stereoselectivity (>99% ee) of the APG4 system. Therefore, instead of using NADPH, the oxidized form of coenyzme ($NADP^+$) and cyclopentanol for the recycling of the coenzyme were used as in the APG4 system. Then the enantioselectivity was improved to an extremely high level (>99% ee) (Table 1, Entry 5). The dramatic improvement was observed in the homogeneous cell-free system as well as in the cell system (APG4 system).

When the S-enzyme and the R-enzyme were used separately for the reduction of **1** in the presence of NADPH, (*S*)-**2** and (*R*)-**2** with high ee's were obtained, respectively (Table 1, Entries 6 and 8). Enantiomerically pure (*S*)-**2** was also obtained by the catalysis of the S-enzyme in the presence of $NADP^+$ and cyclopentanol (Table 1, Entry 7), which indicates that the S-enzyme can recycle $NADP^+$ using cyclopentanol. The formation of cyclopentanone was also confirmed by GC analysis. However, cyclopentanol was not effective for the R-enzyme (Table 1, Entry 9) ((*S*)-**2** was produced with a 12% yield due to the contamination of the S-enzyme in the R-enzyme fraction). To model the condition in

the cell-free extract and APG4, the S-enzyme (0.0011 unit) and Renzyme (0.0059 unit) were mixed and stereoselectivity for the reduction of **1** was examined (Table 1, Entries 10 and 11; one unit of enzyme oxidizes 1 μmol NADPH to $NADP^+$/min in the presence of **1**). When the mixed enzymes were used in the presence of NADPH, an almost racemic product (*R*/*S*)-**2** was obtained (Table 1, Entry 10), while enantiomerically pure (*S*)-**2** was obtained in the presence of $NADP^+$ and cyclopentanol (Table 1, Entry 11). The dramatic improvement in the selectivities was also observed using the mixture of the S-enzyme and the R-enzyme as well as using the cell-free extract and APG4.

The explanation for the difference between using NADPH and using $NADP^+$ and cyclopentanol is as follows: when NADPH is added to the reaction mixture, both enzymes can use NADPH to reduce **1** (Scheme 1, paths B and C), affording a mixture of both enantiomers. However, when $NADP^+$ and cyclopentanol are used for the reduction, the catalytic cycle of the S-enzyme turns (Scheme 1, paths A and B) but that of the R-enzyme does not (Scheme 1, path D). Cyclopentanol is selectively used by the S-enzyme to reduce $NADP^+$ but not by the R-enzyme which leads the high stereoselectivity of the reduction. After all, it was shown that the excellent enantioselectivity can be obtained using the mixture of enzymes of different stereoselectivities because of the selectivity toward cyclopentanol.



Scheme 1.

NADPH is usually considered to freely bind in and dissociate from an active site of enzymes. However, in this case, NADPH produced by the S-enzyme does not dissociate before the reduction of the main substrate **1**; if the NADPH produced by the S-enzyme dissociated into the solution, R-enzyme could use the NADPH to produce (*R*)-**2**, which would result in low stereoselectivity.

The mechanism for stereochemical control is applicable to cell-free extracts of other kinds of *G. candidum* as well as other microorganisms as shown in Table 2. The enantioselectivities using $NADP^+$ and cyclopentanol were much higher than those using NADPH. Similar enzymes are distributed in many kinds of microorganisms. This mechanism is probably applicable to other dehydrogenase systems using secondary alcohol for the recycling of coenzymes[1–7,18–24] although it has not been specified.

In conclusion, the mechanism for stereochemical control by the APG4 system is clarified. Only the S-enzyme but not the R-enzyme can use cyclopentanol for the recycling of NADPH in the homogeneous reaction, which explains the extremely high stereoselectivity of the APG4 system. It is also shown that this system is applicable to other microorganisms.

Table 2
Comparison of the stereoselectivity of the reduction of **1** with NADPH and with $NADP^+$ and cyclopentanol by cell-free extract[a] of various microorganisms

Microorganism	NADPH[b]		$NADP^+$ and Cyclopentanol[c]	
	Yield (%)[d]	ee(%)[d]	Yield (%)[d]	ee(%)[d]
Geotrichum candidum IFO 5767	78	84	77	95
Geotrichum candidum ATCC 34614	75	96	78	98
Galactomyces reessii IFO 1112	65	90	75	97
Endomyces magnusii IFO 4600	7	82	47	98
Endomyces geotrichum IFO 9541	31	77	76	96

[a] A microbial dried-cell preparation (20 mg), dehydrated using acetone prepared as described previously,[12] was homogenized with HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH: pH 7.2, 0.1 M, 2 mL) and centrifugated to obtain the cell-free extract. [b] **1**: 2.5 mM, NADPH: 13 mM, 20 h at 30 °C at 130 rpm. [c] **1**: 2.5 mM, $NADP^+$: 1.5 mM, cyclopentanol: 5.0 mM, 20 h at 30 °C at 130 rpm. [d] determined by GC analysis (Chirasil-DEX CB; 25 m; He 2 mL/ min). Yield is not optimized. The absolute configuration is *S*.

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