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Steady-state kinetics of the oxidation of (*S*)-1-phenyl-1,2-ethanediol catalyzed by alcohol dehydrogenase from *Candida parapsilosis* CCTCC M203011

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Abstract

Kinetic studies of oxidation reaction of (*S*)-1-phenyl-1,2-ethanediol (PED) catalyzed by a NAD^+-dependent alcohol dehydrogenase from *Candida parapsilosis* CCTCC M203011 obtained from China Center for Type Culture Collection (CPADH) were observed for getting insight into the deracemization redox reaction. The data of initial velocity experiments in the absence of product, product (β-hydroxy-hypnone) inhibition experiments and dead-end (pyrazole) inhibition experiments strongly suggest that the reaction follows Theorell-Chance BiBi mechanism in which the coenzymes bind to the free form of the enzyme firstly. The kinetic parameters of this model were estimated by using non-linear regression analysis software.
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Keywords: *Candida parapsilosis* CCTCC M203011; Alcohol dehydrogenase; Enzyme kinetics; Theorell-Chance BiBi mechanism

1. Introduction

In the past few years, the demand for compounds with a high enantiomeric purity has kept increasing for their applications in production of biochemicals, pesticides, flavour chemicals, pigments, liquids crystals, non-linear optical materials and polymers. Oxidoreductase, especially the alcohol dehydrogenases are well known as the suitable catalysts in organic synthesis for its high enantiospecificity, especially chiral synthesis [1–3].

PED is a very important chiral building block for the synthesis of pharmaceuticals and liquid crystals [4,5]. Nie reported that *Candida parapsilosis*, one kind of good producer of dehydrogenase used in chiral synthesis [6–8], have the ability of producing enantiomerically pure (*S*)-PED from the racemates which involved the oxidation of (*R*)-PED to β-hydroxy-hypnone by an NAD^+-linked (*R*)-specific alcohol dehydrogenase and the reduction of ketone to (*S*)-PED by an NADPH-linked (*S*)-specific reductase (Scheme 1) [9,10].

It is a very important way to know the biocatalytic processes by steady-state kinetic studies, especially in chiral building blocks production process. Although there are some kinetic studies about some kinds of dehydrogenases already having been performed [11–14], the kinetic mechanism of the oxidation of PED catalyzed by CPADH is still unknown. And the kinetic studies of oxidation of PED are also the fundamental steps in kinetic modeling of enzymatic deracemization reactions involving the elucidation of the kinetic mechanism of the main reaction.

Some similarly pilot studies of this kind of redox reactions catalyzed by alcohol dehydrogenase followed two mechanisms, sequential ordered BiBi mechanism and Theorell-Chance BiBi mechanism [15–17]. They are both sequential type with the coenzymes binding to the free form of the enzyme, and then binding the substrates. The difference between the types is the sequential ordered BiBi (Scheme 2a) with formation of central ternary complexes and Theorell-Chance BiBi (Scheme 2b) without these complexes. Determination of the type of inhibition produced by the first product (β-hydroxy-hypnone) of the oxidation reaction with respect to the second substrate of the enzyme (PED) is the key factor to conclude the kinetic mechanism [18].

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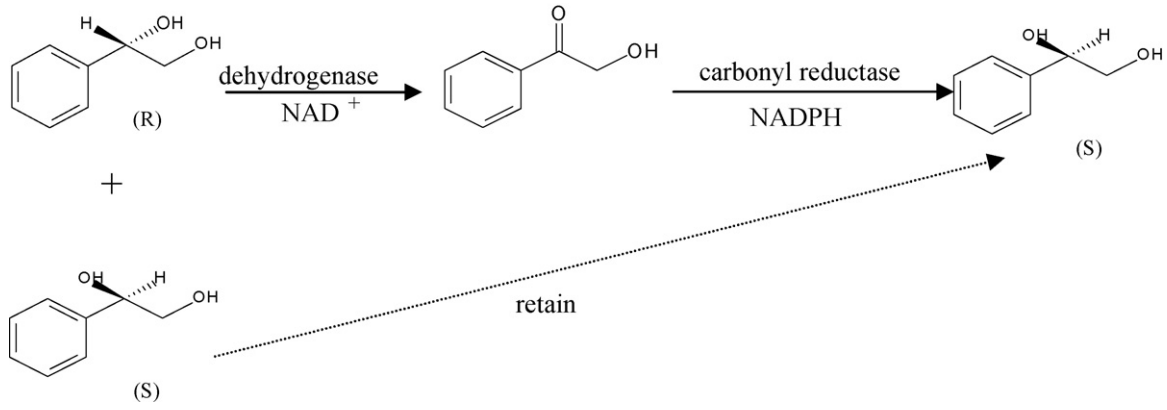
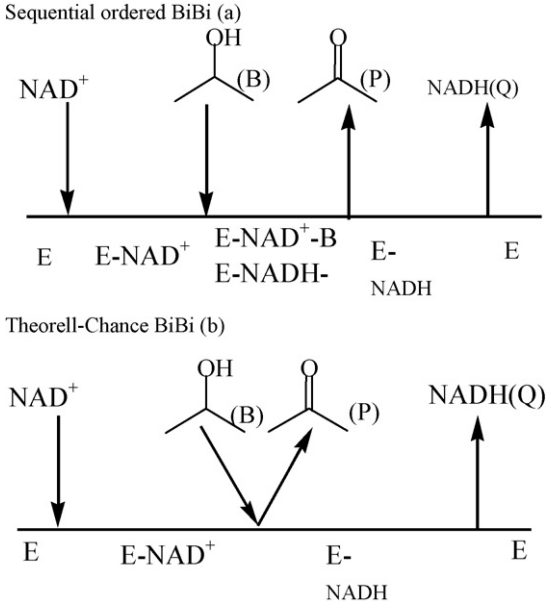


Scheme 1. Deracemization redox reaction of PED catalyzed by enzymes from *Candida parapsilosis*.



Scheme 2. Two common kinetic mechanism of oxidation reaction catalyzed by dehydrogenase. (A) NAD^+, (B) PED, (P) β-hydroxy-hypnone, (Q) NADH, and (E) CPADH.

2. Materials and methods

2.1. Materials

All chemicals used in this work were of analytical grade and commercially available. CPADH was purified after treatment of ethanol precipitation, affinity chromatography (Blue sepharose Fast Flow column, Phamacia) and ion-exchange chromatography (DEAE sepharose Fast Flow column, Phamacia). The purified enzyme was homogeneous checked by SDS-PAGE (unpublished results).

2.2. Methods

2.2.1. Enzyme activity

The standard assay mixture for oxidation comprised, in 250 μl, 5 mM NAD^+, 100 mM PED and 50 μl enzyme solution (8 U/ml) using 0.1 M Tris–HCl buffer, pH 9.0. The reaction mixture was incubated for 2 min without the enzyme at 25 °C, and then the reaction was initiated by the addition of the catalyst. The increase of the amount of the NADH was measured spectrophotometrically at 340 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol of NAD^+ per min under the assay conditions.

2.2.2. Kinetic reaction condition

The determination of the kinetic mechanism of oxidation of (*R*)-PED catalyzed by CPADH was done by initial velocity and product inhibition studies with NAD^+ (0–0.3 mM), 20 μl of the enzyme stock solution, β-hydroxy-hypnone (0–0.8 mM), NADH (0–0.06 mM) as product inhibitor, pyrazole (0–0.1 mM) as dead-end inhibitor and rac-PED (0.5–20 mM) as variable substrate. All the experiments were performed in Tris–HCl buffer, pH 9.0.

2.3. Data processing

The values appearing in the figures were the mean of experimentally determined initial velocities (triplicates), and used in determining the kinetic parameters. Estimates of kinetic parameters, and of their asymptotic standard error (S.E.) were obtained by fitting the appropriate rate equation to experimental initial velocity data obtained as described above using a non-linear least-squares computer program of MATLAB (The MathWorks Inc., MA, USA).

Eq. (1) represents the rate equation for the sequential ordered BiBi mechanism in the presence of product P (ketone) and Eq. (2) represents another Theorell-Chance BiBi mechanism [19,20]:

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_bK_q[P]}{K_pK_{iq}} + \frac{K_bK_q[A][P]}{K_pK_{iq}} + \frac{[A][B][P]}{K_{ip}}} \quad (1)$$

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_bK_q[P]}{K_pK_{iq}} + \frac{K_bK_q[A][P]}{K_pK_{iq}}} \quad (2)$$

where v is the initial velocity, V the maximum velocity, [A] the concentration of NAD^+, [B] the concentration of PED, K_{ia} the dissociation constant of CPADH-NAD^+, K_a the Michaelis constant for NAD^+, K_b the Michaelis constant for PED, K_{ip}

the inhibition constant of β-hydroxy-hypnone, and K_{iq} is the inhibition constant of NADH.

According to Eqs. (1) and (2), the initial velocity equation in the absence of product was:

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad (3)$$

K_p, K_{iq}, and K_q can be written to Eq. (4) and it was subsisted into Eq. (1):

$$K_I = \frac{K_p K_{iq}}{K_q} \quad (4)$$

It gives Eq. (5):

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_b[P]}{K_I} + \frac{K_b[A][P]}{K_I} + \frac{[A][B][P]}{K_{ip}}} \quad (5)$$

On the other hand, from the Haldane equations established for the Theorell-Chance BiBi kinetic mechanism, the following relationship can be introduced into Eq. (2), which is then transformed into Eq. (7):

$$K_{ip} = \frac{K_p K_{iq}}{K_q} \quad (6)$$

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_b[P]}{K_{ip}} + \frac{K_b[A][P]}{K_{ip}}} \quad (7)$$

Eqs. (5) and (7) were fitted to the data set combining initial rate experiments with product inhibition experiments.

Eqs. (8)–(10), which correspond to the linear mixed-type inhibition model, linear non-competitive inhibition rate equation and linear competitive inhibition rate equation, respectively, were fitted to experimental data of initial rate experiments with product inhibition.

$$v = \frac{V_m[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\left(1 + \frac{[I]}{K_{ii}}\right)} \quad (8)$$

$$v = \frac{V_m[S]}{(K_m + [S])\left(1 + \frac{[I]}{K_i}\right)} \quad (9)$$

$$v = \frac{V_m[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (10)$$

where V_m is the maximum velocity, [S] the concentration of substrates, K_m the Michaelis constant of PED, [I] the concentration of inhibitor, K_i the apparent value of the dissociation constant of enzyme-inhibitor complex, and K_{ii} is the apparent value of the dissociation constant of enzyme-varied substrate inhibitor complex.

Eq. (11), which corresponds to the linear dead-end inhibition with formation of a central complex, was fitted to experimental data of initial rate experiments with dead-end inhibitor.

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A]\left(1 + \frac{[I]}{K_{ii}}\right) + K_a[B] + [A][B]} \quad (11)$$

The quality of fittings was evaluated by the determination of statistical parameter, the residual standard error of the estimate of parameters, which is defined by Eq. (12):

$$\mathrm{Syx} = \sqrt{\sum_{i=1}^{n} \frac{(v_i - \overline{v_i})^2}{N - P}} \quad (12)$$

3. Results and discussion

3.1. Initial velocity patterns of the oxidation reaction

As the first step in the determination of the kinetic mechanism of oxidation of PED catalyzed by CPADH, the initial velocity of reaction was determined at fixed concentrations of PED (Fig. 1) showed the fitting of Eq. (1) to the initial velocity data obtained in the absence of products.

The parameters of initial velocity patterns of intersecting lines were obtained when the concentration of PED was varied and the concentration of NAD^+ was fixed at various levels.

The good fitting of these data to Eq. (1) (Fig. 1 and Table 1), as well as the fact that the secondary plots derived from it, such as slopes and ordinate intercepts versus the reciprocal of the fixed substrate concentration were also linear, strongly suggest that the reaction was most probably of the sequential type involving ternary central complexes, not necessary kinetically important. However, these patterns excluded the ping-pong type of mechanism involving more than one stable form of the enzyme.

3.2. Product inhibition patterns

The study of product inhibition was carried out to analyze the kinetic significance of the ternary complexes and also to get the information concerning the sequence of binding of substrates to

Table 1
Estimates of kinetic parameters derived from initial velocity studies on product inhibition of the oxidation of 1-phenyl-1,2-ethanediol with NAD^+ catalyzed by dehydrogenase from *Candida parapsilosis*

Estimates of parameters	NADH		β-Hydroxy-hypnone	
	NAD^+	PED	NAD^+	PED
V_m	6.6 ± 0.4	9.4 ± 0.3	6.6 ± 0.3	9.7 ± 0.3
K_m	0.15 ± 0.04	11.2 ± 0.7	0.18 ± 0.03	9.1 ± 0.5
K_i	0.22 ± 0.04	2.6 ± 0.3	0.89 ± 0.1	1.5 ± 0.1
Inhibition model	Competitive	Non-competitive	Non-competitive	Competitive

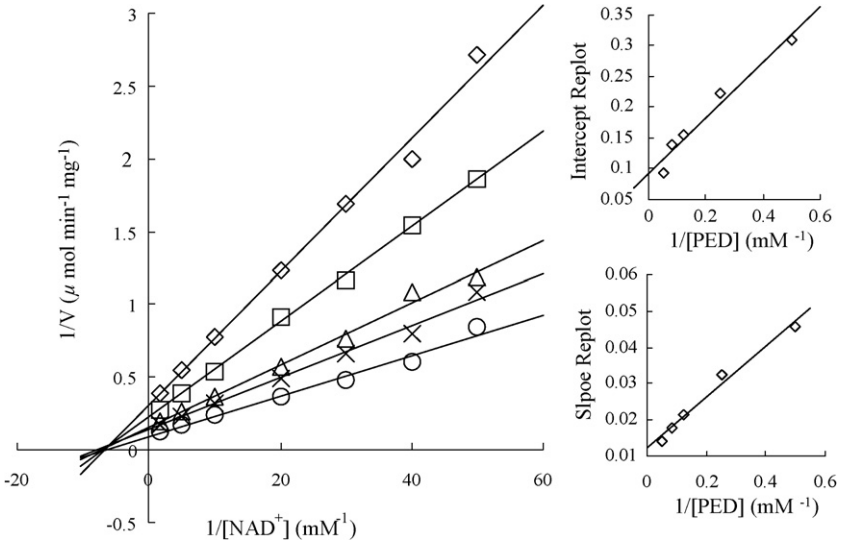


Fig. 1. Effect of PED on the initial velocity of the oxidation reaction of PED with NAD^+ as the variable substrate. The concentration of PED were: (□) 2 mM, (◊) 4 mM, (△) 8 mM, (×) 10 mM, and (◯) 20 mM. The line shows the trend of the data.

the enzyme and the order of release of products from the central ternary complexes. Double reciprocal plots indicate that NADH produced the competitive inhibition in relation to $NAD^+$and linear non-competitive inhibition in relation to PED (Table 1). On the other hand, the ketone, oxidation product of PED, was found to be a linear competitive inhibitor when PED was the varied substrate and a linear non-competitive inhibitor when NAD^+ as the varied substrate (Table 1).

As shown in Table 2, the estimate of K_{ip} of sequential ordered BiBi mechanism is much bigger than it of Theorell-Chance BiBi mechanism. And its standard error also shown that the precision was also very low. After these inhibitions patterns were observed, the sequential ordered mechanism with central complexes kinetically significant can be discarded for the low statistical confidence. For the central complexes are kinetically unimportant, two competitive and two non-competitive inhibitions pattern indicated that the oxidation reaction of PED followed Theorell-Chance BiBi mechanism.

The results shown in Table 3, which was analyzed by fitting the data of product inhibition of ketone with PED as the varied parameter to Eqs. (8) and (10), indicates that both kinetic model are well fitted for the small value of the residual standard error (Syx) and by the fact that this statistical parameter attained almost the same value on fitting both rate equations.

However, the estimate of K_{ip} of sequential order BiBi mechanism is much bigger than it of Theorell-Chance BiBi mechanism. And the asymptotic standard error of the estimate of K_{ii} of sequential order BiBi mechanism is higher than the parameter estimate of K_{is}. So the item of $[I]/K_{ii}$ could be discarded. And then the linear mixed-type inhibition model (Eq. (8)) was transformed into the linear competitive inhibition model (Eq. (9)), which is the Theorell-Chance product inhibition equation.

3.3. Dead-end inhibition studies

Since the product inhibition patterns for varied NAD^+ are symmetrical to those varied PED, product inhibition experiments did not disclose the order of substrate addition and product release.

This information can be observed by adding a dead-end inhibitor. Pyrazole, a well-known irreversible inhibitor of ADH nicotinamide complex for the enzyme which containing zinc atom [21,22], was selected as the dead-end inhibitor.

Fig. 2a and b shows that pyrazole acted as a linear competitive inhibitor when PED was the varied substrate and as a linear

Table 2
Comparison of estimates of kinetic parameters for the sequential ordered BiBi and the Theorell-Chance BiBi mechanism derived from initial velocity studies on the oxidation of 1-phenyl-1,2-ethanediol with NAD^+ catalyzed by dehydrogenase from *Candida parapsilosis*

Estimates of kinetic parameters	Value of sequential order	Value of Theorell-Chance
V (μmol min^{-1} mg^{-1})	10.8 ± 0.5	10.7 ± 0.5
K_{ia} (mM)	0.16 ± 0.02	0.16 ± 0.01
K_b (mM)	4.9 ± 0.4	5.0 ± 0.4
K_a (mM)	0.06 ± 0.01	0.06 ± 0.01
K_I (mM)	7.9 ± 0.2	–
K_{ip} (mM)	568.5 ± 342.5	7.9 ± 0.2
Syx	0.213	0.206

Table 3
Estimates of kinetic parameters derived from initial velocity studies on product (β-hydroxy-hypnone) inhibition of the oxidation of 1-phenyl-1,2-ethanediol catalyzed by dehydrogenase from *Candida parapsilosis* when PED as varied substrate

Estimates of kinetic parameters	Mixed-type competitive inhibition	Competitive inhibition
V_m (μmol min^{-1} mg^{-1})	9.5 ± 0.4	9.7 ± 0.3
K_m (mM)	10.1 ± 0.5	9.1 ± 0.4
K_i (mM)	1.4 ± 0.1	1.5 ± 0.1
K_{ii} (mM)	182.1 ± 112.3	–
Syx	0.183	0.180

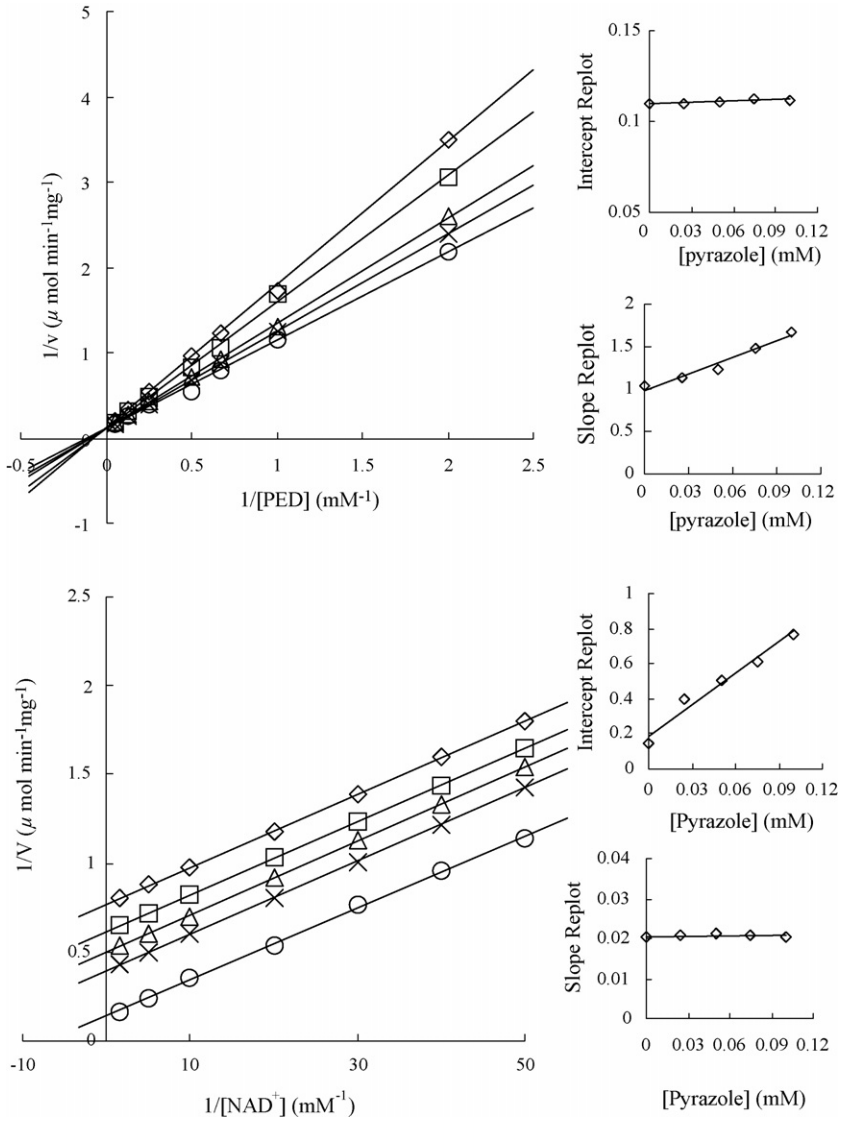


Fig. 2. (a) Inhibition of the reaction of oxidation of PED catalyzed by CPADH by pyrazole with PED as the variable substrate and NAD^+ concentration was kept constant at 0.2 mM. The concentration of pyrazole were: (◯) 0 mM, (×) 0.025 mM, (△) 0.05 mM, (□) 0.075 mM, and (◊) 0.1 mM. The line shows the trend of the data. (b) Inhibition of the reaction of oxidation of PED catalyzed by CPADH by pyrazole with NAD^+ as the variable substrate and PED concentration was kept constant at 8 mM. The concentration of pyrazole were: (◯) 0 mM, (×) 0.025 mM, (△) 0.05 mM, (□) 0.075 mM, and (◊) 0.1 mM. The line shows the trend of the data.

uncompetitive inhibitor when NAD^+ was the varied substrate. The results indicate that the (*S*)-PED ADH binding the coenzyme, NAD^+, firstly and PED being the secondary substrate.

Moreover, since NADH shown the linear competitive inhibition in relation to NAD^+, the reduced coenzyme must be the last product to be released in the reaction, i.e., both coenzymes bind to the free form of the enzyme.

3.4. Conclusion

Determination of the kinetic mechanism of the oxidation of PED catalyzed by CPADH required the combination of several techniques. Double-reciprocal plots of initial velocity data have an intersecting pattern of lines indicative of a sequential mechanism and discharged the possibility of a ping-pong mechanism. Substrate binding order was probed using substrate analogue dead-end inhibitor of NAD^+. Product inhibition studies were initiated to elucidate the order of product release.

Through the experiments data, it suggests that the PED oxidation reaction catalyzed CPADH follows an ordered substrate addition and an ordered product release and supports the occurrence of a Theorell-Chance BiBi mechanism.

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