INHIBITION OF PHENYLALANINE HYDROXYLASE, A PTERIN-REQUIRING MONOOXYGENASE, BY OUDENONE AND ITS DERIVATIVES

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Phenylalanine hydroxylase was shown to be inhibited by oudenone and its derivatives *in vitro*. At a concentration of 2.3×10^{-3} M, oudenone inhibited phenylalanine hydroxylase by 50%, and some of the oudenone derivatives showed more potent inhibition. The kinetic data have shown that the inhibition by oudenone is competitive with a tetrahydropterin cofactor (6,7-dimethyltetrahydropterin, $DMPH_4$) and noncompetitive with phenylalanine and oxygen. Among 12 oudenone derivatives, there was no parallel structure-activity relationship between the inhibitory effect for phenylalanine hydroxylase and that for tyrosine hydroxylase. A derivative of oudenone, [compound No. 142; 2-(3-(3,4-dihydroxyphenyl)-1-oxopropyl)cyclohexan-1,3-dione] showed the most potent inhibition among the oudenone derivatives. It inhibited phenylalanine hydroxylase by 50% at a concentration of 1.8×10^{-5} M. This inhibition was a mixed type with either a tetrahydropterin cofactor, $DMPH_4$, or with the substrate phenylalanine, which was different from the inhibition by oudenone. However, the same noncompetitive inhibition was shown toward oxygen.

Phenylalanine hydroxylase (EC. 1.14.16.1) catalyzes the conversion of L-phenylalanine to L-tyrosine using tetrahydropterin as a reducing agent and molecular oxygen as an oxidizing agent. This is the first and essential step of the degradation of phenylalanine[1,2]. A deficiency of this enzyme leads to the disease, phenylketonuria[3,4].

It is known that two other pterin-requiring enzymes exist. One is tyrosine hydroxylase which produces L-dopa from L-tyrosine and is the rate-limiting enzyme of catecholamine biosynthesis[5], and another is tryptophane hydroxylase which forms L-5-hydroxytryptophan from L-tryptophan and is also the rate-limiting enzyme of serotonin biosynthesis[6,7]. These three pterin-dependent aromatic amino acid hydroxylases catalyze very similar reactions, but it is unknown if these enzymes act through the same reaction mechanism.

Oudenone was discovered as a new tyrosine hydroxylase inhibitor of microbial origin (*Oudemansiella radicata*) by UMEZAWA *et al*[8]. Since the inhibition of tyrosine hydroxylase by oudenone is competitive with a tetrahydropterin cofactor, it is conceivable that oudenone may inhibit phenylalanine hydroxylase which is also a pterin-requiring monooxygenase.

In this paper we studied the mechanism of inhibition of phenylalanine hydroxylase by oudenone and its derivatives, in order to determine if there are differences in the mode of inhibition by oudenone for phenylalanine hydroxylase and tyrosine hydroxylase.

Materials and Methods

6,7-Dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$) was from Aldrich (Wisconsin, USA), catalase was from Boehringer (Mannheim, GFR), L-[U-^{14}C]tyrosine was from Daiichi Pure Chemical Co. (Tokyo, Japan), and Yanapak ODS-T was from Yanagimoto Manufacturing Co. (Kyoto, Japan). (*S*)-Oudenone was purified from the culture filtrate of *Oudemansiella radicata*, as described by UMEZAWA *et al*[8].

Oudenone derivatives were synthesized by the method of OHNO *et al.*, which will be reported elsewhere.

Phenylalanine hydroxylase was purified from rat liver according to the procedure of SHIMAN *et al.*[9] Purified phenylalanine hydroxylase, more than 90% pure, was stored in 0.03 M tris-HCl buffer, pH 7.2, containing 0.037% Tween 80, 50 μM EDTA and 15% glycerol at -80°C until use. Tyrosine hydroxylase was prepared from bovine adrenal medulla according to the procedure of NAGATSU *et al.*,[5] and stored as an ammonium sulfate precipitate at -80°C.

Activity of phenylalanine hydroxylase was assayed by measuring L-tyrosine formed from L-phenylalanine as substrate by high-performance liquid chromatography with a fluorometric detector[10]. The standard incubation mixture (total volume, 250 μl) contained 0.1 M tris-HCl buffer, pH 7.4, 0.98 μg of purified phenylalanine hydroxylase, 10 mM dithiothreitol, 60 μg catalase, 1 mM L-phenylalanine and 50 μM $DMPH_4$. After preincubation for 5 minutes at 30°C, the reaction was started by addition of $DMPH_4$ with dithiothreitol. Incubation was carried out at 30°C for 10 minutes, and the reaction was stopped by adding 50 μl of 30% trichloroacetic acid. After centrifugation at 3,000 rpm for 10 minutes, the supernatant was subjected to high-performance liquid chromatography (Simadzu LC-3A high-performance liquid chromatograph) with fluorometric detection (Shimadzu RF-500 LCA spectrofluorometer) using a column (25 cm$\times$0.4 cm i.d.) of Yanapack ODS-T. The mobile phase was 0.1 M acetate-ammonium buffer (pH 4.5) containing 10% methanol with a flow-rate of 2.0 ml/minute.

The activity of tyrosine hydroxylase was assayed by measuring L-[^{14}C]dopa formed from L-[U-^{14}C]-tyrosine. The incubation mixture contained 0.2 M acetate buffer (pH 6.0), 0.1 mM L-tyrosine containing L-[U-^{14}C]tyrosine (1.1×10^5 cpm), 1 mM $DMPH_4$, 100 mM 2-mercaptoethanol, 2.5 mM $FeSO_4$, and the enzyme solution (1 mg protein) in a total volume of 1.0 ml. The reaction was carried out at 30°C for 15 minutes, and the L-[^{14}C]dopa was measured according to the procedure of NAGATSU *et al.*[5].

For measuring the effects of oudenone and its derivatives on enzyme activity, the potassium salt of (*S*)-oudenone was dissolved in distilled water and added to the incubation mixture. As the control, water was added instead of the oudenone solution. All derivatives of oudenone were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the phenylalanine hydroxylase assay system was 4% (v/v) and that in the tyrosine hydroxylase assay system was 5% (v/v). Then concentrations of DMSO were used for the control.

For kinetic experiments using various oxygen concentrations, various proportions of O_2/N_2 mixed gases in 10 ml-Thunberg tubes were used. After the addition of $DMPH_4$ and dithiothreitol in an ice bath, the air in each tube was immediately replaced by the stream of the mixed gas, and the tubes were closed until the reaction was terminated.

Results

Oudenone inhibited phenylalanine hydroxylase at a concentration of 2.3×10^{-3} M by 50%. The mechanism of this inhibition was further studied by kinetic analysis. LINEWEAVER-BURK plots of velocity *versus* the concentration of $DMPH_4$ (Fig. 1-A), *versus* the concentration of phenylalanine (Fig. 1-B), and *versus* the percentage of O_2 (Fig. 1-C) in the presence or absence of oudenone indicated that the inhibition of phenylalanine hydroxylase by oudenone was competitive with the cofactor, $DMPH_4$, and noncompetitive with the substrates, phenylalanine or molecular oxygen. The *Ki* value of oudenone was calculated to be 1.0×10^{-3} M.

Structure-activity relationship on the effects of oudenone and its derivatives on phenylalanine hydro-

Fig. 1. LINEWEAVER-BURK plots of velocity against (A) 6,7-dimethyltetrahydropterin ($DMPH_4$) concentration, (B) phenylalanine concentration, and (C) O_2 concentration in the presence or absence of oudenone.

Phenylalanine hydroxylase activity was assayed with the standard incubation mixture as described under Methods. (A) and (B) experiments were carried out in air.

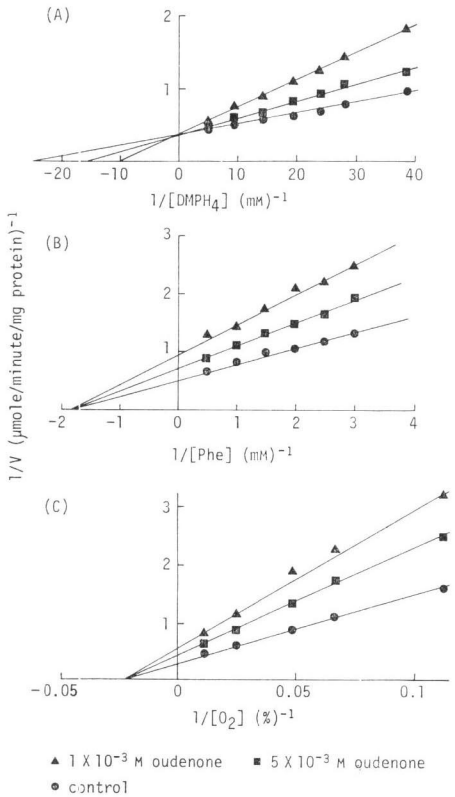
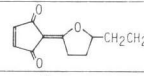
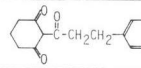
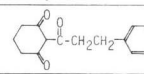
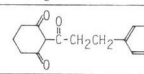
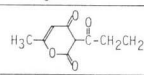
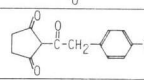
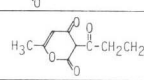
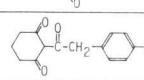
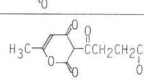
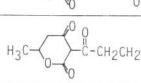
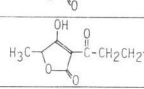
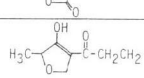


Table 1. Inhibition of phenylalanine hydroxylase and tyrosine hydroxylase by oudenone or its derivatives.

Enzyme activity was assayed with standard incubation mixture and with each inhibitor as described under Methods. IC_{50} means the inhibitor concentration which shows 50% inhibition of enzyme activity, and this value was estimated from each inhibition curve.

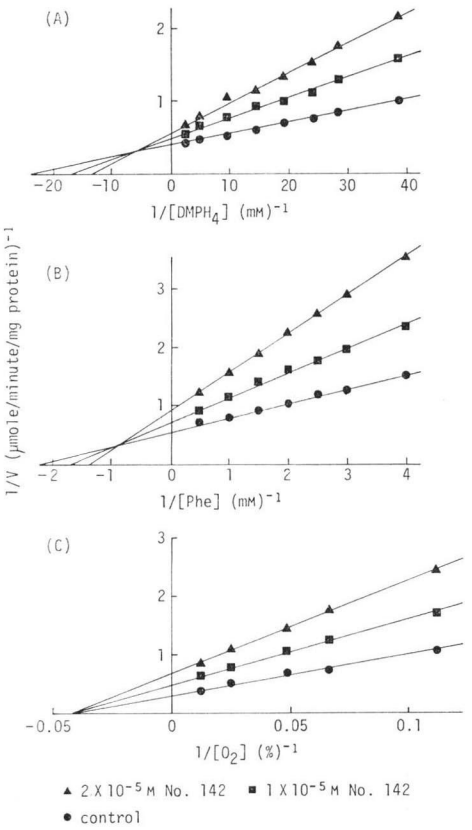
	Structure of oudenone and derivatives	IC_{50} (M)	
		Phe H	Tyr H
Oudenone		2.3×10^{-3}	1.1×10^{-4}
No. 132		3.0×10^{-4}	1.0×10^{-4}
No. 142		1.8×10^{-5}	1.3×10^{-4}
No. 144		9.0×10^{-4}	8.8×10^{-5}
No. 156		1.4×10^{-3}	2.5×10^{-4}
No. 167		3.5×10^{-4}	4.1×10^{-5}
No. 178		$>10^{-3}$	4.9×10^{-5}
No. 187		2.4×10^{-4}	1.5×10^{-5}
No. 255		$>10^{-3}$	7.5×10^{-5}
No. 266		2.6×10^{-4}	1.2×10^{-5}
No. 268		$>10^{-3}$	4.7×10^{-5}
No. 282		$>10^{-3}$	3.2×10^{-5}

xylase and on tyrosine hydroxylase was shown in Table 1. All of the derivatives of oudenone in Table 1 showed 50% inhibition of tyrosine hydroxylase at concentrations of $10^{-5} \sim 10^{-4}$ M. However, the concentrations in 50% inhibition of phenylalanine hydroxylase were much more variable. Compounds, No. 178, No. 255, No. 268, and No. 282, showed more potent inhibition of tyrosine hydroxylase than oudenone. However, they showed only slight inhibition of phenylalanine hydroxylase even at a concentration of 1×10^{-3} M. All inhibitors in Table 1 showed more potent inhibition of tyrosine hydroxylase than of phenylalanine hydroxylase except compound No. 142.

Compound No. 142, 2-(3-(3,4-dihydroxyphenyl)-1-oxopropyl)cyclohexane-1,3-dione, was the most potent inhibitor of phenylalanine hydroxylase. It was 130 times more potent than oudenone. However, the effect of compound No. 142 on tyrosine hydroxylase was similar to that of oudenone.

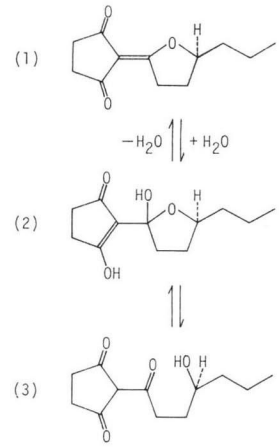
Fig. 2. LINEWEAVER-BURK plots of velocity against (A) $DMPH_4$, (B) phenylalanine concentration, and (C) O_2 concentration in the presence or absence of compound No. 142.

Phenylalanine hydroxylase activity was assayed with the standard incubation mixture as described under Methods except containing 4% of dimethylsulfoxide. (A) and (B) experiments were carried out in air.



xylase was calculated to be 1.4×10^{-5} M.

Fig. 3. Changes in the structure of oudenone in the aqueous solution.



Since this result suggests that the mechanism of inhibition of phenylalanine hydroxylase by compound No. 142 is different from that by oudenone, the mechanism of inhibition by compound No. 142 was further investigated. LINEWEAVER-BURK plots of velocity *versus* the concentration of $DMPH_4$ (Fig. 2-A), *versus* the concentration of phenylalanine (Fig. 2-B), and *versus* the percentage of O_2 (Fig. 2-C) in the presence or absence of compound No. 142 indicated that the inhibition of phenylalanine hydroxylase by compound No. 142 was a mixed type toward either $DMPH_4$ or phenylalanine and noncompetitive toward molecular oxygen. The *Ki* value of compound No. 142 on phenylalanine hydro-

Discussion

Oudenone, of microbial origin, is known as an inhibitor of tyrosine hydroxylase. The inhibition is competitive with $DMPH_4$, and is uncompetitive with the substrate tyrosine[8].

In the present study, we found that oudenone was also an inhibitor of phenylalanine hydroxylase and that it also inhibited the enzyme competitively with the cofactor, $DMPH_4$. However, oudenone showed a noncompetitive inhibition toward the substrate, phenylalanine, in contrast to the uncompetitive inhibition toward the substrate, tyrosine, in the case of tyrosine hydroxylase. The difference in the mode of inhibition by oudenone between these two aromatic amino acid hydroxylases is thought to be due to the difference in the catalytic mechanism. A ping-pong mechanism is proposed as a reaction mechanism of tyrosine hydroxylase with $DMPH_4$[11], but our results on the kinetic analysis of phenylalanine hydroxylase suggest a sequential rather than ping-pong mechanism.

As shown in Fig. 3, oudenone (**1**) undergoes a dynamic structural change and reaches the β-trione

structure (**3**) through the structure (**2**) by simple addition of water[12]. This β-trione structure is thought to be the structure that inhibits hydroxylases, as indicated by the results of Table 1. These data also suggest that phenylalanine hydroxylase requires the β-trione structure more strictly for inhibition than tyrosine hydroxylase.

Compound No. 142 showed especially strong inhibition of phenylalanine hydroxylase. This compound has both the β-trione structure and the catechol structure. Catechol compounds are known to be inhibitors of the two hydroxylases. They inhibit the enzymes competitively with the pterin cofactor[13,14]. The inhibition of phenylalanine hydroxylase by compound No. 142 was a mixed type toward $DMPH_4$ and phenylalanine. Therefore, the strong inhibitory effect of compound No. 142 on phenylalanine hydroxylase may not be only due to the additive effect of the β-trione and catechol moiety. The result suggests that the competitive inhibition with $DMPH_4$ by β-trione is caused by its binding to the enzyme at a different site from where catechol is bound. A difference in the mechanism of hydroxylation by the two enzymes is also supported by the fact that the marked increase of inhibitory action of compound No. 142 on phenylalanine hydroxylase is not observed on tyrosine hydroxylase.

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