THE STEREOSPECIFICITY OF ALCOHOL OXIDASES FROM TANACETUM VULGARE AND CANDIDA BOIDINII

Emilio Cardemil*

Department of Biological Chemistry, The University of Michigan

Ann Arbor, Michigan 48104

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Summary: The stereospecificity of the oxidation of trans-hex-2-en-1-ol and ethanol was determined for alcohol oxidases from Tanacetum vulgare leaves and from the yeast Candida boidinii. It was found that both enzymes remove the pro-1R hydrogen. The enzyme from Candida boidinii also removes the pro-1S hydrogen but to a much lesser degree.

INTRODUCTION

It is known that the oxidation of ethanol catalyzed by alcohol dehydrogenase (1), catalase (2,3), and a microsomal ethanol oxidative system (2) occurs in such a way that the pro-1R hydrogen of ethanol is lost. The same hydrogen atom is removed in the oxidation of glycolic acid catalyzed by glycolic acid oxidase (4,5). In the last 10 years, flavoprotein alcohol oxidases have been isolated from yeast (6,7) and fungi (8), and it has been shown that these enzymes are specific for short chain alcohols. However, the stereospecificity of these enzymes is not known. Recently, a unique alcohol oxidase has been detected and isolated from Tanacetum vulgare leaves (9). In contrast to alcohol oxidases from lower organisms (6-8), this enzyme oxidizes not short chain alcohols but C_5-C_{10} alcohols, trans-hex-2-en-1-ol being the best substrate of those tested (9). It was considered of interest to compare the stereospecificity of these enzymes.

MATERIALS AND METHODS

[1-^{3}H]-Ethanol was obtained by reduction of acetaldehyde (88 μmol) with

*Permanent address: Laboratorio de Bioquímica, Facultad de Ciencias Químicas, Universidad de Chile, Casilla 233, Santiago-1, Chile. All correspondence to be sent to this address.

^{3}H-$NaBH_4$ (74 µmol, 37 mCi, New England Nuclear Corp.). The reaction was carried out in 1.0 ml of 0.10M K_2HPO_4. After stirring for 30 min at 0°, the reaction vessel was put at room temperature and stirred a further 45 min. Then 1 mmole semicarbazide-HCl was added to bind residual acetaldehyde and distilled over a second mmole of semicarbazide-HCl and finally redistilled and stored at 4°. No acetaldehyde remained, as measured spectrophotometrically after the addition of semicarbazide-HCl. No attempt was made to determine the specific radioactivity of the product since ^3H_2O was certainly present in the solution.

(4R)-[4-^{3}H]-NADH was prepared using yeast alcohol dehydrogenase (0.45 mg, Sigma Chem. Co.), [1-^{3}H]-ethanol (109 µmole), NAD^+ (7.5 µmole), semicarbazide-HCl (400 µmole) and sodium pyrophosphate buffer pH 9.2 (250 µmole), all in a final volume of 5 ml. The reaction was carried out at 37° and allowed to proceed until no further change in absorption at 340 nm was detected. The solution was then diluted to 15 ml with cold water and the (4R)-[4-^{3}H]-NADH purified as described by Wilken et al. (10), except that the NaCl gradient was between 0-1%.

(1R)-[1-^{3}H]-Ethanol was generated at 25° in a system containing in a final volume of 10 ml:2.7 µmole (4R)-[4-^{3}H]-NADH ($1.6x10^6$ cpm/µmole), 0.45 mg yeast alcohol dehydrogenase, and 36 µmole acetaldehyde. At the end of the reaction 1 mmole semicarbazide-HCl was added and immediately distilled over another mmole semicarbazide-HCl and redistilled to obtain 1.4 µmole (1R)-[1-^{3}H]-ethanol ($1.5x10^6$ cpm/µmole), as determined according to Bonnichen and Theorell (11).

(1S)-[1-^{3}H]-Ethanol was obtained by exchange of [1-^{3}H]-ethanol with yeast alcohol dehydrogenase-lipoamide dehydrogenase (12). The system used was as follows: yeast alcohol dehydrogenase 0.27 mg, lipoamide dehydrogenase (13) 73 units, albumin 0.21 mg, EDTA 0.72 mg, NAD^+ 0.021 mg, (1-^{3}H)-ethanol 31.5 µmole, all in 0.60 ml of 0.10M phosphate buffer pH 8.2. This was let stand at room temperature for 5 days and then was saturated with NaCl and extracted 4 times with diethyl ether. The ethereal phases were dried with $MgSO_4$, the ether was evaporated off at reduced pressure and the (1S)-[1-^{3}H]-ethanol taken up in 1.0 ml of water. 2.9 µmole ($1.2x10^6$ cpm) of stereospecifically labelled ethanol was obtained.

[1-^{3}H]-Trans-hex-2-en-1-ol was prepared by reduction of 20 µmole of trans-hex-2-enal (Aldrich Chemical Company) with 9.2 µmole (4.7 mCi) of ^{3}H-$NaBH_4$ in 1.0 ml of propan-2-ol. The solution was stirred 2 hours at room temperature and [1-^{3}H]-trans-hex-2-en-1-ol was separated from unreacted aldehyde by thin layer chromatography (conditions as in the legend to Fig. 1). The alcohol zone was eluted with diethyl ether, evaporated to dryness under vacuo and the labelled alcohol dissolved in 4.0 ml of water. Analysis by thin layer chromatography of an ether extract of this solution revealed a single radioactive spot that co-chromatographed with standard trans-hex-2-en-1-ol (Aldrich Chemical Company).

[1-^{3}H]-Trans-hex-2-enal was obtained by the enzymic oxidation of [1-^{3}H]-trans-hex-2-en-1-ol ($5.4x10^6$ cpm, 0.20 µmole) in the presence of alcohol oxidase from T. vulgare (2.8 µg), in 2.0 ml of 0.10M potassium phosphate buffer pH 7.6 at 25°. After a 5 hour incubation, the system was extracted four times with diethyl ether and the labelled aldehyde purified by thin layer chromatography as described for [1-^{3}H]-trans-hex-2-en-1-ol.

(1S)-[1-^{3}H]-Trans-hex-2-en-1-ol was obtained by reduction of 0.84 µmole of [1-^{3}H]-trans-hex-2-enal (specific radioactivity adjusted to $1.9x10^6$ cpm/µmole) with 0.24 mg of liver alcohol dehydrogenase (Worthington Biochemical Company) and 10 µmole NADH in 10 ml of 0.10 M potassium phosphate buffer pH 7.2. The reaction was incubated overnight at 25° and then extracted four times with diethyl ether. The analysis of the ether phases by thin layer chromatography revealed that the reaction had proceeded to completion since no aldehyde could be detected. The ether was evaporated off and the (1S)-[1-^{3}H]-trans-hex-2-en-

l-ol was dissolved in 1.0 ml of water. A total radioactivity of 50,000 cpm was obtained.

(1R)-[1-^{3}H]-Trans-hex-2-en-1-ol was obtained in a similar way except that (4R)-[4-^{3}H]-NADH (0.56 µmole, 8.9x10^5 cpm) and trans-hex-2-en-1-ol (1.27 µmole) were used. The isolated product (2.5x10^5 cpm) was homogenous by thin layer chromatography.

Incubations with catalase were carried out overnight as described by Corral et al. (3). The reactions were stopped and worked up as described in Table 2 except that no acetaldehyde was added at the end of the incubation.

RESULTS

As Table 1 shows, when (1R)-[1-^{3}H]-trans-hex-2-en-1-ol was used as substrate for the alcohol oxidase from T. vulgare leaves, only 4-5% of the radioactivity remaining at the end of the experiment could be extracted into ether, in contrast with almost 100% extraction when (1-S)-[1-^{3}H]-trans-hex-2-en-1-ol was the substrate. These results indicate that the pro-1R hydrogen of trans-hex-2-en-1-ol is eliminated when hydrogen peroxide is formed. The analysis of

TABLE 1

ETHER EXTRACTABLE RADIOACTIVITY AFTER INCUBATION OF (1R)- AND (1S)-[1-^{3}H]-TRANS-HEX-2-EN-1-OL WITH ALCOHOL OXIDASE FROM TANACETUM VULGARE

SUBSTRATE		INITIAL cpmx10^{-3}	FINAL cpmx10^{-3}	RADIOACTIVITY EXTRACTED IN ETHER cpmx10^{-3}	(%)
1R	Exp 1	120	115	5.4	4.7
	Exp 2	43	31	1.2	3.9
	Control	40	37	37.7	100
1S	Exp 1	23	3.9	4.1	100
	Exp 2*	11	8.4	8.2	98
	Control	43	41	39.0	95

Potassium phosphate buffer pH 7.6, 0.20 mmole, 2.8 µg alcohol oxidase from T. vulgare (9), (1R)-[1-^{3}H]-trans-hex-en-1-ol (48,000 cpm/µmole) or (1S)-[1-^{3}H]-trans-hex-2-en-1-ol (236,000 cpm/µmole) were incubated overnight at 25° in a final volume of 2.0 ml. The final radioactivity at the end of the incubation was determined and the mixture extracted 4 times with diethyl ether. The ether extracts were dried over $MgSO_4$ and the extracted radioactivity determined. The ether phases were then concentrated and analyzed by thin layer chromatography. Control experiments were done in the same way but without enzyme.

*incubation 2 hours

the ether phases (Fig. 1) indicates that practically all the radioactivity extracted from the incubation with the 1R isomer is in residual alcohol, but in the case of the 1S isomer the radioactivity is predominantly associated with trans-hex-2-enal. When (1-S)-[1-^{3}H]-trans-hex-2-en-1-ol was incubated overnight (Table 1) approximately 83% of the initial radioactivity was lost, probably due to volatilization of [1-^{3}H]-trans-hex-2-enal. An incubation for 2 hours (shown in the same Table) resulted in the loss of only 23% of the original radioactivity. In this case, the aldehyde represented 35% of the radioactivity recovered in thin layer chromatography, the rest was unchanged (1-S)-[1-^{3}H]-trans-hex-2-en-1-ol (experiment not shown).

When (1R)- and (1S)-[1-^{3}H]-ethanol were incubated with catalase (173 µmole each, specific radioactivity 698 cpm/µmole and 612 cpm/µmole respectively) the specific radioactivity of the acetaldehyde was 6 cpm/µmole when the substrate

TABLE 2

STEREOSPECIFIC OXIDATION OF (1R)- AND (1S)-[1-^{3}H]-ETHANOL BY THE ALCOHOL OXIDASE FROM CANDIDA BOIDINII

Substrate	cpm x 10^{-5}	specific radioactivity	
		acetaldehyde cpm/µmole	acetaldehyde/cpm in ethanol µmole^{-1} x 10^4
1R Exp. 1	1.1	40	3.6
Exp. 2	2.2	86	3.9
Exp. 3	2.2	95	4.3
1S Exp. 4	1.1	204	18.4
Exp. 5	1.2	244	20.4

Potassium phosphate buffer pH 7.6, 1.2 mmole, 5 mg alcohol oxidase from C. boidinii (7), (1R)-[1-^{3}H]-ethanol (1.5 x 10^6 cpm/µmole) or (1S)-[1-^{3}H]-ethanol (4.2 x 10^5cpm/µmole) in 12 ml. Reactions were carried out overnight in 50 ml stoppered conical flasks at room temperature, and stopped by freezing the flasks. Then 177 µmole acetaldehyde was added and the flasks were then warmed up to 0° and 5-6 ml distilled into 10 ml of ice-cold 5,5-dimethyl-1,3-cyclohexanedione (Eastman Kodak Company) 4 mg/ml in 0.10 M sodium acetate buffer pH 4.25 (3). The dimetol-acetaldehyde complex precipitated in a few hours at room temperature, and then was filtered, washed with water and dissolved in 3.0 ml of methanol. The solution was scanned in the UV region and the radioactivity determined in a 1.0 ml aliquot. An extinction coefficient of 8 x 10^3cm^2/mmole at 262 nm (2) was used.

was the 1R isomer and 249 cpm/ mole when the substrate was the 1S isomer, proving that the ^{3}H had been introduced in the right stereochemical position. At the end of the incubation no alcohol remained in any case (11). This result with the (1S)-[1-^{3}H]-ethanol is in agreement with the results of Gang et al. (2) who reported a pronounced isotopic effect with catalase using (R,S)-[1-^{3}H]-ethanol.

Because the alcohol oxidase from Candida boidinii was not very active at the time of the experiments, the specific radioactivity of stereospecifically labelled ethanols had to be much higher. Table 2 shows that when the amount of radioactivity in (1R)- and (1S)-[1-^{3}H]-ethanol was the same, (Exp. 1,4,5) the specific radioactivity of acetaldehyde was 5 times greater for the 1S iso-

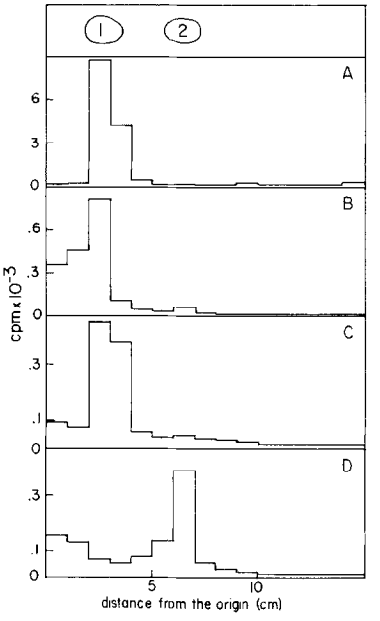

Fig. 1. Analysis in thin layer chromatography of the ether extractable radioactivity after incubation of (1R)- and (1S)- [1-^{3}H]-trans-hex-2-en-1-ol with alcohol oxidase from T. vulgare. For the 1R isomer, A=control and B=enzymic; for the 1S isomer, C=control and D=enzymic. The migration of standards trans-hex-2-en-1-ol [1] and trans-hex-2-enal [2] are shown for comparison; visualization was with I_2 vapors and 2,4-dinitrophenylhydrazine spray respectively. The plates (Kiesel-gel G, 0.25 mm thick) were developed to a uniform height of 15 cm in benzene-ethyl acetate, 9:1 (v/v) and then the radioactivity determined by scraping out 1 cm bands into scintillation vials.

mer than for the 1R isomer. When the amount of radioactivity in (1R)-[1-^{3}H]-ethanol was doubled (Exp. 2 and 3) the specific radioactivity of acetaldehyde was also doubled, showing that the difference in specific radioactivity of acetaldehyde was due to a stereospecific removal of ^{3}H and not to a mass effect. This is clearly demonstrated by the ratio of the specific radioactivity of acetaldehyde to the cpm in ethanol (see Table 2).

DISCUSSION

The synthesis of (1R)- and (1S)-[1-^{3}H]-trans-hex-2-en-1-ol was possible because of the broad substrate specificity of liver alcohol dehydrogenase (14). It is known that this enzyme transfers the pro-1R hydrogen of ethanol (1), geraniol (15), and cis-trans- and trans-trans-farnesol (16), so that it is reasonable to assume the same for trans-hex-2-en-1-ol.

The results with the alcohol oxidase from Tanacetum vulgare (Table 1 and Fig. 1) indicate that the oxidation of trans-hex-2-en-1-ol proceeds with the stereospecific removal of the pro-1R hydrogen. The stereoselectivity of this enzyme is, thus, the same as that of glycolic acid oxidase from tobacco leaves (4,5), which appears to be the only other alcohol oxidase with known stereospecificity.

The enzyme from Candida boidinii shows a 5 to 1 preference for the removal of the pro-1R hydrogen of ethanol (Table 2). As this oxidation must be considered essentially irreversible, this preference probably reflects the difference between the kinetic constants for the removal of the pro-1R and pro-1S hydrogen of ethanol.

As a general conclusion of this work, it can be said that enzymes as different as alcohol dehydrogenase (1), catalase (2,3) a microsomal ethanol-oxidative system (2), and alcohol oxidases from Tanacetum vulgare leaves and Candida boidinii have the same steric requirements for the oxidation of alcohols, which are also the same as those of glycolic acid oxidase from tobacco leaves (4,5). It wo be interesting to look at the stereospecificity of alcohol oxidases from bacteria, enzymes that appear to contain a pteridine derivative as prosthetic group (17).

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