SUBSTRATE SPECIFICITY OF COENZYME B_{12}-DEPENDENT DIOL DEHYDRASE: GLYCEROL AS BOTH A GOOD SUBSTRATE AND A POTENT INACTIVATOR*

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SUMMARY

The substrate specificity of adenosylcobalamin-dependent diol dehydrase was further studied in detail using an enzyme preparation that appears homogeneous by ultracentrifugal and gel electrophoretical criteria. Besides 1,2-propanediol and 1,2-ethanediol, glycerol, 1,2- and 2,3-butanediol were found to serve as substrate for the enzyme, whereas 1,3-propanediol was not. Of the substrate analogs tested, glycerol displayed some striking features: it was dehydrated to β-hydroxypropionaldehyde with concomitant inactivation of the enzyme. Although the initial velocity with glycerol was comparable to that with 1,2-propanediol, the dehydration reaction ceased almost completely within 3 min accompanying rapid, irreversible inactivation of the holoenzyme. 1,2- and 2,3-Butanediol were converted to butyraldehyde and methyl ethyl ketone, respectively, at a rate much lower than that with 1,2-propanediol. 2,3-Butanediol is the only compound, other than 1,2-diols, known at present to show a considerable substrate activity.

Diol dehydrase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) from Aerobacter aerogenes ATCC 8724 is an adenosylcobalamin-requiring enzyme which catalyzes the conversion of 1,2-propanediol and 1,2-ethanediol to propionaldehyde and acetaldehyde, respectively (2). It has been reported that glycerol, 1,2- and 2,3-butanediol show neither substrate nor inhibitor activity (2). We have described in the previous paper that 1,2-butanediol and styrene glycol having no significant substrate activity behave as weak competitive inhibitors, and that they protect holodiol dehydrase against inactivation by oxygen through forming an oxygen-resistant ternary complex between apoenzyme, coenzyme and a substrate analog (3). However, the substrate specificity of this enzyme remains somewhat ambiguous.

Recently, in cooperation with Dr. K. Soda's group in Institute of Chemical Research, Kyoto University, and Dr. A. Poznanskaja (on leave from All Union Vitamin Research Institute, Moscow, USSR), we have developed a new

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*Paper VII in the series concerning with coenzyme B_{12}-dependent diol dehydrase. The preceding paper in this series is Toraya et al.(1).

procedure for purifying diol dehydrase to a homogeneous protein[1]. The present communication deals with the reaction of this enzyme with several substrate analogs, which was studied in detail using a homogeneous enzyme preparation.

MATERIALS AND METHODS

Materials. 1,2-Butanediol was prepared by acid hydrolysis of 1,2-butylene oxide, as described before (3). Crystalline adenosylcobalamin was purchased from Glaxo Ltd., Greenford, U. K.. All other chemicals were reagent grade commercial products and were used without further purification. Diol dehydrase apoenzyme was purified from the cells of A. aerogenes grown statically in a glycerol-1,2-propanediol medium, according to our new procedure. The enzyme preparation was established to be homogeneous by the criteria of ultracentrifugation and polyacrylamide disc gel electrophoresis.

Enzyme Assay. The activity of diol dehydrase was assayed by either the DNPH[2] method of Lee and Abeles (2) or the MBTH method which has been newly applied for the assay of this enzyme[1]. When glycerol was used as a substrate, the tryptophan method of Smiley and Sobolov (4) was also employed: β-hydroxypropionaldehyde formed was dehydrated and converted to the acrolein-tryptophan complex by heating with L-tryptophan in 7 N HCl, and determined colorimetrically.

Protein Assay. Protein concentration was determined spectrophotometrically by measuring the absorbance at 278 nm. An E value of 5.27 for 10 mg of diol dehydrase per ml and for a 1-cm light path was used throughout[1].

Identification of the Products. The products formed by reaction of diol dehydrase with substrate analogs were either converted to the 2,4-dinitrophenylhydrazone derivative or reduced to alcohol with $NaBH_4$, and identified by comparing their behaviors in thin layer chromatography on silica gel G with those of the derivatives of the corresponding authentic samples. Polyhydroxy compounds were located by spraying with chromic acid-sulfuric acid (5 g $K_2Cr_2O_7$ in 100 ml 40 % H_2SO_4) as described by Prey et al. (5).

RESULTS

Reaction of Diol Dehydrase with Substrate Analogs and Identification of the Reaction Products. During the course of a study on the mechanism of protection of holodiol dehydrase by substrate analogs against oxygen-inactivation, we found that carbonyl compounds are formed by reaction of the enzyme with some substrate analogs. Table I summarizes reactivities of the carbonyl products toward DNPH, MBTH and the tryptophan reagent and behaviors of the 2,4-dinitrophenylhydrazone derivatives in thin layer chromatography on silica gel G. From these data, the products formed from 1,2- and 2,3-butanediol can be undoubtedly identified as butyraldehyde and methyl ethyl ketone, respectively. On the other hand, the product formed from glycerol was not only reactive with MBTH but also convertible to acrolein by heating in 7 N HCl. As shown in Table II, glycerol was enzymatically converted to a compound which was reducible with $NaBH_4$ to 1,3-propanediol. Hence, the product formed by reaction of glycerol with diol dehydrase can be concluded to be β-hydroxy-

[1] A. Poznanskaja, K. Tanizawa, K. Soda, T. Toraya and S. Fukui, manuscript in preparation.

[2] Abbreviations used are: DNPH, 2,4-dinitrophenylhydrazine; MBTH, 3-methyl-2-benzothiazolinone hydrazone.

Table I. Identification of the Reaction Products.

Thin layer chromatography was carried out on silica gel G layer.

Compound	Reactivity with			R_F of 2,4-dinitrophenylhydrazone	
	DNPH	MBTH	tryptophan reagent	Solvent I[a]	Solvent II[b]
Product from 1,2-butanediol	+	+		0.57	0.61
Product from 2,3-butanediol	+	+[c]		0.52	0.56
Product from glycerol		+	+		
Authentic butyraldehyde	+	+		0.56	0.61
Authentic methyl ethyl ketone	+	+[c]		0.52	0.56

[a]Solvent I; benzene-petroleum ether (75:25 by vol). [b]Solvent II; benzene-dimethylacetamide (90:10 by vol). [c]Less reactive than aldehydes.

Table II. Reduction of the Reaction Product from Glycerol with $NaBH_4$.

The resulting compound was identified by thin layer chromatography on silica gel G. The spray reagent used is described in the text.

Compound	R_F			
	Solvent I[a]	Solvent II[b]	Solvent III[c]	Solvent III[d]
$NaBH_4$-reduction product	0.58	0.61	0.66	0.64
Glycerol	0.40	0.47	0.60	0.54
1,2-Propanediol	0.66	0.66	0.74	0.70
1,3-Propanediol	0.56	0.61	0.68	0.65

[a]Solvent I; 1-butanol-water (75:25 by vol). [b]Solvent II; 1-butanol-water (90:10 by vol). [c]Solvent III; dioxane. [d]The silica gel G layer impregnated with 0.1 N boric acid was used.

propionaldehyde. It is established from these data that diol dehydrase catalyzes the reactions [1]-[3] whose type is formally the same as that of

$$CH_3CH_2\underset{\underset{OH}{|}}{CH}CH_2OH \longrightarrow CH_3CH_2CH_2CHO + H_2O \quad [1]$$

$$HOCH_2\underset{\underset{OH}{|}}{CH}CH_2OH \longrightarrow HOCH_2CH_2CHO + H_2O \quad [2]$$

$$CH_3\underset{\underset{OHOH}{|\ \ |}}{CHCH}CH_3 \longrightarrow CH_3CH_2\overset{\overset{O}{\|}}{C}CH_3 + H_2O \quad [3]$$

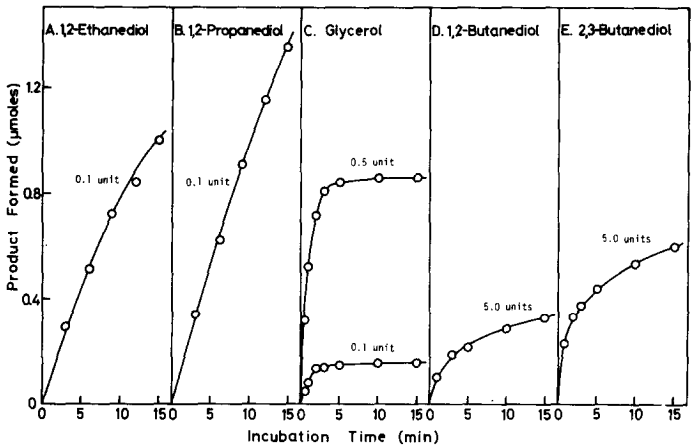

Fig. 1. Time course of reaction of diol dehydrase with various substrate analogs.
The amount of the reaction products formed from 1,2-diols and 2,3-butanediol was determined by the MBTH method and the DNPH method, respectively.

reactions with 1,2-propanediol and 1,2-ethanediol (dehydration of vic-diols), except that dehydration of 2,3-butanediol leads to the ketone formation.

The following compounds did not appreciably served as a substrate for this enzyme to form carbonyl compounds: 1,3-propanediol, β-mercaptoethanol and ethanolamine.

Time Course of Reaction of Diol Dehydrase with Substrate Analogs. Fig. 1 illustrates the time course of reaction of diol dehydrase with various substrate analogs. When 1,2-propanediol is used as a substrate, the reaction rate was linear within about 20 min (Fig. 1B), whereas the rate slightly deviated from a straight line with 1,2-ethanediol (Fig. 1A). In the cases of 1,2-butanediol (Fig. 1D) and 2,3-butanediol (Fig. 1E) dehydration, such deviation from a straight line was more significant. Although the initial velocity with glycerol was comparable to that with 1,2-propanediol, rapid inactivation of the enzyme was accompanied with the catalytic reaction, resulting in complete cessation of the diol dehydrase reaction in about 3 min (Fig. 1C). Such an inactivated enzyme was found to be unreactive even with 1,2-propanediol, and the enzyme activity was not restored by gel filtration on Sephadex G-25 (data not shown). Hence, it is clear that the enzyme is irreversibly modified by reaction with glycerol.

Substrate Activity of the Analogs and Their Affinity for Diol Dehydrase. The kinetic constants for various substrate analogs were determined by the conventional double reciprocal plots (Table III). The affinity of the analogs for the enzyme decreases in the following order: 1,2-propanediol > 2,3-butane-

Table III. Substrate Activity of Several Diols (at 37°C).

Substrate analog		Product	Km (mM)	Turnover number (sec^{-1})
$RCHCH_2OH$ (OH)	R = H	RCHO	1.08	286
	R = CH_3		0.18	337
	R = CH_2OH		1.54	371
	R = CH_2CH_3		2.5	7.2
$CH_3CHCHCH_3$ (OHOH)		$CH_3CH_2CCH_3$ (O)	0.64	15.1

diol > 1,2-ethanediol > glycerol > 1,2-butanediol. The turnover number obtained from the initial velocity was calculated on the basis of a molecular weight and equivalent weight of 2.3×10^5 daltons (6) and the highest specific activity of 88 units per mg. The turnover number of about 337 sec^{-1} with 1,2-Propanediol indicates that diol dehydrase catalysis is 2.4-fold more efficient than ethanolamine ammonia-lyase catalysis (turnover number, 143 sec^{-1} per active site with ethanolamine (7)). It seems interesting that 2,3-butanediol shows a considerable initial velocity and the high affinity for the enzyme although it does not belong to 1,2-diols. It is also obvious that glycerol serves as a good substrate as well as a potent inactivator for this enzyme.

DISCUSSION

It was clearly demonstrated that a homogeneous preparation of diol dehydrase acts not only on 1,2-propanediol and 1,2-ethanediol but also on glycerol, 1,2-butanediol and 2,3-butanediol. 2,3-Butanediol is the only compound, other than 1,2-diols, known at present to show a considerable substrate activity and a high affinity for diol dehydrase. It is of much interest that glycerol causes the rapid, irreversible inactivation, although it is dehydrated at a rate comparable to that with 1,2-propanediol. Probably for this reason, it would have been considered that glycerol possesses no appreciable substrate activity in this system. The enzyme inactivated by reaction with glycerol had lost the ability to catalyze the conversion of 1,2-propanediol to propionaldehyde, indicating that the dehydration of glycerol and 1,2-propanediol is catalyzed at the same site of the enzyme. A detailed study on the mechanism of this inactivation process is in progress, and will be published elsewhere.

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