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ISOLATION AND CHARACTERIZATION OF GLYCOLIC ACID OXIDASE FROM HUMAN LIVER

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Summary

Glycolic acid oxidase has been isolated from human liver and purified over 3000-fold to a specific activity of 123 U/mg protein by a 5-step procedure. The preparation gave a single protein band on polyacrylamide gel electrophoresis, required flavin mononucleotide for catalytic activity, had a pH optimum between 8.2—8.8 depending on the substrate, and had a molecular weight of 105 000. The enzyme has a broad specificity towards α-hydroxy acids. Glycolate ($K_m = 3.3 \cdot 10^{-4}$ M) was the most effective substrate. The enzyme was stable for several months when stored as an $(NH_4)_2SO_4$ precipitate or in 15% glycerol. Since glycolate inhibits the oxidation of glyoxylate to oxalate by glycolic acid oxidase, it is suggested that glycolic acid oxidase contributes to the synthesis of oxalate in vivo when the glyoxylate concentration is high and the glycolate concentration is low.

Introduction

The biosynthesis of oxalate has been shown to be a contributing factor in primary hyperoxaluria [1—3], kidney stone formation [4,5], and possibly ethylene glycol toxicity [6]. Glyoxylic acid has been identified as the major immediate precursor of oxalate [3] and three enzymes, glycolic acid oxidase (glycollate:oxygen oxidoreductase, EC 1.1.3.1) [7,8], lactate dehydrogenase (EC 1.1.1.27) [9,10] and xanthine oxidase (EC 1.2.3.2) [11], catalyze this

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reaction in vitro [12,13]. However, only lactate dehydrogenase and xanthine oxidase have been isolated and characterized from human tissues [11,14,15], although glycolic acid oxidase has been isolated from plant and animal tissues and studied extensively [7,8,16—20]. Lactate dehydrogenase and glycolic acid oxidase also catalyze the oxidation of glycolate to glyoxylate [8,21] which appears to be the major catalytic function of the latter enzyme. In this investigation, glycolic acid oxidase has been isolated and purified from human liver. The significance of this enzyme in oxalate synthesis in vivo is also considered.

Methods

Assays of glycolic acid oxidase. Spectrophotometric assays were performed according to the procedures of Schuman and Massey [22]. The reduction of potassium ferricyanide was used to follow enzyme activity throughout the isolation procedure. One unit of activity was defined as the amount of enzyme which causes a decrease in absorbance of 0.01 U/min at 420 nm. The reduction of 2,6-dichlorophenolindophenol was used to locate enzyme activity in the dilute fractions obtained from chromatographic columns. This assay could not be used in the early stages of purification because crude preparations reduced 2,6-dichlorophenolindophenol in the absence of glycolate.

Glycolic acid oxidase was also assayed by following the consumption of oxygen using a 'Clark type' oxygen electrode [23].

Protein determination. Protein concentration was determined by the method of Warburg and Christian [24]. Protein homogeneity was determined by polyacrylamide gel electrophoresis [25]. The protein bands were located by staining with Coomassie Blue followed by destaining in 7% acetic acid. Glycolic acid oxidase activity in the gels was determined by the method of Grodzinski and Colman [26].

Chromatographic identification of the products of the oxidation of [1-^{14}C]-glycolate. The reaction medium consisted of 200 μmol pyrophosphate buffer (pH 8.8), 20 μmol [1-^{14}C]glycolate (0.5 μCi), enzyme and distilled water in a total volume of 3 ml. The reaction was started by the addition of enzyme. 1-ml aliquots were removed at the beginning of the reaction and after 30 min incubation at 37° C. The aliquots were placed in 1 ml 50 mM citrate buffer (pH 2.88) and sucrose was added to 12.5% (w/v). The samples were adjusted to pH 2.5 and labeled intermediates were separated, identified and quantitated by column chromatography [27]. The intermediates produced were corrected for their possible presence in the initial [1-^{14}C]glycolate sample by subtracting the amount present in the aliquot removed at zero time.

Tissue distribution. 1 g each of human liver, kidney, heart, brain, adrenal gland, testis, thyroid, pancreas, spleen, lung, skeletal muscle and lymph node was homogenized for 1 min with a Super Dispax Tissuizer (Teckmar Co.) in 10 ml of cold 0.1 M sodium pyrophosphate buffer (pH 8.8). The presence of glycolic acid oxidase activity was determined by the rate of oxygen consumption. Since other enzymes, glycolic acid dehydrogenase and lactic dehydrogenase, which oxidize glycolate do not utilize oxygen directly as an electron acceptor, they do not interfere with the assay of glycolic acid oxidase.

Subcellular distribution. The procedure described by Mahler and Cordes

[28] was used to isolate cellular components. Enzyme activity was assayed as described above.

Flavin requirement. The apoenzyme of glycolic acid oxidase was prepared by the method of Ushijima [16]. The apoenzyme was reconstituted by dissolving the apoenzyme in 10 mM pyrophosphate buffer (pH 8.8) and adding 1.5 μM flavin mononucleotide.

Determination of molecular weight. The molecular weight of glycolic acid oxidase was estimated by the method of Zwaan [29]. Electrophoresis gels consisting of 4% and 8% polyacrylamide were prepared by the method of Davis [25]. Catalase, lactate dehydrogenase, bovine serum albumin and ovalbumin were used as standards.

Enzyme purification. Human liver was removed from accident victims and stored at -20°C until used. No measurable loss of enzyme activity was detected when the liver was stored in this manner for three months or more. All steps in the purification were performed at 4°C. All buffers contained 1 mM EDTA.

300 g of liver were thawed and minced with scissors. The minced tissue was washed with several volumes of 0.15 M KCl and homogenized in 0.1 M sodium citrate/phosphate buffer (pH 6.1) for 1 min with a Waring Blendor. The homogenate was diluted to 3000 ml with buffer and strained through several layers of cheese cloth to remove fibrous material. After adjusting to pH 6.1 with 1 N HCl the homogenate was centrifuged at 16 500 $\times g$ in a refrigerated centrifuge for 30 min and the supernatant fractions were pooled. The precipitate forming at 35—60% satn. of $(NH_4)_2SO_4$ was dissolved in a minimal amount of 5 mM sodium phosphate buffer (pH 6.1). The solution was dialyzed for 36 h against 3 changes of 5 mM phosphate buffer (pH 6.1), 1 mM EDTA (buffer 1).

The protein solution was applied to a column (5 × 15 cm) of DEAE-cellulose (0.71 mEq/g) (Bio-Rad) equilibrated and eluted with buffer 1 until the absorbance at 280 nm in the eluate was less than 0.005. This removed lactate dehydrogenase. 50 mM sodium phosphate buffer (pH 6.1), 1 mM EDTA, was then used to elute the glycolic acid oxidase.

This preparation was then applied directly to a column (3 × 9 cm) prepared with hydroxyapatite (Bio-Rad) previously equilibrated with buffer 1. The column was then eluted with 0.1 M sodium phosphate buffer (pH 6.1)/0.4 M $(NH_4)_2SO_4$ until the absorbance at 280 nm in the eluate was less than 0.005. This removed xanthine oxidase. The column was eluted with 0.1 M sodium phosphate buffer (pH 6.1)/0.8 M $(NH_4)_2SO_4$. Glycolic acid oxidase passed through the column as a light yellow band.

The eluate was concentrated by adding crystalline $(NH_4)_2SO_4$ to 100% saturation and stirring for 1 h. The precipitate was collected by centrifugation at 20 000 $\times g$ for 20 min, dissolved in a minimal amount of buffer 1 and dialyzed overnight against 2 changes of the same buffer. This fraction was applied to a second hydroxyapatite column (1 × 25 cm) equilibrated with buffer 1 and was then eluted with a linear gradient of 500 ml each of 0.1 M sodium phosphate buffer (pH 6.1)/0.3 M $(NH_4)_2SO_4$ and 0.1 M sodium phosphate buffer (pH 6.1)/0.7 M $(NH_4)_2SO_4$ (10-ml fractions). Glycolic acid oxidase was recovered from the column in fractions 60—80. The fractions containing glycolic acid oxidase were pooled and dialyzed against two changes of 10 mM

sodium pyrophosphate buffer (pH 8.8). The procedure provides an enzyme preparation that appears homogeneous when subjected to polyacrylamide gel electrophoresis.

Results

Enzyme purification

A typical purification with 41% recovery of initial activity is summarized in Table I. This enzyme preparation gave a single protein band after electrophoresis on polyacrylamide gels which oxidized glycolate and glyoxylate and suggests that the preparation is homogeneous. The preparation represents a 3000-fold purification and had a specific activity of 123 units which is similar to that reported by Schuman and Massey [22] for homogenous glycolic acid oxidase purified from pig liver.

Enzyme distribution

Glycolic acid oxidase activity was detected only in the liver in man. No activity was found in kidney, heart, brain, adrenal gland, testis, thyroid, pancreas, spleen, lung, skeletal muscle or lymph node. The major amount of the activity in the liver, 92% was found in the supernatant fraction. No activity was found in the nuclear or microsomal fractions, and less than 1% was found associated with the mitochondria.

Substrate specificity

The highest rate of oxidation among 13 potential substrates was observed with glycolate which also had the lowest K_m and the highest V_m (Table II). Significant oxidation of L-α-hydroxyisocaproate and DL-α-hydroxyvalerate also occurred. The relative rate of oxidation of glyoxylate compared to glycolate was much lower than has previously been reported for rat liver glycolic acid oxidase [16]. Among optically active substrates, the L-configuration appears to be the most effective, since L-lactate is oxidized about 3 times faster than

TABLE I

PURIFICATION OF GLYCOLIC ACID OXIDASE FROM HUMAN LIVER *

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Percent recovery
Supernatant	2750	44 550	2100	0.041	100
35–60% Ammonium sulfate precipitate after dialysis	167	8 601	2100	0.242	100
DEAE-cellulose eluate	55	736	1796	2.44	86
First hydroxyapatite eluate **	5	111	1554	14.0	74
Second hydroxyapatite eluate **	5	7	861	123	41

* Enzyme activity was determined by the reduction of potassium ferricyanide. One unit of activity is defined as that amount of enzyme which causes a decrease in absorbance at 420 nm of 0.01 per min.

** After concentration and dialysis.

TABLE II

SUBSTRATE SPECIFICITY OF GLYCOLIC ACID OXIDASE FROM HUMAN LIVER *

Substrate	Relative rate	K_m (nM)	Maximum velocity **
Glycolate	100	0.33	8.86
L-α-Hydroxyisocaproate	54	1.93	5.48
DL-α-Hydroxyvalerate	41	n.d.	—
Glyoxylate	16	3.54	3.62
DL-α-Hydroxybutyrate	14	—	—
DL-α-Hydroxycaproate	13	—	—
L-Lactate	8	4.52	2.33
DL-α-Hydroxyisovalerate	7	—	—
D-Lactate	3	—	—

* Activity was assayed by following oxygen utilization. The reaction medium consisted of 200 μmol sodium pyrophosphate buffer (pH 8.8), 30 μmol glycolate or 10 mM other potential substrates, enzyme and distilled water in a total volume of 3 ml. The oxygen content was 0.25 mM.

** Maximum velocity is expressed as μmol glycolate oxidized per min per mg protein.

n.d., not determined.

D-lactate. The oxidation of both D and L-lactate by glycolic acid oxidase has been reported previously [16]. DL-β-phenyllactate (a competitive inhibitor of glycolic acid oxidase), glycolaldehyde, DL-glycerate, and DL-α-hydroxycaprylate were not oxidized.

pH

The pH optimum varied between 8.2 and 8.8, depending on the substrate (Fig. 1). Glycolate had a pH optimum at 8.8, glyoxylate had a broad pH optimum between 8.4 and 8.8, and lactate was oxidized most rapidly at pH 8.2.

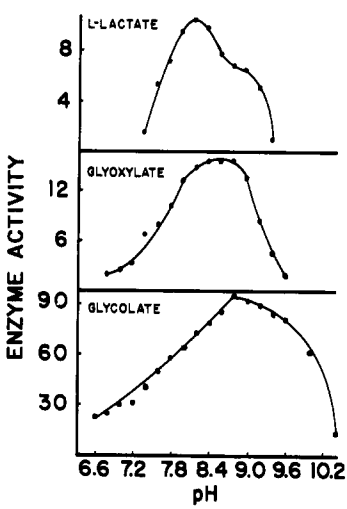

Fig. 1. Effect of pH on glycolic acid oxidase activity. The reaction medium consisted of 200 μmol sodium phosphate buffer at the specified pH , 30 μmol substrate indicated in the upper left corner, enzyme and distilled water in a total volume of 3 ml. Enzyme activity is expressed as nmol oxygen consumed per min.

Coenzyme requirement

Removal of the coenzyme produced a clear, colorless apoenzyme solution that had no catalytic activity towards glycolate. The apoprotein was not activated by riboflavin and was only slightly activated by flavin adenine-dinucleotide. 35% activity was restored by the addition of flavin mononucleotide indicating that the enzyme has the same coenzyme requirement as glycolic acid oxidase isolated from other animal sources [16,30]. The limited recovery of the initial activity suggests that some denaturation occurred during the preparation of the apoenzyme.

Enzyme stability

Purified glycolic acid oxidase retained 74—76% of its activity when stored at 4°C and between pH 8.0—8.5. The loss in activity was significantly increased when the pH was either higher or lower. The enzyme was stable for 10 min at temperatures up to 40°C, lost activity at higher temperature, and was completely inactivated at 65°C. The enzyme was stable for several months if stored as an $(NH_4)_2SO_4$-precipitate or in the presence of 15% glycerol. 1 mM EDTA also had a protecting effect. Freezing at −20°C caused considerable loss of activity, however, stability was enhanced if the enzyme was stored in 10^{-4} M flavin mononucleotide.

Molecular weight

A molecular weight of 105 000 was determined by electrophoresis.

Reaction products

The products of the oxidation of [1-^{14}C]glycolate by glycolic acid oxidase were identified by column chromatography (Fig. 2). [^{14}C]Glyoxylate accumulated although glycolic acid oxidase can oxidize glyoxylate to oxalate. No [^{14}C]oxalate was detected. This does not seem unreasonable when considering that glycolate has a 10-fold higher affinity for the glycolic acid oxidase and would completely inhibit the oxidation of glyoxylate.

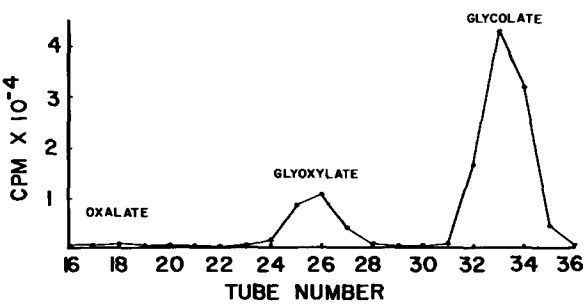

Fig. 2. Identification of products of glycolate oxidation by glycolic acid oxidase. The reaction medium consisted of 200 μmol sodium pyrophosphate buffer (pH 8.8), 20 μmol (0.5 μCi) of [1-^{14}C]glycolate, enzyme and distilled water in a total volume of 3 ml. Aliquots were removed at zero time and after 30-min incubation at 37°C. Intermediates were identified by column chromatography.

Inhibitors

p-Chloromercuribenzoate was the most effective of the 26 inhibitors evaluated, causing 100% inhibition at a concentration of 10^{-5} M (Table III) (Tris, $CuSO_4$, EDTA and thioglycollate were not inhibitory). Iodoacetate, DL-phenyllactate, heptanoate and hexanoate were the most potent of the remaining compounds, causing 32—36% inhibition at concentrations of 10^{-3} M. Copper, which has been reported to inhibit glycolic acid oxidase from other animal sources [16], caused no inhibition. The inhibition by dicarboxylic acid increased as the length of the carbon chain decreased, with oxalate producing the greatest inhibition. The straight chain dicarboxylic acids were more effective inhibitors than branched chain dicarboxylic acids (methylmalonate caused no inhibition). On the other hand, the inhibition by monocarboxylic acids increased as the length of the carbon chain increased. The presence of hydroxyl groups and methyl groups lowered the inhibition by monocarboxylic acids. Inhibition was also observed with aldehydes and with amines.

TABLE III

EFFECT OF POTENTIAL INHIBITORS ON GLYCOLIC ACID OXIDASE *

Inhibitor **	Percent inhibition
General	
0.001 mM *p*-chloromercuribenzoate	85
0.01 mM *p*-chloromercuribenzoate	100
0.1 mM iodoacetate	23
Iodoacetate	36
KCN	20
m-Hydroxy-benzyl-alcohol	23
Diethyldithiocarbamate	22
o-Hydroxy-benzyl-alcohol	12
Dicarboxylic acids	
Oxalate	17
Malonate	12
Succinate	6
Monocarboxylic acids	
Acetate	19
Propionate	21
Valerate	27
Hexonoate	32
Heptonoate	33
Phenyllactate	35
β-Hydroxypropionate	7
α-Hydroxy-α-methylbutyrate	17
Amines	
α-Methyl-benzylamine	13
Hydroxylamine	11
Aldehydes	
Glyoxylate	21
Methylglyoxal	15
L-Aminopropanal	11

* The reaction media consisted of 200 μmol sodium pyrophosphate buffer (pH 8.8), 10 μmol glycolate, inhibitor at the specified concentration, enzyme and distilled water to 3 ml. Activity was determined by measuring the rate of oxygen consumption.

** The concentration of each inhibitor was 1.0 mM unless otherwise indicated.

Kinetics

Parallel lines resulted when a plot of the reciprocal of the velocity versus the reciprocal of the glycolate concentration at different fixed concentrations of oxygen (0.0523 mM, 0.0912 mM and 0.262 mM) was prepared. Similar results were obtained when the fixed and variable substrates were reversed. When dichlorophenolindophenol was used as the electron acceptor and the reaction medium was equilibrated with air, a plot of the reciprocal of the velocity versus the reciprocal of the glycolate concentration at different concentrations of dichlorophenolindophenol (0.05 μM, 0.10 μM and 0.20 μM) resulted in converging lines. If oxygen was purged from the reaction medium and the assays performed under anaerobic conditions the reciprocal plots with dichlorophenolindophenol as the electron acceptor resulted in parallel lines. With O_2 as the electron acceptor, glycolic acid oxidase had a K_m for glycolate and O_2 of 0.34 mM and 0.16 mM, respectively. With 2,6-dichlorophenolindophenol as the electron acceptor, the K_m for glycolate and 2,6-dichlorophenolindophenol were 0.22 mM and 0.14 mM, respectively. The V_m with oxygen as the electron acceptor was 8.86 μmol glycolate oxidized/min/mg protein and with 2,6-dichlorophenolindophenol as the electron acceptor 13.76 μmol.

Discussion

The properties of human liver glycolic acid oxidase are very similar to the properties of the enzyme isolated from other mammalian sources [7,8,10]. The location in the liver, the broad substrate specificity towards α-hydroxy acids, the preference for the L configuration, and the pH optimum of 8.8 for glycolate are common for most mammalian glycolic acid oxidases. The molecular weight of 105 000 determined by disc electrophoresis is very similar to the value of 100 000 obtained by molecular sieve chromatography by Dickinson [33] for pig liver glycolic acid oxidase. The inhibitor studies in which the inhibition by monocarboxylic acids is directly proportional to chain length while inhibition by dicarboxylic acids is inversely proportional to chain length are in agreement with observations made on pig liver glycolic acid oxidase [32]. The stability of the homogenous glycolic acid oxidase preparations from pig and human liver are essentially identical. Both enzymes are stable for several months if stored as an $(NH_4)_2SO_4$-precipitate or in the presence of 15% glycerol.

While flavin mononucleotide was the only coenzyme capable of activating the apoenzyme from human liver, rat liver glycolic acid oxidase was activated by either flavin mononucleotide or flavin adeninedinucleotide [8]. This variance might be due to an intrinsic difference in the two enzymes or might be related to the crude state of the rat liver enzyme used in the study. When the rate of glycolate oxidation is taken as the reference, rat liver glycolic acid oxidase is more effective in catalyzing the oxidation of glyoxylate to oxalate than human liver glycolic acid oxidase. This suggests that glycolic acid oxidase may make less of a contribution to oxalate synthesis in man than it does in the rat and must be taken into consideration when relating metabolic studies between the two species.

When glycolate is used as the substrate and oxygen as the electron acceptor glycolic acid oxidase shows parallel line kinetics. This suggests a mechanism

involving only binary complexes. When 2,6-dichlorophenolindophenol is used as the electron acceptor converging line kinetics are observed suggesting a mechanism involving a tertiary complex. At first it appears that the type of kinetics depends on the electron acceptor. However, it must be taken into consideration that oxygen is usually present in aqueous solutions unless specifically eliminated. If oxygen is present when rates are followed as the reduction of 2,6-dichlorophenolindophenol, the reduced enzyme may combine with either electron acceptor. This results in a rate equation that predicts converging line kinetics even though only binary complexes are involved [36]. If oxygen is purged from the reaction medium the enzyme again shows parallel line kinetics. It thus appears that the mechanism for the oxidation of glycolate is similar using either oxygen or 2,6-dichlorophenolindophenol as the electron acceptor, and that the dissimilarities are due to the presence of oxygen in the 2,6-dichlorophenolindophenol system. This may explain why Dickinson [33] observed parallel line kinetics with the oxygen system and Schuman and Massey [32] observed converging lines with the 2,6-dichlorophenolindophenol system using pig liver glycolic acid oxidase.

The contribution that glycolic acid oxidase makes to oxalate biosynthesis may be less than previously thought considering the recent discovery of glycolic acid dehydrogenase [34]. Liao and Richardson [12] have shown that oxalate synthesis from glycolate is totally abolished in the isolated perfused rat liver by DL-phenyllactate. This suggests that lactate dehydrogenase and xanthine oxidase play only a minor role, if any, in oxalate synthesis. However, it would not exclude glycolic acid dehydrogenase since this enzyme is also inhibited by DL-phenyllactate.

The question arises as to the contribution glycolic acid oxidase and glycolic acid dehydrogenase make to oxalate synthesis in vivo. Although glycolic acid oxidase catalyzes the oxidation of glyoxylate to oxalate, no oxalate formation occurred when [1-^{14}C]glycolate was used as the substrate for the enzyme. When glycolate was provided to the isolated perfused rat liver which normally oxidized [^{14}C]glyoxylate to [^{14}C]oxalate, the synthesis of [^{14}C]oxalate was completely inhibited, but significant amounts of unlabeled oxalate were formed from the oxidation of glycolate by glycolic acid dehydrogenase [25]. This suggests that with oxalate precursors such as glycolaldehyde, hydroxypyruvate, phenylalanine, tyrosine, ethanolamine and hydroxyproline, which are metabilized via glycolate, glycolic acid dehydrogenase would be the major enzyme involved. On the other hand, with oxalate precursors such as glycine and tryptophan which are metabolized via glyoxylate, glycolic acid oxidase would be the major contributing enzyme. Since glycolate inhibits the oxidation of glyoxylate by glycolic acid oxidase and glyoxylate inhibits the oxidation of glycolate by glycolic acid dehydrogenase, the contribution of these two enzymes to endogenous oxalate synthesis would be determined by the relative concentrations of glycolate and glyoxylate. While not definitive, the results reported here confirm the need for considering the role of both glycolic acid oxidase and glycolic acid dehydrogenase in developing methods for controlling oxalate metabolism in primary hyperoxaluria and kidney stone formation.

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