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GLYCOLATE OXIDOREDUCTASE IN *ESCHERICHIA COLI*

J. M. LORD

Division of Natural Sciences, University of California, Santa Cruz, Calif. 95060 (U.S.A.)

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SUMMARY

Escherichia coli cells growing on glycolate as a sole source of carbon synthesise an enzyme which catalyses the oxidation of this compound to glyoxylate. The formation of glyoxylate from glycolate by extracts of such cells is dependent upon the presence of the artificial electron acceptors phenazine methosulphate and 2,6-dichlorophenolindophenol. The enzyme catalysing this conversion has been partially purified from cell extracts. The enzyme readily oxidised glycolate (K_m $4 \cdot 10^{-5}$ M) and D(—)-lactate (K_m $7 \cdot 10^{-4}$M), but L(+)-lactate was oxidised only slowly. Maximum rates of glycolate oxidation were observed in the pH range 8.0–8.8.

The enzyme was relatively insensitive to metal complexing agents but was sensitive to sulphydryl inhibitors. Addition of glycolate to the enzyme produced spectral changes characteristic of the reduction of flavin and cytochrome components, but no cofactors have been positively identified.

INTRODUCTION

Enzymes which catalyse the oxidation of glycolate to glyoxylate have been isolated from a variety of plant[1–3] and animal[4–6] tissues. Characterization of these enzymes has established that they are FMN flavoproteins and that O_2 functions as hydrogen acceptor for glycolate electrons. These enzymes, designated glycolate oxidase (glycolic acid:oxygen oxidoreductase, EC 1.1.3.1) catalyse the reaction

$$CH_2OH \cdot CO_2H + O_2 \rightarrow CHO \cdot CO_2H + H_2O_2$$

Although these enzymes show maximum activity with glycolate as substrate, they are generally not specific for this compound and can also oxidise other short chain aliphatic α-hydroxy acids. A feature of these enzymes, however, is their stereospecificity for the L-α-hydroxy acid isomers.

Recently an enzyme catalysing the oxidation of glycolate to glyoxylate has been observed in extracts of several unicellular green algae[7,8]. The enzyme from several algal species differs from the higher plant and animal oxidase in that O_2 does not function as the acceptor for glycolate electrons[9,10].

In addition the algal oxidoreductase oxidises D(—)-lactate preferentially over L(+)-lactate[11]. Glyoxylate formation from glycolate in the presence of algal ex-

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate; PCMB, *p*-chloromercuribenzoate.

tracts is dependent on the addition of artificial electron acceptors such as 2,6-dichlorophenolindophenol (DCIP), and is not stimulated by the addition of FMN[11].

When bacteria grow on glycolate as a sole source of carbon and energy, the first step in the synthesis of cell constituents is the oxidation of this compound to glyoxylate[12]. The enzyme catalysing this step, adaptively synthesized by bacteria growing on glycolate, has been assumed to be an oxidase[12]. However, Kornberg and Gotto[13] observed that extracts of glycolate grown *Pseudomonas* showed appreciable glycolate-dependent O_2 uptake only in the presence of added electron acceptors such as phenazine methosulphate (PMS) or DCIP.

The present paper is a report on the further characterization of the bacterial glycolate oxidising enzyme. In particular, evidence is presented that the enzyme from glycolate grown *Escherichia coli* does not link directly to O_2 and thus is a glycolate oxidoreductase of the type previously found in green algae rather than a typical glycolate oxidase.

MATERIALS AND METHODS

Maintenance and growth of organism

Escherichia coli K_{12} was maintained on slopes of glycolate growth medium which had been solidified with 2 % (w/v) agar. The medium contained 25 mM sodium–potassium phosphate (pH 7.2), 50 mM glycolic acid (neutralized with NaOH), 50 mM NH_4Cl and salts (200 mg $MgSO_4 \cdot 7H_2O$; 10 mg $CaCl_2 \cdot 2H_2O$; 0.5 mg $ZnSO_4 \cdot 7H_2O$; 0.5 mg $MnSO_4 \cdot 4H_2O$; 0.1 mg $CuSO_4$; 0.1 mg $CoSO_4 \cdot 7H_2O$; 0.1 mg $Na_2B_4O_7 \cdot 10H_2O$; 2 mg $Na_2MoO_4 \cdot 2H_2O$ and 0.1 ml FeEDTA solution per l of medium). Cultures of the organism were grown at 28 °C and stored at 2 °C. They were subcultured at intervals of 2–3 weeks.

For experimental use, organisms from the agar slopes were used to inoculate 250 ml of growth medium in 1-l Erlenmeyer flasks. The cultures were shaken at 30 °C for 16 h and were then used to inoculate larger cultures of 3 l of medium in 6-l Erlenmeyer flasks. The inoculated medium was rigorously aerated at 30 °C. The bacteria were harvested during the exponential phase of growth by centrifuging at 2000 × *g* for 10 min.

Preparation of cell extracts

The packed cells were washed once in 10 mM potassium phosphate (pH 8.0) and then were resuspended in the same buffer to give a 15–20 % (v/v) cell suspension.

Cells were broken by passing this suspension through an ice-cold French pressure cell[14] at 20000 lb/inch^2.

The extract was centrifuged at 20000 × *g* for 10 min at 0.4 °C. The supernatant was decanted and used for experimental purposes. The pellet contained negligible glycolate oxidoreductase.

Glycolate oxidoreductase assays

(*a*) *Manometric assay*. The main compartment of a Warburg flask contained 100 μmoles of potassium phosphate (pH 8.0) cell extract (containing 1.6 mg of protein) and water to 2.4 ml, with 0.2 ml of 2 M KOH and a filter paper wick in the centre well. The side arm contained 0.2 ml of 0.1 M glycolate and 0.2 of 1 % (w/v) PMS.

After thermal equilibration at 30 °C, the contents of the side arm were tipped and O_2 uptake was measured.

(*b*) *Spectrophotometric assay.* Glycolate dependent reduction of DCIP was measured in the presence of catalytic amounts of PMS. The reaction mixture contained, in a final volume of 3.0 ml, 200 μmoles of potassium phosphate (pH 8.0) 0.2 μmole of DCIP, 0.1 ml of 1 % (w/v) PMS and 0.01–0.1 ml of cell extract. The reaction was started by the addition of 10 μmoles of glycolate (neutralized with KOH) and was followed by measuring a decrease in extinction at 600 nm on a Hitachi Perkin Elmer double-beam recording spectrophotometer. When this assay was performed anaerobically the reactants were contained in a 3-ml Thunberg cuvette with glycolate in the sidearm.

The cuvette was evacuated and flushed with O_2-free N_2 several times.

Under the conditions of the assay, a decrease in extinction of 0.01 corresponds to the oxidation of 1.8 nmoles of glycolate.

(*c*) *Glyoxylate formation from glycolate.* The effect of the electron acceptors PMS and DCIP on the rate of glyoxylate formation from glycolate was assayed by measuring the rate of glyoxylate phenylhydrazone formation. Because of interactions between the dyes and phenylhydrazine under the assay conditions used, glyoxylate formation could not be continuously monitored.

This problem was overcome by incubating 100 μmoles of potassium phosphate (pH 8.0) 0.2 μmole of DCIP, 0.1 ml of 1 % (w/v) PMS, cell extract (containing 0.1 mg of protein) and 10 μmoles of potassium glycolate in a final volume of 2.4 ml. At fixed time intervals, individual assays were terminated by the addition of 0.1 ml of 12 M HCl. After standing for 10 min, in which time the blue DCIP colour had disappeared, 0.5 ml of 0.1 M phenylhydrazine·HCl was added. The mixture was allowed to stand a further 10 min, then the extinction due to glyoxylate phenylhydrazone was measured at 324 nm.

Partial purification of glycolate oxidoreductase

Step 1. The crude extract from 20 g wet weight of glycolate grown *E. coli* was obtained as described previously. All subsequent operations were carried out at 0–5 °C.

Step 2. The crude enzyme extract was further centrifuged at 48000 × *g* for 1 h and the pellet was discarded.

Step 3. A 2 % (w/v) protamine sulphate solution was added slowly with stirring to the supernatant from the previous step to give a final ratio of 1.5 mg of protamine sulphate for each 10 mg of soluble protein. After standing for 10 min the precipitate was removed by centrifuging at 20000 × *g* for 10 min and was discarded.

Step 4. Solid $(NH_4)_2SO_4$ was added with constant stirring to the enzyme solution (27.7 g of $(NH_4)_2SO_4$ per 100 ml of supernatant) until the solution was 45 % saturated with respect to $(NH_4)_2SO_4$. After 15 min stirring, the precipitated proteins, which contained all the enzyme activity, were collected by centrifugation and the yellow coloured supernatant was discarded. The precipitate was then extracted successively with $(NH_4)_2SO_4$ solutions of decreasing concentration. The extractions were made with 50-ml vol. each of 40, 36, 33, 30 and 26 % $(NH_4)_2SO_4$ solution. The bulk of the enzyme extracted was recovered in the 30 and 26 % solutions which were combined.

Step 5. Sufficient solid $(NH_4)_2SO_4$ was added to the enzyme solution from the previous step to make it 45% saturated. The precipitated proteins were collected by centrifugation and were dissolved in 5 ml of 10 mM potassium phosphate (pH 8.0), and this solution was dialysed overnight against 5 l of 10 mM potassium phosphate (pH 8.0). The dialysed enzyme solution was applied to the top of a Sephadex G-200 column. The gel had been swollen in 10 mM potassium phosphate (pH 8.0) and degassed with a vacuum pump before the slurry was poured into a Pharmacia chromatographic column. The diameter and length of the gel bed were 25 and 270 mm, respectively. The column was eluted with 10 mM potassium phosphate (pH 8.0) at a flow rate of 15 ml/h and fractions, each of 5 ml, were collected. The bulk of the enzyme eluted was recovered in the 12th and 13th fractions which were combined.

Protein determination

Protein was measured by the method of Lowry *et al.*[15], using a standard curve prepared for crystalline bovine serum albumin.

RESULTS

The addition of glycolate to extracts of glycolate-grown *E. coli* resulted in only a slight stimulation of O_2 uptake above the observed endogenous rate (Fig. 1).

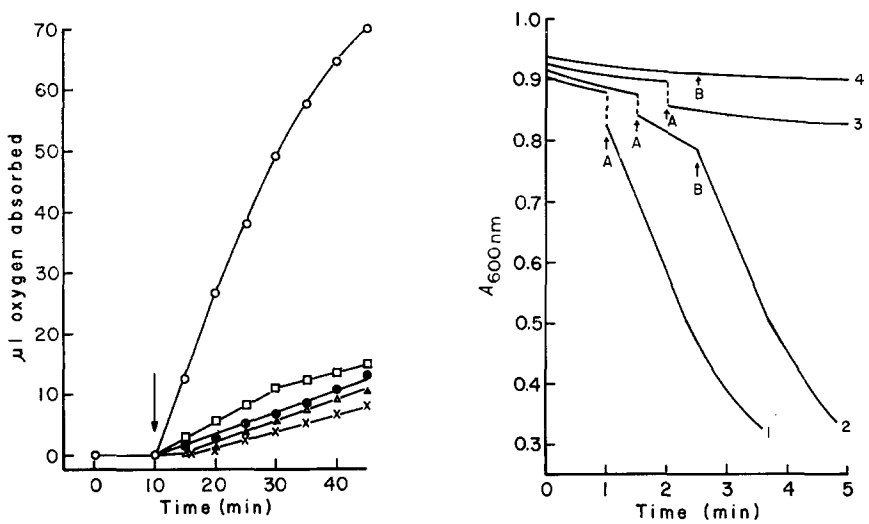

Fig. 1. Oxidation of glycolate by *E. coli* extracts. Rates of oxygen uptake were measured by Warburg manometry. For details of the reaction mixture see Materials and Methods. The reaction was initiated by adding glycolate at the time indicated by the arrow. ○—○, complete system; □—□, phenazine methosulphate omitted; ●—●, glycolate omitted; △—△, complete system, but cell extract boiled before incubation; ×—×, glycolate and phenazine methosulphate omitted.

Fig. 2. Effect of PMS on glycolate-dependent DCIP reduction by *E. coli* extracts. The complete reaction mixture contained, in 3 ml, 200 µmoles of potassium phosphate (pH 8), 0.2 µmole of DCIP, 10 µl of *E. coli* extract (containing 0.12 mg of protein), 0.1 ml of 1% (w/v) PMS and 10 µmoles of glycolate. Reaction 1, extract, DCIP and PMS were initially incubated; Reaction 2, extract and DCIP; Reaction 3, boiled extract, DCIP and PMS; Reaction 4, extract and DCIP. Arrow A indicates addition of glycolate and Arrow B addition of PMS. Taken from a tracing made with a Hitachi Perkin Elmer recording spectrophotometer.

The rate of glycolate-dependent O_2 uptake was greatly stimulated by the addition of PMS (Fig. 1). This indicated that O_2 did not function as primary electron acceptor during glycolate oxidation by the enzyme in these extracts.

Such extracts also catalysed a slow rate of glycolate dependent DCIP reduction. Again the addition of catalytic amounts of PMS greatly stimulated the rate of DCIP reduction (Fig. 2). The slow rate of aerobic DCIP reduction observed in the absence of PMS was not due to competition between DCIP and O_2 for glycolate electrons, since this rate was approximately the same when measured anaerobically. The PMS-stimulated rate of DCIP reduction was also virtually the same whether measured aerobically or anaerobically (8.7 and 8.9 μmoles per mg of protein per h, respectively), which suggested that PMS preferentially transferred glycolate electrons to DCIP rather than to O_2. The failure of O_2 to compete with DCIP for glycolate electrons during the aerobic assay further indicated that the *E. coli* glycolate oxidising enzyme was not a typical glycolate oxidase, since such competition is a feature of the oxidase isolated from spinach leaves[16].

Other redox compounds were tested for their ability to replace PMS in stimulating electron transfer from glycolate to DCIP. FMN, FAD, NAD, NADP, oxidised glutathione, KNO_3, $K_3Fe(CN)_6$, $FeCl_3$ (at 1 mM final concentration) and cytochrome *c* (at 10 μM final concentration) were all without effect. With the exception of cytochrome *c* and $K_3Fe(CN)_6$, the above compounds were also without effect on the rate of PMS stimulated DCIP reduction. Cytochrome *c* inhibited the rate of PMS stimulated DCIP reduction about 60%. This inhibition was explained as a result of competition between cytochrome *c* and DCIP for glycolate electrons transferred *via*

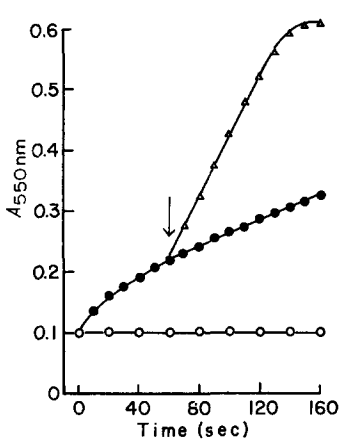

Fig. 3. PMS-dependent reduction of cytochrome *c*. The reaction mixture contained, in a final volume of 3.0 ml, 100 μmoles of Tris buffer (pH 8), 0.1 μmole of cytochrome *c*, 50 μl of cell extract (containing 0.36 mg of protein) and 50 μl of 1% (w/v) PMS. The reaction was started by adding glycolate (10 μmoles) at the time indicated by the arrow and was measured at 550 nm. △—△, complete system; ●—●, glycolate or PMS omitted; ○—○, extract omitted.

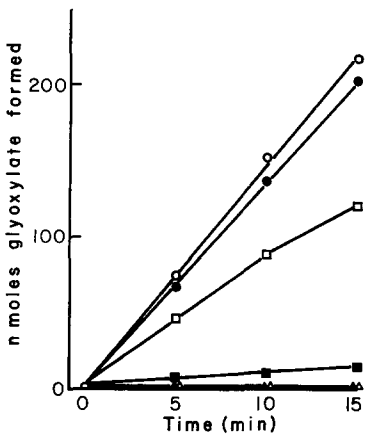

Fig. 4. Requirement of added electron acceptors for glyoxylate formation from glycolate. The complete system contained 100 μmoles of potassium phosphate (pH 8), 0.2 μmole of DCIP, 0.1 ml of 1% (w/v) PMS extract (containing 0.10 mg of protein) and 10 μmoles of glycolate. Glyoxylate phenylhydrazone was formed and measured as described in the Materials and Methods. ○—○, complete system; ●—●, DCIP omitted; □—□, PMS omitted; ■—■, DCIP and PMS omitted; △—△, glycolate omitted; ▲—▲, complete, but extract boiled before incubation.

PMS, since it was observed that the extracts catalysed a glycolate-dependent, PMS-dependent reduction of cytochrome *c* (Fig. 3). $K_3Fe(CN)_6$ completely inhibited DCIP reduction.

The failure of O_2 alone to function as an effective electron acceptor during glycolate oxidation by *E. coli* extracts was confirmed by measuring the aerobic rate of glyoxylate formation from glycolate. Appreciable glyoxylate formation was only observed in the presence of the added electron acceptors DCIP and PMS, with PMS being most stimulatory (Fig. 4). The rate of glyoxylate formation in the presence of PMS was 8.3 μmoles per mg of protein per h, in good agreement with the rate of glycolate oxidation of 8.8 μmoles per mg of protein per h calculated on the basis of DCIP reduction. The effect of other artificial electron acceptors on the rate of glyoxylate formation was not determined, however, FMN, FAD and NAD were without effect.

The glycolate oxidising enzyme was adaptively synthesized when *E. coli* was grown on glycolate. Using the PMS stimulated DCIP reduction assay, glycolate was oxidised 100 times as fast by extracts of glycolate grown cells as by extracts of succinate grown cells containing comparable amounts of protein.

The enzyme was partially purified from extracts of glycolate grown cells (Table I).

TABLE I

SUMMARY OF THE PURIFICATION OF *E. coli* GLYCOLATE OXIDOREDUCTASE

The enzyme was assayed by measuring the rate of PMS stimulated DCIP reduction. One unit of enzyme catalyses the oxidation of 1 μmole of glycolate per min.

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Total enzyme (units)*	*Spec. act. (units/mg)*	*% Recovery*
1. French press extract	120	2260	339	0.15	100
2. Supernatant after 1 h centrifugation at 48000 × *g*	110	1385	317	0.23	93.5
3. Supernatant from protamine sulphate treatment	115	1125	310	0.28	91.5
4. Material precipitated with 45% $(NH_4)_2SO_4$ and extracted with 30 and 26% $(NH_4)_2SO_4$ solutions	100	130	157	1.21	46.3
5. Active fractions following Sephadex G-200 gel filtration	10	30	93	3.10	27.4

Properties of the partially purified enzyme

Enzyme activity was measured by the PMS stimulated DCIP reduction assay.

(*a*) *pH optimum.* No significant difference in the rate of DCIP reduction by the partially purified enzyme was observed when Tris·HCl or phosphate buffers were tested at equivalent pH values. The pH optimum for glycolate oxidation in Tris·HCl buffer extended over a broad range between 8.0 and 8.8 (Fig. 5) and was similar to that observed for glycolate oxidase from leaves[2] and glycolate oxidoreductase from *Chlamydomonas*[11].

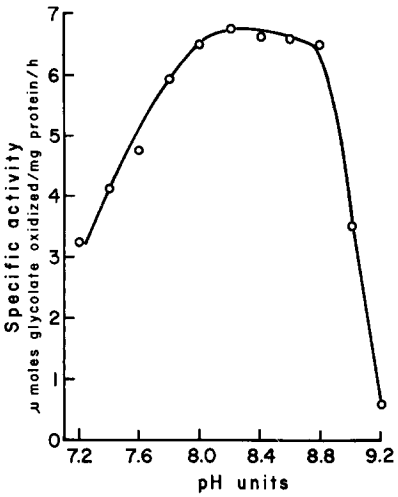

Fig. 5. The effect of pH on the rate of glycolate oxidation by partially purified *E. coli* glycolate oxidoreductase. The specific activity of the enzyme was 1.21 units/mg of protein.

(*b*) *Substrate specificity. E. coli* glycolate oxidoreductase, in addition to oxidising glycolate, slowly oxidised L(+)-lactate, DL-malate, phosphoglycolate and glyoxylate (Table II). It is not clear whether these slow rates represent oxidation of these substrates, or rather indicate the presence of impurities in the substrates (for example, glycolate in the phosphoglycolate). More significantly, the partially purified enzyme showed higher activity with D(—)-lactate as substrate than with glycolate. This high rate of D(—)-lactate oxidation further emphasized that the *E. coli* enzyme differs from the typical glycolate oxidase isolated from plant and animal sources which is stereospecific for L-α-hydroxy acid isomers.

When glycolate and D(—)-lactate were simultaneously added to the *E. coli*

TABLE II

SUBSTRATE SPECIFICITY OF *E. coli* GLYCOLATE OXIDOREDUCTASE

Rates of PMS stimulated DCIP reduction are expressed as percentage of the rate with glycolate. 10 μmoles of each substrate was assayed. The specific activity of the enzyme was 3.1 units/mg of protein.

Substrate	*Relative rate*
Glycolate	100
D(−)-Lactate	114
Glycolate + D(−)-lactate	114
L(+)-Lactate	15.7
DL-Malate	2.8
L-Malate	0
DL-Glycerate	0
Glyoxylate	6.4
Phosphoglycolate	9.3
Glycine	0
L-Hydroxyisocaproate	0

glycolate oxidoreductase in mixed substrate assays, the rates observed for each substrate alone were not additive (Table II) suggesting that both substrates were being oxidised by the same enzyme. This conclusion was supported by the observation that the peak of glycolate oxidising activity eluted from the Sephadex G-200 column during the purification procedure coincided exactly with the peak of D(—)-lactate oxidising activity.

The apparent K_m for glycolate oxidation by glycolate oxidoreductase from *E. coli* was $4 \cdot 10^{-5}$ M. With D(—)-lactate as substrate, a higher apparent K_m of $7 \cdot 10^{-4}$ M was obtained.

(*c*) *Effect of ionic strength.* Glycolate oxidoreductase was inhibited by increasing ionic strength of NaCl (Fig. 6). In this respect the *E. coli* enzyme differs from purified glycolate oxidase from spinach which, when assayed by the DCIP reduction assay, is stimulated by increasing ionic strengths[3].

(*d*) *Effect of inhibitors. E. coli* glycolate oxidoreductase was relatively insensitive to the metal complexing agents sodium azide and phenanthroline up to a concentration of 1 mM and EDTA up to a concentration of 10 mM (Table III). These observations suggest that the *E. coli* enzyme does not have a metal cofactor. Assay of the enzyme in the presence of the sulphydryl inhibitors $CuSO_4$ and *p*-chloromercuribenzoate (PCMB) showed the enzyme to be readily inhibited by these agents. The

TABLE III

EFFECT OF INHIBITORS ON *E. coli* GLYCOLATE OXIDOREDUCTASE

Inhibitors were added directly to the DCIP reduction assay to give the final concentrations listed below. The specific activity of the enzyme was 3.1 units/mg of protein.

Inhibitor	*Concn (M)*	% *Inhibition*
NaN_3	$1 \cdot 10^{-5}$	0
	$1 \cdot 10^{-4}$	2.0
	$1 \cdot 10^{-3}$	5.5
Phenanthroline	$1 \cdot 10^{-5}$	9.5
	$1 \cdot 10^{-4}$	5.5
	$1 \cdot 10^{-3}$	11.3
EDTA	$1 \cdot 10^{-5}$	5.5
	$1 \cdot 10^{-4}$	3.8
	$1 \cdot 10^{-3}$	7.3
	$1 \cdot 10^{-2}$	13.3
KCN	$1 \cdot 10^{-5}$	3.8
	$1 \cdot 10^{-4}$	13.3
	$1 \cdot 10^{-3}$	58.5
	$2 \cdot 10^{-3}$	79.3
$CuSO_4$	$1 \cdot 10^{-6}$	37.8
	$5 \cdot 10^{-6}$	69.8
	$1 \cdot 10^{-5}$	88.7
PCMB	$1 \cdot 10^{-5}$	11.3
	$5 \cdot 10^{-5}$	79.3
	$1 \cdot 10^{-4}$	95.3

enzyme was also sensitive to cyanide. Because the enzyme is relatively insensitive to metal complexing agents but is susceptible to attack by sulphydryl inhibitors, it was concluded that the observed cyanide inhibition probably represented sulphydryl reactions rather than metal complexing. The ability of the enzyme to oxidise D(—)-lactate was inhibited at the same rate as the glycolate oxidising activity at corresponding cyanide concentrations (data not shown). Glycolate oxidase from higher plants is not affected by cyanide[3].

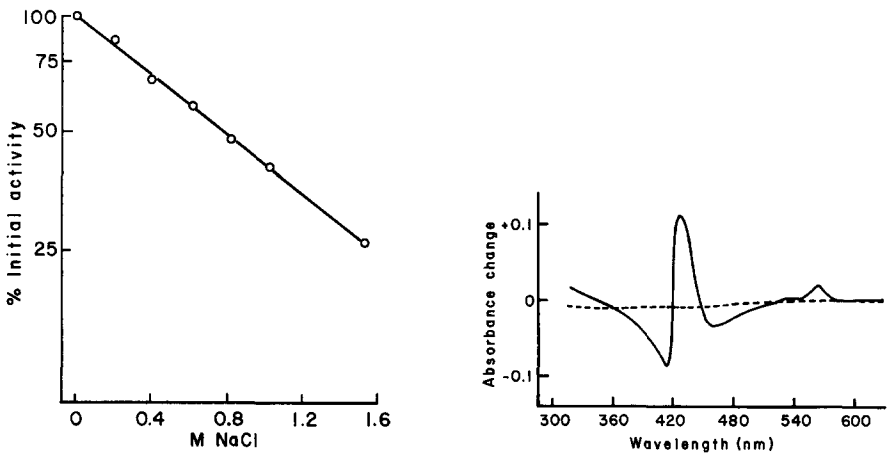

Fig. 6. The effect of increasing ionic strength on the activity of *E. coli* glycolate oxidoreductase. NaCl solutions were added directly to the assay mixture to give the final concentrations indicated. The specific activity of the enzyme was 3.1 units/mg of protein.

Fig. 7. Aerobic difference spectrum obtained by adding glycolate to *E. coli* glycolate oxidoreductase. Enzyme solution (2.5 ml of specific activity 1.21 units/mg of protein) was added to two 3-ml silica spectrophotometer cuvettes. The cuvettes were placed in a Hitachi Perkin Elmer double-beam recording spectrophotometer and a wavelength scan over the range indicated was made to obtain a base line (------). Then 0.1 ml of water was added to the reference cuvette and 0.1 ml of 0.1 M glycolate was added to the test cuvette and the wavelength scan was repeated (———).

(*e*) *Effect of cations.* Glycolate oxidoreductase activity was unaffected by 10 mM $MgCl_2$ and $CaCl_2$, but was completely inhibited by 10 mM $CuSO_4$, $HgCl_2$, and $ZnCl_2$. Inhibition by these cations probably resulted from interaction with sulphydryl groups.

(*f*) *Glycolate induced difference spectrum.* The aerobic difference spectrum observed when glycolate was added to the enzyme solution which had been purified as far as the $(NH_4)_2SO_4$ step (specific activity 1.21 units /mg of protein) is shown in Fig. 7. A marked Soret band at 427 nm and additional peaks at 529 and 558 nm were observed, indicating the reduction of a cytochrome component, possibly a *b*-type cytochrome. The absence of a peak in the region of 600 nm suggests that *a*-type cytochromes were not present. The bleaching in the 450-nm region indicates flavin reduction. Addition of dithionite in place of glycolate produced an identical difference spectrum although the peaks were more pronounced and an additional intense absorption increase peaked at 365 nm (data not shown). When dithionite was added to a diluted enzyme preparation obtained after Sephadex G-200 chromatography

(specific activity 3.1 units /mg of protein), a difference spectrum typical of reduced cytochrome was again observed (data not shown). This indicated a possible association of a cytochrome component with the enzyme, since free cytochromes should have been held back on the gel column.

(*g*) *Stability of the enzyme.* Storage of the enzyme which had been purified through the $(NH_4)_2SO_4$ step (specific activity 1.21 units /mg of protein) at 2 °C for 10 days resulted in approx. 50 % loss of activity. No compounds which might have protected the enzyme, such as sulphydryl compounds, were added. Incubation of the partially inactivated enzyme for up to 90 min at 0–2 °C with 10 mM FMN or 10 mM FAD did not result in any recovery of enzyme activity.

DISCUSSION

The results reported in this paper establish that the glycolate oxidising enzyme synthesized by *E. coli* growing on glycolate as a sole carbon source does not function as a typical glycolate oxidase, as is found, for example, in higher plant leaves. The *E. coli* enzyme was dependent on the addition of PMS to stimulate an appreciable transfer of glycolate electrons to O_2 (Fig. 1). Extracts of glycolate grown *Pseudomonas*[13] and *Micrococcus denitrificans*[17] are likewise dependent on added electron-acceptors to promote significant glycolate dependent O_2 uptake. These observations do not eliminate the possibility that O_2 functions as the terminal acceptor for glycolate electrons '*in vivo*', but if this is so it may be concluded that the natural acceptor or carriers of the internal electron transport chain have been lost or inactivated during the extraction procedure. This conclusion was supported by the finding that added artificial electron acceptors, such as PMS or DCIP, were essential to the demonstration of glyoxylate formation from glycolate (Fig. 4). In this respect the *E. coli* enzyme is similar to the glycolate oxidoreductase previously isolated from unicellular green algae[11]. The natural acceptor in such algae, in lieu of O_2, is as yet unknown. Similarities between glycolate oxidoreductase from green algae and *E. coli* were further established by comparing the effects of sulphydryl inhibitors and cyanide on enzyme activity, and the ability of the enzyme from both sources to oxidise D(—)-lactate preferentially over L(+)-lactate.

The algal[11] and bacterial enzymes (Table III) were inhibited by $CuSO_4$, PCMB and KCN, while the purified glycolate oxidase from spinach leaves is relatively insensitive to PCMB[3] and is not inhibited by KCN.

During the purification and storage of both plant[2] and animal[4] glycolate oxidase, some inactivation occurs due to dissociation of the FMN prosthetic group of the enzyme. Addition of FMN to such preparations readily restores the enzyme to full activity. However, addition of FMN or FAD to partially inactivated glycolate oxidoreductase from *Chlorella*[18] or *E. coli* had no effect.

A most striking difference between the *E. coli* enzyme and the plant or animal oxidase is the substrate specificity with respect to the oxidation of the D and L isomers of lactic acid. Glycolate oxidase shows absolute stereospecificity for L-α-hydroxy acids. D(—)-Lactate is not oxidised by this enzyme. In contrast the *E. coli* enzyme, in common with that from algae[11], preferentially oxidises the D isomer of lactate (Table II). Indeed the partially purified *E. coli* enzyme was more active towards D(—)-lactate as substrate than glycolate under the conditions of assay used here

(Table II). On the basis of the limited evidence mentioned in the results section, it is suggested that one enzyme is acting on both substrates. However, the lower apparent K_m observed with glycolate as substrate ($4 \cdot 10^{-5}$ M) than with D(—)-lactate (K_m $7 \cdot 10^{-4}$ M) suggests that glycolate is the preferred substrate for the enzyme.

The aerobic difference spectrum obtained when glycolate was added to the partially purified enzyme provided some evidence for glycolate-dependent reduction of a flavin and a cytochrome. In this respect the high activity of the enzyme towards D(—)-lactate is of interest, since a form of D(—)-lactate dehydrogenase isolated from yeast has been identified as a flavohaemoprotein[19]. Such enzymes contain FMN and cytochrome *c* as prosthetic groups[20]. The yeast enzyme can transfer electrons from D(—)-lactate to cytochrome *c* or DCIP. It has been suggested that some intermediate carrier is involved between the enzyme and cytochrome *c*, and that PMS can replace this factor in purified preparations[21].

The *E. coli* enzyme catalyses a PMS-dependent reduction of DCIP (Fig. 2) or cytochrome *c* (Fig. 3) in the presence of glycolate and further purification of the enzyme is necessary to examine for potential prosthetic groups. The results obtained here do, however, provide evidence for fundamental differences between the bacterial glycolate oxidoreductase and glycolate oxidase from other tissues, and in particular indicate that alternative acceptors other than O_2 function in the primary electron transfer steps during glycolate oxidation.

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