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The intracellular localization of glycolate oxidoreductase in *Escherichia coli*

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Distribution of glycolate oxidoreductase in cell-free extracts of *E coli* grown in mineral medium containing sodium glycolate has been studied The enzyme was found to be largely associated with the cytoplasmic membranes Homologous antiserum to the membranes inhibited the enzyme activity completely after the solubilization of the intact membranes with Triton X-100 Results on the effect of pronase on glycolate oxidoreductase activity confirmed that part of the enzyme is exposed to the surface of the membrane

Cytoplasmic membrane, Glycolate oxidoreductase, (*Escherichia coli*)

1. INTRODUCTION

Glycolate formation and metabolism were studied in various plants, algae, cyanobacteria, photosynthetic bacteria and chemolithotrophic bacteria [1–6]. Heterotrophic bacteria such as *E. coli* and *Pseudomonas* were found to grow on glycolate as a sole source of carbon and energy through the synthesis of an adaptive enzyme which oxidizes glycolate to glyoxylate [7–9]. The only study which characterized the enzyme from *E. coli* was by Lord [9]; he found that it does not link directly to oxygen. However, glyoxylate formation by extracts of *E.coli*, *Micrococcus denitrificans* and *Pseudomonas* was shown to be dependent on the addition of an artificial electron acceptor such as 2,6-dichlorophenolindophenol (DCPIP) or phenazine methosulphate (PMS) [8–10].

Glycolate oxidoreductase was partially purified from *E.coli* extract using a 20000 × *g* supernatant [9]. The intracellular distribution of glycolate oxidoreductase in *E.coli* is discussed.

2. MATERIALS AND METHODS

2 1. *Organism and growth conditions*

Escherichia coli ATCC 11775 was grown on glycolate medium [9] which contained 25 mM sodium potassium phosphate (pH 7 2), 50 mM sodium glycolate, 50 mM NH_4Cl and salts (200 mg $MgSO_4$, 10 mg $CaCl_2$ $2H_2O$, 0 5 mg $ZnSO_4$ $7H_2O$, 0.5 mg $MnSO_4$ $4H_2O$, 0 1 mg $CuSO_4$, 0 1 mg $CoSO_4$ $7H_2O$, 0.1 mg $Na_2B_4O_7$ $10H_2O$, 2 mg Na_2MoO_4 $2H_2O$ and 0.1 ml FeEDTA solution per liter of medium)

The organism was grown axenically in 10 l aspirators equipped with magnetic stirrer and sparged with sterile air at 30°C It was subcultured at intervals of 2–3 weeks

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2.2. *Preparation of cytoplasmic membranes*

This was carried out according to Haddock [11]. Log phase cultures were harvested by centrifugation at 2500 × *g* for 15 min. The cell pellet was suspended in 30 ml of 10 mM potassium phosphate buffer pH 8 0. Cells were disrupted by four 15-s periods of ultrasonication punctuated with 15-s rest periods in an ice bath. The resulting material was immediately diluted with an equal volume of potassium phosphate buffer containing 0 4 M sucrose. All subsequent centrifugation was performed at 4°C. The cell-free extract was centrifuged at 12000 × *g* for 15 min The supernatant was respun at 226000 × *g* for 60 min using a Beckman ultracentrifuge. The pellet containing the cytoplasmic membranes was gently resuspended and washed once by repeated spin at 226000 × *g* for 60 min Aliquots of all fractions were retained for assay

2.3. *Density gradient centrifugation*

Aliquots, 2 ml each, of the washed cytoplasmic membranes and the cell-free extract were layered onto 20 ml linear gradients of 10 to 60% (w/w) sucrose in potassium phosphate buffer in polycarbonate tubes. Centrifugation was done in a Beckman ultracentrifuge at 80000 × *g* for 4 h 1 0 ml fractions were collected from the bottom of the tubes

2 4 *Glycolate oxidoreductase assay*

Glycolate-dependent reduction of DCPIP was measured in the presence of catalytic amounts of PMS, according to the method of Lord [9] The reaction mixture contained in a final volume of 3 0 ml 200 μmol potassium phosphate buffer pH 8.0, 0.2 μmol DCPIP, 0.1 ml of 1% (w/w) PMS and 0.1 ml cell-free extract. The reaction was started by the addition of 10 μmol sodium glycolate. A decrease in extinction at 600 nm was recorded using a Pye Unicam SP 800 spectrophotometer.

2 5. *Lactate dehydrogenase assay*

D-Lactate dehydrogenase was assayed as described by Wittenberger [12].

2 6. *Preparation of antiserum*

Cytoplasmic membranes of *E coli* were emulsified with an equal volume of Freund's Complete Adjuvant and 1 0 ml of this mixture (containing 1 0 mg membrane protein) was injected s c. into the hind foot of a New Zealand white rabbit After one week a booster injection containing 1.5 mg protein but without adjuvant was injected subcutaneously into the neck. Blood was collected 2 weeks later and serum was prepared and then stored at −20°C

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2 7 *Treatment with pronase*

Cytoplasmic membranes of *E coli* were incubated with pronase (1 mg/ml) for 15 min at 8°C and then washed with potassium phosphate buffer containing 0 4 M sucrose pH 8 0 by centrifuging at 226 000 × *g* for 60 min The resulting pellet was also rewashed All pellet fractions were retained for assay of glycolate oxidoreductase

2 8 *Protein determination*

Protein was measured according to Lowry et al [13] using crystalline bovine serum albumin as a standard

3. RESULTS AND DISCUSSION

Glycolate oxidoreductase was detected in all fractions of *E.coli* cell extracts by differential centrifugation as shown in table 1. The highest specific activity was consistently exhibited by the 226 000 × *g* × 60 min pellet. Washing of this pellet resulted in further increase in specific activity (table 1). Characterization and purification of the enzyme from *E.coli* was reported by Lord [9]. He found that addition of glycolate to extracts of glycolate-grown *E.coli* resulted in slight stimulation of oxygen uptake and the extracts also catalyzed a slow rate of glycolate-dependent DCPIP reduction aerobically and anaerobically. However, these reactions were greatly stimulated by the addition of PMS [9]. This is similar to the glycolate oxidoreductase isolated from unicellular green algae [14]. The enzyme was adaptively synthesized when *E.coli* was grown on glycolate as the sole carbon source and no enzyme activity was detected when the organism was grown on brain heart infusion medium.

Fig.1 shows the sucrose density gradient of the 226 000 × *g* × 60 min fraction and the cell-free extract of *E.coli*. The highest glycolate oxidoreductase activity was correlated with the maximal protein concentrations of both fractions (fig.1A,B). The 226 000 × *g* × 60 min fraction is the cytoplasmic membrane which was iso-

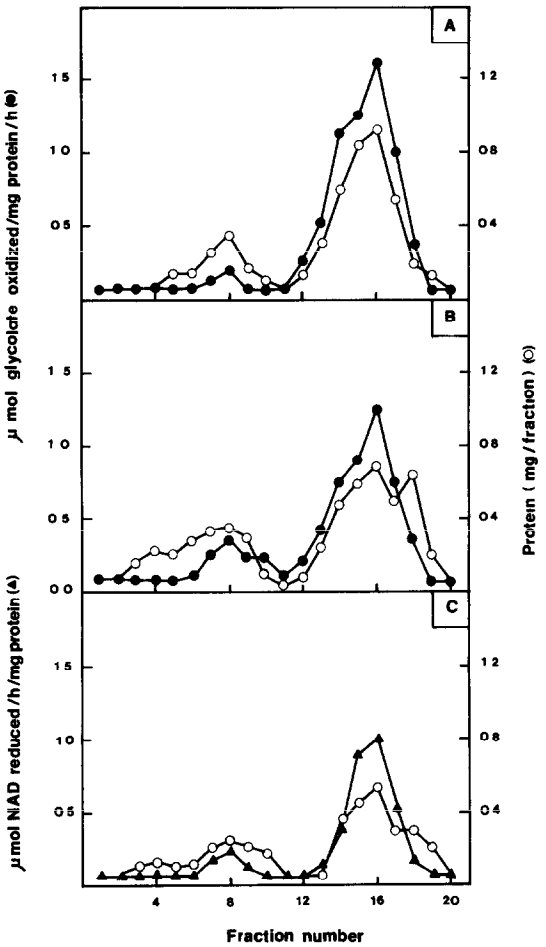

Fig 1 Distribution of glycolate oxidoreductase in (A) washed 226 000 × *g* × 60 min pellet (cytoplasmic membrane) and (B) cell free extract of *E coli* Lactate dehydrogenase in cytoplasmic membrane fractions (C) was also assayed after linear sucrose density gradient ○---○, Protein, measured as optical density at 280 nm, ●---●, glycolate oxidoreductase activity, ▲---▲, lactate dehydrogenase activity

Table 1

Distribution of glycolate oxidoreductase and glycolate dehydrogenase activity in centrifuged fractions of *E coli*

Fraction	Glycolate oxidoreductase		Glycolate dehydrogenase	
	Spec act [a]	% Act [b]	Spec act.[c]	% Act [b]
12 000 × *g* × 15 min pellet	0 00	0	0 00	0
12 000 × *g* × 15 min supernatant	1 23	100	1 16	100
226 000 × *g* × 60 min pellet	1 30	65	1 01	76
226 000 × *g* × 60 min supernatant	0 03	1 3	0 09	2 0
Washing of 226 000 × *g* × 60 min pellet	1 95	58 3	2 10	71 4
Washing of 226 000 × *g* × 60 min supernatant	0.00	0	0 00	0

[a] Specific activity measured as μmol glycolate oxidized/h per mg protein
[b] Percentage activity originally present in 12 000 × *g* × 15 min supernatant
[c] Specific activity measured as μmol glyoxylate/h per mg protein

Table 2

Effect of inhibitors on *E coli* glycolate oxidoreductase in the 226 000 × *g* × 60 min pellet

Inhibitor[a]	Concentration	% Inhibition[b]
Potassium cyanide	$1\ 10^{-5}$ M	0.00
	$1\ 10^{-4}$ M	8 00
	$1\ 10^{-3}$ M	55.00
	$2\ 10^{-3}$ M	67.31
Cupric sulfate	$1\ 10^{-6}$ M	40.00
	$5\ 10^{-6}$ M	72.43
	$1\ 10^{-5}$ M	84 61
EDTA	$1 \cdot 10^{-5}$ M	0.00
	$1\ 10^{-4}$ M	2 01
	$1\ 10^{-3}$ M	4 00
	$1\ 10^{-2}$ M	10 11
Sodium azide	$1\ 10^{-5}$ M	0 00
	$1\ 10^{-4}$ M	0 20
	$1\ 10^{-3}$ M	1 30

[a] Inhibitors added to the enzyme assay mixture to give the final concentrations used

[b] Specific enzyme activity was 1 8 μmol glycolate oxidized/h per mg protein

lated by Haddock [11] from *E.coli*. For further characterization of this cytoplasmic membrane fraction, a marker enzyme, lactate dehydrogenase, was assayed using all the fractions from the sucrose density gradient as presented in fig.1C. The highest lactate dehydrogenase activity coincided with the maximal protein peaks of both cytoplasmic membranes and crude extract (fig.1). Electron microscopic observation of a negatively stained 226 000 × *g* fraction also revealed a closed membrane vesicle (unpublished observation).

The enzyme activity in the cytoplasmic membranes of *E.coli* was inhibited by the sulphydryl inhibitor $CuSO_4$ ($1 \cdot 10^{-5}$ M), while the enzyme was insensitive to the metal complexing agents such as sodium azide

Table 3

Effects of homologous antiserum to the washed 226 000 × *g* × 60 min pellet (cytoplasmic membranes) from *E coli* upon glycolate oxidoreductase activity

Reaction	Spec. act	% Inhibition
Glycolate oxidoreductase of the 226 000 × g × 60 min[a]	1 80	0.00
+0.2 ml null serum	1 80	0 00
+0 2 ml antiserum	0 75	58 33
Glycolate oxidoreductase of the solubilized 226 000 × *g* × 60 min[b]	1 79	0 00
+0 2 ml null serum	1 76	1 70
+0 2 ml antiserum	0 00	100 00

[a] Specific enzyme activity measured as μmol glycolate oxidized/h per mg protein

[b] Solubilisation of the cytoplasmic membranes was by the addition of 1% (v/v) Triton X-100 to the final concentration

Table 4

Glycolate oxidoreductase of the 226 000 × *g* × 60 min pellet of *E coli* treated with pronase enzyme

	Spec act [a]	% Activity
226 000 × g × 60 min original pellet	1 80	100 00
226 000 × *g* × 60 min washings of cytoplasmic membranes after treatment with pronase[b]		
First pellet	0 73	40 56
Second pellet	0 70	38 89

[a] Specific activity measured as μmol glycolate oxidized/h per mg protein

[b] Pronase was added in a final concentration of 1 mg/ml and incubated for 15 min at 8°C

and EDTA as presented in table 2. 76% inhibition of the enzyme in *E.coli* was due to the addition of $2 \cdot 10^{-3}$ M potassium cyanide. This agrees with the finding of Lord [9], where it is concluded that the cyanide inhibition of the enzyme is probably due to sulphydryl reaction rather than metal complexing. An antiserum raised against the washed cytoplasmic membranes caused 58% inhibition of the enzyme activity as indicated in table 3. However, after solubilization of the membranes with 1% (v/v) Triton X-100, complete inhibition of the enzyme was obtained with homologous antiserum (table 3). In addition, pronase enzyme was added to *E.coli* cytoplasmic membranes to destroy all the accessible glycolate oxidoreductase; after washing of the membranes from pronase enzyme, the activity of glycolate oxidoreductase left inside the membranes was about 38–40% as shown in table 4. This indicates that part of the glycolate oxidoreductase in *E.coli* is exposed to the surface of the cytoplasmic membrane while the other part is embedded inside (tables 3 and 4). No previous studies on the intracellular distribution of the enzyme are reported. However, Lord [9] has purified the enzyme partially from the 48 000 × *g* × 60 min supernatant fraction.

We can conclude from in vitro studies that glycolate oxidoreductase in *E.coli* is firmly associated with the cytoplasmic membranes.

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