Regulation of Glyoxylate Metabolism in *Escherichia coli* K-12

L. N. ORNSTON[1] AND M. K. ORNSTON[1]

Biochemistry Division, University of Illinois, Urbana, Illinois 61801

The relative contributions of the dicarboxylic acid and the tricarboxylic acid cycles to the oxidative catabolism of glyoxylate in *Escherichia coli* K-12 were deduced by analysis of mutant strains that were blocked in the formation of glyoxylate carboligase and of malate synthase G (the "glycolate form" of malate synthase). Mutant strains unable to form malate synthase G were unimpaired in their ability to oxidize glyoxylate. Hence, the dicarboxylic acid cycle does not appear to play an essential role in this process. Organisms blocked in the synthesis of glyoxylate carboligase did not oxidize glyoxylate at a detectable rate, indicating that wild-type organisms convert glyoxylate to acetyl-coenzyme A and oxidize it via the tricarboxylic acid cycle. The foregoing evidence indicates that malate synthase G plays an anaplerotic role during growth with glycolate or acetate as the carbon source. The in vivo activity of malate synthase G was not detectable when the intracellular concentration of acetyl-coenzyme A was low, suggesting that this substrate or a closely related metabolite exerts a sensitive positive control over the enzyme. The synthesis of malate synthase G appears to be induced directly by glycolate which may be formed by a constitutive reduced nicotinamide adenine dinucleotide phosphate-dependent glyoxylate reductase in glyoxylate- or acetate-grown cells.

Glycolate or glyoxylate can serve as a sole carbon source for *Escherichia coli* K-12. When one of these compounds is the growth substrate, a series of specialized reactions converts it to common intermediary metabolites. These reactions and the enzymes that mediate them are depicted in Fig. 1. The interconversion of glycolate and glyoxylate can be catalyzed by two distinct enzymes: a flavine mononucleotide-dependent glycolate oxidase (19) and a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-requiring glyoxylate reductase (7). As shown in Fig. 1, a point of metabolic divergence occurs at the level of glyoxylate, which can participate in either of two condensing reactions: malate synthase [EC 4.1.3.2, L-malate glyoxylate-lyase (coenzyme A-acetylating)] joins glyoxylate and acetyl coenzyme A (CoA) to give rise to L-malate (18); glyoxylate carboligase catalyzes the conversion of two molecules of glyoxylate to tartronic semialdehyde and carbon dioxide (13). The latter reaction is the first step in the glycerate pathway, a series of reactions that results in the synthesis of 3-phosphoglyceric acid from glyoxylate (6).

The divergent condensation reactions have fostered a divergence of views regarding their role in the oxidation of glyoxylate by *E. coli*. According to one proposal (6), depicted in Fig. 2a, the glycerate pathway plays a major oxidative role: glyoxylate is converted to acetyl-CoA via 3-phosphoglycerate (3-PGA) and is oxidized via the tricarboxylic acid cycle. According to the scheme presented in Fig. 2a, malate synthase replenishes those intermediates of the tricarboxylic acid cycle that are used for biosynthesis and hence may be termed an anaplerotic enzyme (10). Evidence indicating that this mechanism may be operative has been presented by Hansen and Hayashi (6), who demonstrated the presence of high levels of all of the enzymes requisite for its operation in cultures of glycolate grown *E. coli* (Crooke's strain). These authors concluded that glyoxylate could be oxidized entirely via the tricarboxylic acid cycle.

Another viewpoint was presented by Kornberg and Sadler (12), who studied a citrate synthaseless mutant strain derived from *E. coli* W. When the growth medium was supplemented with a low level of glutamate, mutant cultures grew at the expense of glycolate at the same rate and to the same extent as wild-type cells. In addition, mutant cells oxidized glycolate as well as wild-type cells.
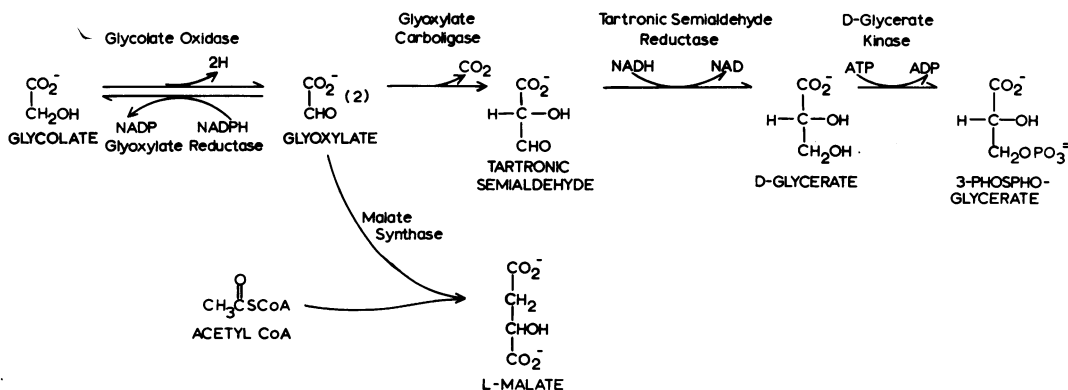
FIG. 1. *Reactions responsible for conversion of glycolate to common intermediary metabolites.*

On the basis of this evidence, Kornberg and Sadler concluded that the tricarboxylic acid cycle is not essential for the oxidation of glycolate by *E. coli*. To account for their observations, the authors proposed a dicarboxylic acid cycle, depicted in Fig. 2b. In this scheme, malate synthase and the enzymes of the glycerate pathway undergo a reversal of the roles that they are assigned in Fig. 2a: malate synthase initiates the oxidation of glyoxylate, and the enzymes of the glycerate pathway perform an anaplerotic function. Kornberg and Sadler (12) presented several lines of evidence to demonstrate the operation of the dicarboxylic acid cycle in the citrate synthaseless mutant. Glycolate-grown cultures of the mutant rapidly incorporated ^{14}C glycolate into malate, indicating that malate synthase was active in the cells. Nonradioactive glycolate stimulated the release of $^{14}CO_2$ from acetate by cultures of the citrate synthaseless mutant, indicating that the dicarboxylic acid cycle permitted these cells to oxidize some acetate. This oxidation was inhibited by arsenite, and, under these conditions, labeled pyruvate accumulated. Therefore, Kornberg and Sadler (12) proposed that the dicarboxylic acid cycle represents a quantitatively important route in the oxidation of glyoxylate by *E. coli*.

Recent studies of the regulation and genetics of malate synthase in *E. coli* K-12 have renewed interest in its role during growth with glycolate. Falmagne et al. (3) reported that there are two forms of malate synthase, and Vanderwinkel and De Vlieghere (16) showed that independent genetic loci govern their synthesis. One form, termed malate synthase A, is found in extracts of cells after growth with acetate, but not with glycolate (16). The structural gene governing the synthesis of malate synthase A is closely linked with the isocitrate lyase locus; the two enzymes appear to be members of a single operon (16), which is governed primarily by repressive control exerted

by a metabolite closely related to pyruvate or phosphoenolpyruvate (9). The second form, malate synthase G, contributes about 40% of the activity observed in extracts of acetate-grown cells and over 90% of the activity observed in glycolate-grown cells (16). The genetic region controlling malate synthase G formation has been identified (15), but the regulation of its synthesis has remained obscure.

The experiments reported in this paper were undertaken to determine the role of malate synthase G during growth with glycolate and the regulatory mechanisms governing the formation of this enzyme. The relative importance of the tricarboxylic acid and dicarboxylic acid cycles in the oxidation of glyoxylate by *E. coli* K-12 has been estimated by isolation and study of mutant strains blocked in the synthesis of the two enzymes that initiate utilization of glyoxylate, malate synthase G and glyoxylate carboligase; the influence of these genetic lesions on the oxidative capacity of the mutant strains has been examined. The properties of mutants blocked in the synthesis of glycolate oxidase have given an indication of the means by which the synthesis of malate synthase G is controlled.

MATERIALS AND METHODS

Bacterial strains. The properties of the strains of *E. coli* K-12 used in this study are summarized in Table 1. Strain K6G was isolated by selecting for a recombinant that could grow at the expense of glycolate in the presence of proline, histidine, arginine, and shikimate, and in the absence of methionine and thymine, after a standing mating of the Hfr strain WT K1 and the F⁻ strain K6. Mutants were obtained by mutagenesis with ethylmethanesulfonate (Kodak Ltd., Liverpool, England) and were selected for inability to grow at the expense of glycolate, when penicillin (Glaxo Laboratories Ltd., Greenford England) was present (4).

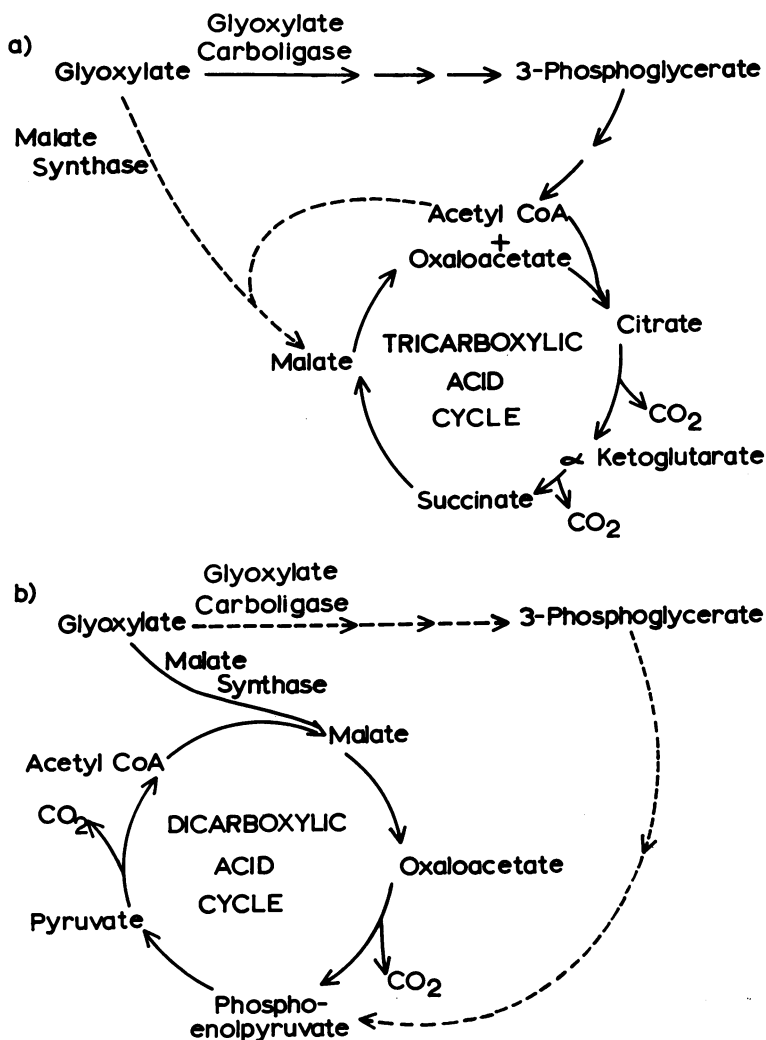**Growth conditions.** All cultures were grown in

FIG. 2. *Dicarboxylic acid and tricarboxylic acid cycles proposed for glyoxylate oxidation by Escherichia coli. Oxidative reactions are represented by solid lines; anaplerotic reactions, by dashed lines. If glyoxylate is oxidized via the tricarboxylic acid cycle, glyoxylate carboligase plays an oxidative role (a). If the dicarboxylic acid cycle is used for glyoxylate oxidation, malate synthase is an oxidative enzyme (b).*

mineral medium (2) supplemented when necessary with growth factors in the following concentrations (mg/liter): thymine, 40; methionine, 40; histidine, 40; proline, 50; arginine, 150; and shikimic acid, 20. Solid media were prepared by separately sterilizing equal volumes of double-strength mineral base and 3% (w/v) Ionagar No. 1 (Oxoid, Oxo Ltd., London, England). The solutions were combined after they had cooled to less than 55 C. Carbon sources and growth factors were added aseptically to the mineral media from concentrated sterile stock solutions. Growth media contained carbon sources at the following concentrations: 30 mM sodium glycolate, 20 mM sodium glyoxylate, 30 mM sodium acetate, 10 mM

glucose, and 0.5% Casamino Acids (Oxoid). Cultures were grown in a volume of 100 ml in 500-ml Erlenmeyer flasks which were aerated at 30 C in a Gallenkamp Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

In most cases, cells were harvested during exponential growth. When the inducing substrate (such as glycolate) was supplemented with Casamino Acids, cultures were allowed to grow almost into stationary phase before harvesting: an inoculum of 5 ml was added to 100 ml of medium and shaken for 16 to 20 hr to permit the enzymes to be induced to the highest possible level.

Preparation of extracts. Cells were harvested by

TABLE 1. *Properties of strains of E. coli K-12 employed*

| Strain | Source | Ability to grow at the expense of | | Apparent dysfunction in glycolate metabolism | Sex | Growth requirements[a] |
		Glycolate	Glyoxylate			
WT K1	H. L. Kornberg	+	+	None	Hfr	met, thy
Glc-2	Mutant of WT Kl	−	−	Glyoxylate carboligase	Hfr	met, thy
K6	H. L. Kornberg	−	−	Glyoxylate carboligase	F⁻	pro, his, arg, shi
K6G	Recombinant of WT K1 and K6	+	+	Malate synthase G	F⁻	pro, his, arg, shi
WT R4	H. L. Kornberg	+	+	None	Hfr	met
Glc-102	Mutant of WT R4	−	+	Glycolate permease	Hfr	met
Glc-103	Mutant of WT R4	−	+	Glycolate oxidase	Hfr	met
Glc-104	Mutant of WT R4	−	+	Malate synthase G and a glycolate enzyme[b]	Hfr	met

[a] Growth requirements are abbreviated as follows: L-methionine, met; thymine, thy; L-proline, pro; L-histidine, his; L-arginine, arg; shikimate, shi.

[b] Mutant GlC-104 has apparently undergone a single mutation which has rendered it unable to form malate synthase G and, in addition, has impaired the synthesis of glycolate permease or glycolate oxidase, or both of these enzymes.

centrifugation for 10 min in an MSE 18 centrifuge (Measuring and Scientific Equipment Co. Ltd., London, England), washed, and resuspended in 20 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 2 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.9), and stored at −20 C until extracted. All of the enzymes examined were stable for at least 1 month when frozen in whole cells.

Extracts were prepared by thawing a frozen suspension of cells, treating the suspension for 2 min with an MSE 60-w ultrasonic oscillator (Measuring and Scientific Equipment Co. Ltd.), and centrifuging the preparation at 10,000 × g for 10 min. The supernatant liquid was used as a crude extract for the assay of enzymes. Protein concentration was determined with biruet reagent (17).

Manometry. When both the oxidative capacity and enzymatic constitution of a culture were to be examined, the cells were harvested and resuspended in 10 mM potassium phosphate buffer, pH 7.0. The resuspended cells were divided into two portions, one of which was used for manometry, and the second of which was centrifuged, washed with 20 mM Tris buffer (pH 7.9), and extracted by standard procedures.

In manometric experiments, each Warburg vessel contained 200 μmoles of Na₂HPO₄-KH₂PO₄ buffer (pH 7.0) and 4 mg (dry weight) of cells. The center well contained 0.2 ml of 10% NaOH and the side arm contained 20 μmoles of substrate. The total liquid volume was 3.2 ml. After equilibration at 37 C, the substrate was added to the main compartment, and the rate of oxygen consumption was determined.

Enzyme assays. Continuous spectrophotometric assays were performed at 30 C in 1.0-ml cuvettes with a light path of 1.0 cm. Absorbancy was measured with a Unicam SP800 recording spectrophotometer (Unicam Instruments Ltd., Cambridge, England). For the discontinuous assay of malate synthase at 25 C,

3-ml cuvettes with a light path of 1.0 cm were employed; absorbancy was measured with a Unicam SP600 spectrophotometer. In all cases, an enzyme unit is defined as the amount of enzyme that catalyzes the removal from the assay mixture of 1 μmole of substrate per min.

Published assay procedures were used for determination of the activity of glycolate oxidase (19) and glyoxylate carboligase (11). Glyoxylate reductase was determined by measuring the rate of NADPH oxidation in the presence of glyoxylate. Assay mixtures contained 100 μmoles of potassium phosphate buffer (pH 7.0), 0.2 μmole of NADPH (Sigma Chemical Co., St Louis, Mo.), 1 mM sodium glyoxylate, and extract, in a final volume of 1.0 ml. Rates were measured against a control cuvette which contained no enzyme. Under these conditions, a change of 6.22 absorbance units at 340 nm corresponds to the reduction of 1 μmole of glyoxylate per cuvette. It should be noted that in wild-type cells the activity of glyoxylate reductase can be obscured by the combined action of glyoxylate carboligase and tartronic semialdehyde reductase (Fig. 1). Accordingly, measurements of the level of glyoxylate reductase are most accurately obtained with mutants that cannot form glyoxylate carboligase.

The activity of malate synthase was measured by determining the rate of glyoxylate-dependent release of free CoA from acetyl-CoA at 25 C. Acetyl-CoA was prepared from CoA (Sigma Chemical Co.) and acetic anhydride by the method of Stadtman (14). Each assay mixture contained 10 μmoles of MgCl₂, 0.4 μmole of acetyl-CoA, 1.0 μmole of glyoxylate, and 100 μmoles of Tris-hydrochloride buffer (pH 7.9), in a volume of 0.9 ml. The reaction was initiated by addition of enzyme and stopped by addition of 2.0 ml of 6 M urea, followed by 0.1 ml of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid). After 10 min, the absorbance at 412 nm was determined; an increment of 4.53

absorbance units at this wavelength corresponded to the release of 1 μmole of CoA per cuvette. The difference in CoA concentration in assay mixtures that had been incubated for 0 and 10 min was normally used for calculation of malate synthase activity. When necessary, the rate of CoA release in the absence of glyxoylate was determined; extracts did not catalyze this reaction at an appreciable rate in the absence of phosphate.

The relative concentrations of the two forms of malate synthase, G and A, were determined by thermal inactivation of malate synthase A by the procedure of Vanderwinkel and De Vlieghere (16).

RESULTS

Influence of carbon source on enzyme levels in wild-type cells. The influence of carbon source on the levels of some of the enzymes directly associated with glycolate metabolism is shown by the data presented in Table 2. Glycolate oxidase, glyoxylate carboligase, and malate synthase were not present at significant levels in extracts of wild-type cells after growth with Casamino Acids alone. Growth with acetate increased the rate of formation of malate synthase, but did not enhance appreciably the rate of synthesis of either glycolate oxidase or glyoxylate carboligase. Glyoxylate-grown cells contained undetectable levels of glycolate oxidase, but high levels of glyoxylate carboligase and malate synthase; the specific activity was five times that found in extracts of cells grown with Casamino Acids. About 80% of the malate synthase found in extracts of glyoxylate-grown cells was malate synthase G. Glycolate was the sole carbon source that elicited the synthesis of glycolate oxidase. The malate synthase in extracts of glycolate-grown cultures was over 90% malate synthase G, in accord with the observations of Vanderwinkel and De Vlieghere (16). The high specific activity of malate synthase in extracts of glycolate-grown cells (almost four times the specific activity found in extracts of glyoxylate-

grown cells) suggests that glycolate may be more directly responsible for the enzyme's induction than is the enzyme's substrate, glyoxylate. Furthermore, the inductive patterns presented in Table 2 indicate that the syntheses of glycolate oxidase, glyoxylate carboligase, and malate synthase G are not coordinately controlled.

Casamino Acids did not strongly repress the glycolate-induced formation of glycolate oxidase or of malate synthase (Table 2). On the other hand, the levels of glyoxylate carboligase were repressed over 50% by addition of Casamino Acids to glyoxylate or glycolate growth medium. A sharp repressive effect exerted on the synthesis of glyoxylate carboligase in *E. coli* by acetogenic compounds has been reported (8); the repression described in Table 2 was probably due to this phenomenon. Despite its repression, glyoxylate carboligase was induced to a readily detectable level in the presence of Casamino Acids. Accordingly, growth medium supplemented with Casamino Acids was used for induction of enzymes in cultures of mutants that had lost the ability to utilize glycolate or glyoxylate as a sole carbon source.

Characterization of glyoxylate carboligaseless mutant strains. Strains Glc-2 and K6 were unable to grow at the expense of either glycolate or glyoxylate. When glycolate-induced cultures of these mutant strains were shaken with glycolate, glyoxylate accumulated in the medium, indicating that glycolate could penetrate their cell membrane. When cultures of Glc-2 or K6 were grown with acetate and exposed to glyoxylate, they removed the aldehyde from the growth medium at a rate (0.85 μmoles of glyoxylate removed/mg of cells formed) identical to that of wild-type cells grown under the same conditions. Hence, it appeared that strain Glc-2 and K6 had not been altered in their permeability to glyoxylate.

The nature of the enzymatic lesion in Glc-2 and K6 was deduced from the data presented in Table 3. Although glycolate- or glyoxylate-induced cultures of the mutants formed malate synthase at a specific activity comparable to that found in wild-type cells, the mutant strains did not form detectable levels of glyoxylate carboligase under conditions which elicited at least a 15-fold induction of the enzyme in wild-type cultures. This evidence indicates that strains Glc-2 and K6 have undergone mutations which have rendered them unable to form glyoxylate carboligase.

Mutants lacking glyoxylate carboligase cannot form tartronic semialdehyde, and consequently the activity of the NADPH-dependent glyoxylate reductase is not obscured by tartronic semialdehyde reductase (5) in extracts of these organisms. Studies with the glyoxylate carboligaseless mu-

TABLE 2. *Influence of carbon source on the levels of glycolate enzymes in E. coli WT K1*

Inducing growth substrate	Cas-amino Acids[a]	Specific activity (μmole per min per mg of protein)		
		Malate synthase	Glyoxylate carboligase	Glycolate oxidase
—	+	0.02	<0.004	<0.001
Acetate	—	0.09	<0.004	<0.001
Glyoxylate	—	0.10	0.21	<0.001
Glyoxylate	+	0.09	0.09	<0.001
Glycolate	—	0.38	0.21	0.020
Glycolate	+	0.40	0.08	0.018

[a] Presence (+) or absence (−) of 0.5% Casamino Acids.

TABLE 3. *Characterization of strains Glc-2 and K6[a]*

Strain	Grown with Casamino Acids and	Specific activity (µmole per min per mg of protein)	
		Malate synthase	Glyoxylate carboligase
WT Kl	Glycolate	0.33	0.062
	Glyoxylate	0.08	0.093
Glc-2	Glycolate	0.26	<0.004
	Glyoxylate	0.12	<0.004
K6	Glycolate	0.29	<0.004
	Glyoxylate	0.06	<0.004

[a] Enzyme levels in extracts after growth with 0.5% Casamino Acids and either 30 mM glycolate or 20 mM glyoxylate.

tants Glc-2 and K6 indicated that the rate of synthesis of glyoxylate reductase is not influenced greatly by glycolate or glyoxylate in the growth medium: the specific activity of the enzyme was 0.010 ± 0.002 µmole per min per mg of protein in extracts of the mutant strains after growth with Casamino Acids in the presence or the absence of glycolate or glyoxylate. In contrast, exposure to glycolate induced the synthesis of glycolate oxidase in mutant cultures to the level found in wild-type strains (Table 2). The specific activity of glyoxylate reductase was not influenced by passage through a column (1.0 × 10 cm) of Sephadex G-25 at room temperature, indicating that if the enzyme requires cofactors it binds them tightly.

Characterization of mutants impermeable to glycolate and mutants blocked in the formation of glycolate oxidase and of malate synthase G. Mutant strains Glc-102, Glc-103, and Glc-104 all grow with glyoxylate, but not with glycolate, as sole carbon source. As shown in Table 4, none of the three mutants formed detectable amounts of glycolate oxidase, when grown with glycolate and Casamino Acids. Mutants Glc-102 and Glc-104 formed very little malate synthase, but extracts of mutant Glc-103 possessed as much malate synthase as wild-type extracts (Table 4). Since glycolate entered Glc-103 cells at a rate sufficient to elicit full induction of malate synthase, the absence of glycolate oxidase in extracts of this strain strongly suggests that mutant Glc-103 has been altered in a gene governing its synthesis. The presence of full levels of malate synthase in extracts of Glc-103 cells indicates that glycolate itself can induce the synthesis of malate synthase G without being converted to glyoxylate. The same cannot be said of the influence of glycolate on the synthesis of glyoxylate carboligase: the

TABLE 4. *Characterization of mutants Glc-102, Glc-103, and Glc-104[a]*

Strain	Specific activity (µmole per min per mg of protein)		
	Glycolate oxidase	Malate synthase	Glyoxylate carboligase
WT R4	0.0160	0.39	0.080
Glc-102	<0.0003	0.01	<0.004
Glc-103	<0.0003	0.45	<0.004
Glc-104	<0.0003	0.02	<0.004

[a] Enzyme levels in extracts after growth with 30 mM glycolate supplemented with 0.5% Casamino Acids.

absence of glyoxylate carboligase in extracts of mutant Glc-103 indicates that glycolate must be oxidized to glyoxylate in order to elicit the synthesis of this enzyme.

Since neither mutant Glc-102 nor mutant Glc-104 formed appreciable amounts of glycolate oxidase, of glyoxylate carboligase, or of malate synthase during growth with glycolate (Table 4), it might be concluded that both of these strains have suffered a similar genetic lesion that has rendered them impermeable to glycolate, but not to glyoxylate. Examination of malate synthase levels in glyoxylate-grown cultures of these strains (Table 5) indicated that this is not the case. As shown in Table 5 the specific activity of malate synthase in glyoxylate-grown cultures of strain Glc-102 was comparable to that found in wild-type cultures, but the specific activity of malate synthase found in cultures of strain Glc-104 was considerably less than that found in cultures of the wild type. Most of the low activity of malate synthase in extracts of glyoxylate-grown cells of strain Glc-104 is labile at 61 C under the conditions described by Vanderwinkel and De Vlieghere (16), and hence is attributable to the "acetate" form of malate synthase. Thus, it appears that mutant Glc-104 is blocked in the synthesis of malate synthase G. In contrast, 70% of the malate synthase found in extracts of glyoxylate-grown cultures of Glc-102 or of WT R4 resists heat treatment and thus appears to be malate synthase G (Table 5). Accordingly, mutants Glc-102 and Glc-104 differ in that the former organism can form malate synthase G, but the latter cannot.

Mutant Glc-102 was able to form malate synthase G (Table 5), but neither this enzyme nor glyoxylate oxidase was synthesized when the organism was grown in the presence of glycolate (Table 4). This phenotype probably results from a mutation which has rendered Glc-102 cells impermeable to glycolate and thus prevents induc-

TABLE 5. *Levels of malate synthase in mutants Glc-102 and Glc-104 after growth at the expense of 20 mM glyoxylate*

Strain	Specific activity (μmole per min per mg of protein)		
	Total malate synthase	Malate synthase A	Malate synthase G
WT R4	0.070	0.022	0.048
Glc-102	0.060	0.018	0.042
Glc-104	0.026	0.023	0.003

TABLE 6. *Levels of malate synthase and of glycolate oxidase in extracts of mutant Glc-104 and revertants thereof after growth with 0.5% Casamino Acids and 30 mM glycolate*

Strain	Specific activity (μmole per min per mg of protein)	
	Malate synthase	Glycolate oxidase
WT R4	0.30	0.012
Glc-104	0.02	<0.0005
Glc-104 R1	0.25	0.0104
Glc-104 R2	0.09	0.0038

TABLE 7. *Levels of malate synthase and of glyoxylate carboligase in extracts of WT K1 and of K6G after growth at the expense of 30 mM glycolate*

Strain	Specific activity (μmole per min per mg of protein) of	
	Malate synthase	Glyoxylate carboligase
WT K1	0.31	0.20
K6G	0.014	0.19

tion of either glycolate oxidase or malate synthase by this compound.

The inability of mutant Glc-104 to form glycolate oxidase during growth in the presence of glycolate (Table 4) indicates that, in addition to its defect in the formation of malate synthase G, it is also impaired in the synthesis of glycolate oxidase. The latter defect may be the indirect result of an alteration in permeability which prevents glycolate from entering the cell and inducing the synthesis of glycolate oxidase. Spontaneous revertants of strain Glc-104, selected for ability to grow with glycolate, have regained the ability to form both glycolate oxidase and malate synthase G in the presence of this compound (Table 6). Thus, it appears that both of the defects in strain Glc-104 result from a single mutation.

Strain K6G can utilize either glycolate or glyoxylate as a sole growth substrate. Nevertheless, after growth with glycolate, cultures of K6G contained less than 5% of the malate synthase activity found in wild-type cells (Table 7); the malate synthase foond in extracts of K6G is unstable under the conditions described by Vanderwinkel and De Vlieghere (16), indicating that it is malate synthase A. Hence, it appears that strain K6G cannot form malate synthase G, but that this deficiency does not prevent growth of the organism at the expense of either glycolate or glyoxylate.

Glyoxylate oxidation by wild-type and mutant cells. Strain K6G grew at the expense of glycolate without forming significant quantities of malate synthase (Table 7). Similarly, mutant Glc-104 formed very little malate synthase during growth at the expense of glyoxylate (Table 5). These observations suggested that malate synthase does not play an essential role in the oxidation of glyoxylate. The validity of this viewpoint was tested by direct examination of the ability of wild-type cells and of mutant cells to oxidize glyoxylate. After growth with glycolate or glyoxylate, cultures were divided into two portions, one of which was

used to determine the ability of whole cells to oxidize glyoxylate and the other of which was used to measure the specific activity of malate synthase and of glyoxylate carboligase. The results of these studies are summarized in Tables 8 and 9.

As can be seen in Table 8, the specific activity of malate synthase in extracts of glyoxylate-grown cultures of strain Glc-104 was 6% of that found in extracts of glycolate-grown cultures of WT R4; the specific activity of glyoxylate carboligase in the mutant was 86% of that found in the wild type. Despite the reduced level of malate synthase, the mutant cells oxidized glyoxylate at 88% of the rate of glycolate-induced wild-type cells (Table 8). Hence, it appeared that malate synthase was not essential for the oxidation of glyoxylate by these cultures.

The relative importance of malate synthase and of glyoxylate carboligase in the oxidation of glyoxylate was investigated by comparing the glyoxylate-oxidizing ability of two closely related strains: strain K6G, which was deficient in the synthesis of malate synthase, and strain K6 which was impaired in the synthesis of glyoxylate carboligase. As is shown in Table 9, glycolate-induced cultures of the malate synthase-deficient strain K6G oxidized both glycolate and glyoxylate at a rapid rate. Thus, it appeared that in this strain, as in mutant Glc-104, malate synthase was not necessary for glyoxylate oxidation. On the other hand,

TABLE 8. *Oxidation of glyoxylate by 20 mM glyoxylate-grown cultures of Glc-104 and by 30 mM glycolate-grown cultures of WT R4*

Strain	Growth substrate	Specific activity (μmole per min per mg of protein)		QO$_2$ (μliters of O$_2$ per hr per mg of cells) with glyoxylate[a]
		Malate synthase[a]	Glyoxylate carboligase[a]	
WT R4	Glycolate	0.416 (100)	0.21 (100)	32 (100)
Glc-104	Glyoxylate	0.026 (6)	0.18 (86)	28 (88)

[a] The values in parentheses indicate the activity as a percentage of that observed in glycolate-grown WT R4 cells.

TABLE 9. *Glycolate and glyoxylate oxidation by cultures of K6 and K6G*[a]

Strain	Inducing substrate	Specific activity (μmole per min per mg of protein)		QO$_2$ (μliters of O$_2$ per hr per mg of cells) with	
		Malate synthase[b]	Glyoxylate carboligase[c]	Glycolate[c]	Glyoxylate[c]
K6G	Glycolate	0.016 (6)	0.09 (100)	60 (100)	19 (100)
K6	Glycolate	0.285 (100)	<0.004 (<5)	6.3 (10)	<2 (<12)
K6G	Glyoxylate	0.003 (11)	0.08 (89)	—	17.5 (90)
K6	Glyoxylate	0.061 (21)	<0.004 (<5)	—	<2 (<12)

[a] Cultures were grown with 0.5% Casamino Acids supplemented with either 30 mM glycolate or 20 mM glyoxylate.

[b] The values in parentheses indicate the activity as a percentage of that observed in glycolate-induced K6 cells.

[c] The values in parentheses indicate the activity as a percentage of that observed in glycolate-induced K6G cells.

glycolate-induced cultures of the glyoxylate carboligase-deficient strain K6 did not oxidize glyoxylate at a detectable rate, despite the presence of high levels of malate synthase in these cells (Table 9). Glycolate is oxidized by strain K6 at 10% of the rate of oxidation in strain K6G. After 2.3 μmoles of oxygen had been consumed by the K6 culture in the presence of glycolate, the contents of the Warburg vessel were analyzed for glyoxylate: 3.84 μmoles of glyoxylate were recovered, indicating that over 80% of the oxygen consumed by these cells was used to convert glycolate to glyoxylate. These results strongly suggested that glyoxylate carboligase plays an essential role in the oxidation of glyoxylate.

The oxidative role of glyoxylate carboligase and of malate synthase was further studied with mutant Glc-2, which differs from its parent strain WT K1 in that it cannot form glyoxylate carboligase. The results of these investigations are summarized in Table 10. After growth in the presence of glycolate, cells of both strain WT K1 and strain Glc-2 contained high levels of malate synthase, and cultures of both strains oxidized malate at a rapid rate. Hence, all of the enzymes of the dicarboxylic acid cycle (Fig. 2b) were present, and the enzymes necessary for conversion of malate to acetyl-CoA were operative in both cul-

tures. Nevertheless, the suspension of mutant Glc-2 cells did not oxidize glyoxylate at a detectable rate. These observations indicate that the dicarboxylic acid cycle was not functional in mutant Glc-2, because malate synthase, the enzyme that introduces glyoxylate into the cycle, was not catalytically active although it was present at high levels in this organism.

DISCUSSION

Pathway of glyoxylate oxidation. One of the major purposes of this investigation was to establish the degree to which the tricarboxylic acid cycle (Fig. 2a) and the dicarboxylic acid cycle (Fig. 2b) contribute to the oxidation of glyoxylate by *E. coli*. The results presented in Tables 8 and 9 indicate that malate synthase G does not play an essential role in the oxidation of glyoxylate and that a functional dicarboxylic acid cycle is not necessary for this process. On the other hand, the data contained in Tables 9 and 10 demonstrate that glyoxylate carboligase does play an essential role in the oxidation of its substrate. These results indicate that the product of glyoxylate carboligase, tartronic semialdehyde, is an intermediate in the oxidative pathway, but they do not demonstrate conclusively that the entire glycerate pathway and the tricarboxylic acid cycle are essential

TABLE 10. Glyoxylate and malate oxidation by cultures of WT K1 and of mutant Glc-2[a]

Strain	Inducing substrate	Specific activity (μmole per min per mg of protein)		QO_2 (μliters of O_2 per hr per mg of cells) with	
		Malate synthase[b]	Glyoxylate carboligase[c]	Glyoxylate[c]	Malate[c]
WT K1	Glyoxylate	0.07 (18)	0.085 (100)	31 (100)	102 (100)
Glc-2	Glyoxylate	0.12 (30)	<0.002 (<3)	<2 (<7)	97 (95)
WT K1	Glycolate	0.40 (100)	0.062 (73)	22.5 (73)	—
Glc-2	Glycolate	0.26 (65)	<0.002 (<3)	<2 (<7)	—

[a] Cultures were grown with 0.5% Casamino Acids and supplemented with either 30 mM glycolate or 20 mM glyoxylate.

[b] The values in parentheses indicate the activity as a percentage of that observed in glycolate-induced WT K1 cells.

[c] The values in parentheses indicate the activity as a percentage of that observed in glyoxylate-induced WT K1 cells.

for glyoxylate oxidation. Conceivably, tartronic semialdehyde might be oxidized via a cycle containing hydroxymalonate and glycolaldehyde as intermediates. Such a hypothetical cycle could not contain glycolate as an intermediate, since mutant Glc-103, which cannot form glycolate oxidase, grows well at the expense of glyoxylate. We have sought, but not found, enzymatic systems catalyzing the oxidation of tartronic semialdehyde in crude extracts of E. coli. Thus, our present results favor the view that glyoxylate is converted to glycerate and is oxidized by the tricarboxylic acid cycle via acetyl-CoA in E. coli (Fig. 2a).

Roles of malate synthase. Our results indicate that malate synthase G is not required for the oxidation of glyoxylate. Nevertheless, mutants deficient in the synthesis of malate synthase G grow more slowly (with a mean generation time of 300 min) than wild-type cells (with a mean generation time of 200 min) at the expense of glyoxylate, indicating that the enzyme does serve an important function. An observation of Ashworth and Kronberg (2) suggests the nature of this function: they have shown that mutants lacking phosphoenolpyruvate carboxylase (and thus unable to grow at the expense of glucose or glycerate) grow well with glycolate as sole carbon source. This evidence indicates that, during growth with glycolate, malate synthase G is an anaplerotic enzyme, forming malate at a rate sufficient to replenish those intermediates of the tricarboxylic acid cycle that are depleted for biosynthetic purposes. A similar role must be filled in cells during growth with acetate; significantly, such cells contain substantial quantities of malate synthase G. Acetate-grown cells posed an additional problem: formation of three-carbon acids such as phosphoenolpyruvate for gluconeogenesis. This biosynthetic role is played in glycolate-grown cells by the enzymes of the glycerate

pathway. Acetate-grown cells, on the other hand, possess a second form of malate synthase which can replenish intermediates depleted for this biosynthetic function.

Mechanism of induction of malate synthase in E. coli. The genes governing the synthesis of malate synthase A and of isocitrate lyase appear to be members of a single operon (16) and thus subject to identical control. Kornberg (9) has shown that this control is largely a repression exerted by a metabolite closely related to pyruvate and phosphoenolpyruvate. In addition, phosphoenolpyruvate inhibits the activity of isocitrate lyase (1). Thus, isocitrate lyase and malate synthase A are governed by end-product repression and inhibition, mechanisms commonly employed to regulate biosynthetic sequences.

The synthesis of malate synthase G appears to be mediated by induction. Growth with glycolate induced malate synthase G in E. coli to four times the level found in glyoxylate-grown cells (Table 2). This evidence indicated that glycolate may induce the synthesis of malate synthase G more directly than glyoxylate. Support for this view derives from the observation that mutant Glc-103, which lacked glycolate oxidase and hence could not convert glycolate to glyoxylate, formed fully induced levels of malate synthase when exposed to glycolate (Table 4).

If glycolate is the only metabolite which can elicit the synthesis of malate synthase G, then cells must have a mechanism for forming glycolate during growth with glyoxylate. E. coli extracts contain low levels of a NADPH-dependent glyoxylate reductase (7); studies with mutant Glc-2 showed that the rate of synthesis of this enzyme is not influenced by the presence or absence of glyoxylate or glycolate in the growth medium. A likely role for this constitutive glyoxylate reductase is the formation of glycolate, the apparent true inducer of malate synthase, from glyoxylate.

Control of the activity of malate synthase. After growth in the presence of glycolate, mutants unable to form glyoxylate carboligase possess high levels of malate synthase G and of all of the enzymes necessary for conversion of malate to acetyl-CoA. Nevertheless, such cells do not oxidize glyoxylate at an appreciable rate (Table 10). The simplest interpretation of this inactivity is that the intracellular concentration of acetyl-CoA is too low to permit malate synthase G to catalyze rapid formation of malate. The same interpretation can account for the apparent absence of a contribution by malate synthase G to the oxidative metabolism of cells that contain glyoxylate carboligase (Tables 8 and 9): presumably, the activity of citrate synthase maintains the pool of acetyl-CoA at a low level and thus restricts the activity of malate synthase G. However, when cells are exposed to both glyoxylate and an acetogenic substrate, the activity of malate synthase G can increase appreciably in order to replenish oxalacetate and thus increase the rate of utilization of acetyl-CoA via the tricarboxylic acid cycle. In this regard, the citrate synthaseless strain of *E. coli* W studied by Kornberg and Sadler (12) is of particular interest. This mutant accumulated acetate under a variety of growth conditions because the lesion in citrate synthase synthesis prevented utilization of acetyl-CoA. Unlike strains deficient in glyoxylate carboligase, the citrate synthaseless organism was able to oxidize glyoxylate via the dicarboxylic acid cycle. The dramatic difference in the oxidative capabilities of these strains is most probably due to differences in their intracellular concentration of acetyl-CoA during exposure to glyoxylate. High concentrations of acetyl-CoA in the citrate synthaseless mutant could have stimulated the operation of the dicarboxylic acid cycle; the relatively low concentrations of acetyl-CoA in the glyoxylate carboligaseless strain prevented glyoxylate from being introduced to the dicarboxylic acid cycle by malate synthase G. Since the concentration of malate synthase G does not influence the oxidative capacity of cells which contain glyoxylate carboligase, it seems likely that the level of acetyl-CoA in these (and wild-type) strains is too low to permit the dicarboxylic acid cycle to operate at a significant rate.

Properties of mutants defective in the metabolism of glycolate. Vanderwinkel and De Vlieghere (16) isolated a strain of *E. coli* K-12 which was unable to form malate synthase G and also had lost the ability to grow at the expense of glycolate. The properties of this mutant indicated that malate synthase G might be essential for growth with glycolate. That this is not the case was shown by strain K6G which can grow with glycolate while forming no malate synthase G. Thus, it seems probable that the mutant of Vanderwinkel and De Vlieghere is similar to mutant Glc-104, which cannot form malate synthase G and also has lost the ability to form glycolate oxidase (although the second defect may result from an alteration in its permeability to glycolate). The pleiotropic effects in mutant Glc-104 seem to result from a single mutation: revertants selected for the ability to grow with glycolate regain both glycolate oxidase and malate synthase G.

The genetic basis for the pleiotropic effects observed in mutant Glc-104 is unknown. The syntheses of malate synthase G and of glycolate oxidase do not appear to be coordinately controlled, since the former, but not the latter, enzyme is induced in glyoxylate-grown cells. Despite the lack of strict regulatory linkage, the syntheses of glycolate oxidase and of malate synthase G may both be governed by the same regulatory gene: this gene could be altered so that the enzymes cannot be formed by strain Glc-104. On the other hand, it is possible that glycolate oxidase and malate synthase G share a protein subunit which cannot be formed by strain Glc-104. Evidently, the two enzymes are not dependent on each other for their synthesis or their stability, since glycolate oxidase can be formed in the absence of malate synthase G (in strain K6G) and malate synthase G can be synthesized in the absence of glycolate oxidase (in mutant Glc-103).

The genetic nature of strain K6G is curious. Its F⁻ parent, strain K6, lacked glyoxylate carboligase but formed malate synthase G. A standing mating of K6 with the Hfr strain WT K1 (which can form both glyoxylate carboligase and malate synthase G) produced a recombinant, K6G, which had lost the ability to form malate synthase G as it gained the ability to form glyoxylate carboligase. The mechanism by which the cross between two malate synthase G-positive strains led to formation of a malate synthase G-negative recombinant is unknown, but analysis of this process may reveal a genetic entity that regulates the malate synthase G.

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