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Heterogeneity of the Glyoxylate-Condensing Enzymes

WARNER S. WEGENER, HENRY C. REEVES, AND SAMUEL J. AJL
Research Laboratories, Albert Einstein Medical Center, Philadelphia, Pennsylvania

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ABSTRACT

WEGENER, WARNER S. (Albert Einstein Medical Center, Philadelphia, Pa.), HENRY C. REEVES, AND SAMUEL J. AJL. Heterogeneity of the glyoxylate-condensing enzymes. J. Bacteriol. 90:594-598. 1965.—Evidence is presented that the enzymatic condensations of glyoxylate with acetyl-CoA (malate synthase), propionyl-CoA (α-hydroxyglutarate synthase), butyryl-CoA (β-ethylmalate synthase), and valeryl-CoA (β-*n*-propylmalate synthase) are catalyzed by different enzymes. The possibility that these activities resulted from a single enzyme possessing a broad fatty acid acyl-CoA substrate specificity was ruled out. The latter was suggested by the fact that cells grown on a number of short-chain fatty acids exhibited all the above activities. The conclusion that these reactions are catalyzed by different enzymes is based on the following considerations: (i) the enzymes can be differentially inactivated by heat; (ii) under various growth conditions, where all the condensing enzymes are present, their respective activities do not show a constant ratio, as would be expected if they were catalyzed by a single enzyme; and (iii) under appropriate growth conditions, one or more of these enzymes has been shown to be present to the exclusion of others.

For the past several years, our laboratory has been studying the role of glyoxylate in the growth of microorganisms with various short-chain fatty acids as the source of carbon. The condensation of glyoxylate with acetyl-coenzyme A (CoA) to form malic acid is catalyzed by malate synthase (Wong and Ajl, 1956). In addition, enzymatic condensations of glyoxylate with propionyl-CoA, butyryl-CoA, and valeryl-CoA to form, respectively, α-hydroxyglutarate, β-ethylmalate, and β-*n*-propylmalate have been described (Reeves and Ajl, 1962; Rabin, Reeves, and Ajl, 1963; Imai, Reeves, and Ajl, 1963). Malate synthase, β-ethylmalate synthase, and β-*n*-propylmalate synthase affect α condensations between glyoxylate and the respective fatty acid acyl-CoA substrates. In contrast, the condensation of glyoxylate with propionyl-CoA occurs via a β condensation mechanism. In light of the similarities among these reactions, it was considered essential to determine whether they were catalyzed by different enzymes or by a single enzyme possessing broad fatty acid acyl-CoA substrate specificity.

MATERIALS AND METHODS

Materials purchased commercially were: sodium glyoxylate-monohydrate, Sigma Chemical Co., St. Louis, Mo.; 1-C^{14}-sodium glyoxylate-monohydrate, Nuclear-Chicago Corp., Des Plaines, Ill.; CoA, P-L Biochemicals, Inc., Milwaukee, Wis.; and *p*-nitrophenylhydrazine, Eastman Chemical Products, Inc., Kingsport, Tenn. All other chemicals and reagents were of the highest quality commercially available.

Growth of cells and preparation of enzyme extracts. Escherichia coli, strain E-26, and *Pseudomonas aeruginosa* were used in this investigation. Cells were grown as previously described (Reeves and Ajl, 1962) in a mineral salts medium containing sodium acetate, valerate, propionate, butyrate, or glycolate as the sole carbon source. Glucose-citrate cells were grown at 30 C under stationary conditions in 8,000 ml of medium in 8-liter carboys as previously described (Wheat and Ajl, 1955). Trypticase Soy Broth (BBL) cultures were grown at 37 C under stationary conditions in 2,000 ml of medium in 2-liter Erlenmeyer flasks. Cell-free extracts were prepared by sonic treatment as previously described (Reeves and Ajl, 1962).

Optical and chemical methods. Protein concentrations were determined by the method of Lowry et al. (1951). Acetyl-CoA, propionyl-CoA, butyryl-CoA, and valeryl-CoA were prepared according to the method of Simon and Shemin (1953), and assayed by the method of Lipmann and Tuttle (1945).

Assay of glyoxylate-condensing enzyme activity. The condensing reactions were assayed by measuring the fatty acid acyl-CoA-dependent disappearance of glyoxylate. The assay method measures residual 1-C^{14}-glyoxylate as the *p*-nitrophenylhydrazone derivative (Wegener, Reeves, and Ajl, 1965). Specific activities are expressed as millimicromoles of glyoxylate utilized per 15 min per milligram of protein.

Heat-inactivation studies. Heat inactivation of the glyoxylate-condensing enzymes were studied with a cell-free enzyme extract prepared from valerate-grown cells. A 2-ml amount of extract was placed in glass test tubes (16 by 125 mm)

which had been preincubated in a water bath maintained at the desired temperature. Temperature was monitored with a YS1-model 42SC Tele-Theromometes (Arthur H. Thomas Co., Philadelphia, Pa.). The tubes were incubated without shaking for the desired time, removed, and immediately chilled in an ice-salt bath at −5 C for 1 min. They were then transferred to an ice bath, and coagulated protein was removed by centrifugation at 4 C for 10 min at 27,000 × *g*.

RESULTS

Influence of carbon source on formation of the glyoxylate-condensing enzymes. In many microorganisms, the formation of malate synthase appears to be an adaptive response to growth on acetate (Reeves and Ajl, 1960). Since *E. coli*, when transferred from a complex medium (Trypticase Soy Agar) to a propionate, butyrate, or valerate mineral salts medium, grows only after a lag period of 3 to 5 days, it was thought that the other glyoxylate-condensing enzymes were likewise formed adaptively in response to growth on these fatty acids. However, as is seen in Table 1, this appears not to be the case. For example, propionate-grown cells catalyzed not only the condensation of glyoxylate with propionyl-CoA, but also with acetyl-CoA, butyryl-CoA, and valeryl-CoA. Similarly, cells grown on butyrate, valerate, and glucose contained all of the glyoxylate-condensing enzymes.

In light of these findings, it seemed essential to determine whether these reactions were catalyzed by different enzymes or by a single enzyme possessing a broad specificity for the various fatty acid acyl-CoA substrates. In this regard, it had been shown by Dixon, Kornberg, and Lund (1960) that malate synthase purified from yeast did not catalyze the condensation of glyoxylate with either propionyl-CoA or butyryl-CoA. The authors assayed for these enzymes by use of the spectrophotometric method reported by Dixon and Kornberg (1959). This procedure has been found unsatisfactory in assaying for α-hydroxyglutarate, β-ethylmalate, and β-*n*-propylmalate synthases, since the products of the latter-condensations apparently are formed as the acyl-CoA esters and not as the free acids (Wegener et al., 1965; Megraw et al., *in press*). Hence, these enzymes cannot be assayed at 232 mμ by measuring the glyoxylate-dependent cleavage of the fatty acid acyl-CoA substrates. The results obtained by Kornberg and co-workers (Dixon et al., 1960) need, therefore, be re-evaluated in light of our published method (Wegener et al., 1965).

Differential heat inactivation of glyoxylate-condensing enzymes. The question of enzyme heterogeneity was investigated by use of the technique of differential heat inactivation. This technique has been used in demonstrating the heterogeneity of the mitochondrial lactic dehydrogenase isozymes (Plagemann, Gregory, and Wroblewski, 1961). The rate of thermal inactivation was also used to differentiate D-lactic from L-lactic dehydrogenase in crude enzyme preparations of *P. natriegens* (Walker and Eagon, 1964). Thermal inactivation has been used to distinguish two malate synthase enzymes in *E. coli*. Wiame (1963) showed that malate synthase in crude enzyme extracts prepared from either acetate- or glycolate-grown cells exhibited dissimilar rates of heat inactivation. These data are in support of conclusions drawn from growth experiments that the formation of malate synthase is under two cellular control mechanisms. The synthesis of one enzyme is considered to be regulated by derepression mechanisms, whereas the formation of a second isozyme appears to be controlled by a glyoxylate-induction mechanism (Vanderwinkel et al., 1963).

Thermal inactivation studies of the glyoxylate-condensing enzymes were performed with crude extracts prepared from valerate-grown *E. coli*. Cells grown under these conditions were selected since they had been shown to possess high specific

TABLE 1. *Influence of carbon source on occurrence of glyoxylate-condensing enzymes*

Inoculum	Carbon source	Specific activity				Ratio of activities*
		Malate synthase	β-Ethyl-malate synthase	α-Hydroxy-glutarate synthase	β-*n*-Propyl-malate synthase	
Propionate-adapted *Escherichia coli*	Propionate	59.0	4.8	10.5	15.7	1.0:2.2:3.3
Butyrate-adapted *Pseudomonas aeruginosa*	Butyrate	56.8	11.8	9.3	15.8	1.0:0.8:1.3
Valerate-adapted *E. coli*	Valerate	44.6	38.1	40.1	38.4	1.0:1.1:1.0
Glycolate-adapted *E. coli*	Glycolate	68.6	3.6	8.7	16.5	1.0:2.4:4.6
Valerate-adapted *E. coli*	Glucose	58.2	10.3	7.9	21.8	1.0:0.8:2.1

* Ratio of activities of β-ethylmalate synthase to α-hydroxyglutarate synthase to β-*n*-propylmalate synthase.

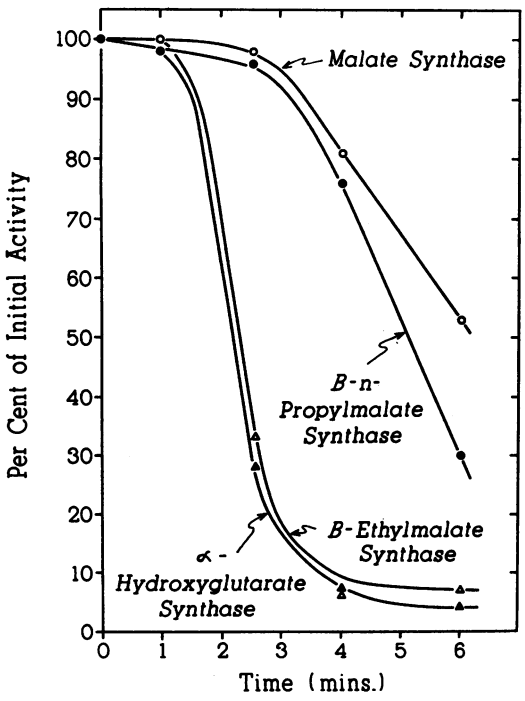

FIG. 1. *Thermal inactivation of glyoxylate-condensing reactions at 55 C.*

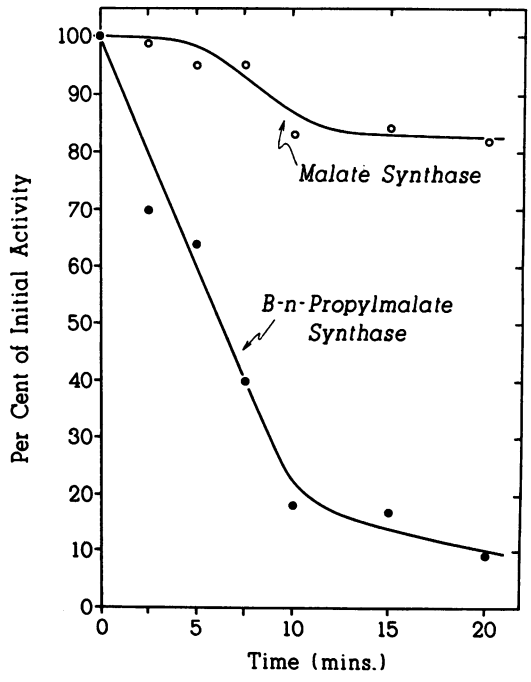

FIG. 2. *Thermal inactivation of malate synthase and β-n-propylmalate synthase at 51 C.*

activities for all the condensing reactions. Figure 1 shows the results of heating this extract at 55 C. At this temperature, malate synthase and β-*n*-propylmalate synthase are inactivated at approximately the same rates, whereas β-ethylmalate synthase and α-hydroxyglutarate synthase also show similar rates of heat inactivation; the rates of the latter two being greater than the former. After 4 min at 55 C, 75 to 80% of the activity of malate synthase and β-*n*-propylmalate synthase remained, whereas α-hydroxyglutarate synthase and β-ethylmalate synthase retained only 7 to 8% of their initial activities. Hence, heating at 55 C differentiates the acetyl-CoA and valeryl-CoA condensing enzymes from the butyryl-CoA and propionyl-CoA enzymes.

Figure 2 shows the results of heat inactivation at 51 C. This experiment was performed in an attempt to differentiate malate synthase from β-*n*-propylmalate synthase. At this temperature, the rate of heat inactivation of *n*-propylmalate synthase is greater than that of malate synthase. Whereas incubation for 10 min at 51 C destroyed only 10 to 20% of malate synthase, there was 70 to 80% inactivation of β-*n*-propylmalate synthase.

Figure 3 shows the heat inactivation of β-ethylmalate synthase and α-hydroxyglutarate

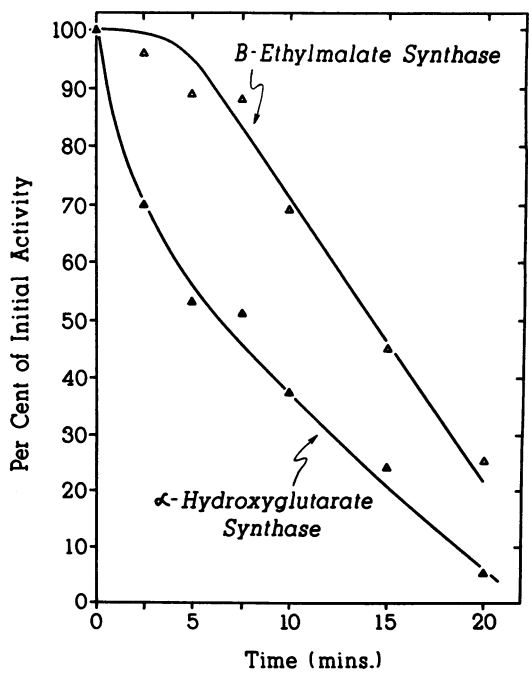

FIG. 3. *Thermal inactivation of β-ethylmalate synthase and α-hydroxyglutarate synthase at 48 C.*

TABLE 2. *Differential formation of glyoxylate-condensing activities*

Inoculum	Growth medium and conditions	Specific activity			
		Malate synthase	β-Ethylmalate synthase	α-Hydroxyglutarate synthase	β-*n*-Propylmalate synthase
Valerate-adapted *Escherichia coli*	Trypicase Soy Broth (restricted oxygen)	15.5	No activity	No activity	No activity
Propionate-adapted *E. coli*	Glucose-citrate (restricted oxygen)	29.7	No activity	No activity	10.4
Acetate-adapted *E. coli*	Acetate mineral-salts (aerobic)	61.2	No activity	No activity	16.0

synthase at 48 C. It is seen that β-ethylmalate synthase is significantly more stable than α-hydroxyglutarate synthase. Incubation for 5 min at 48 C resulted in a 40 to 50% inactivation of α-hydroxyglutarate synthase, but only a 5 to 10% loss of β-ethylmalate synthase activity. These results indicate that the activities of malate, α-hydroxyglutarate, β-ethylmalate, and β-*n*-propylmalate synthases can be differentiated from one another by differential thermal inactivation.

Differential formation of the glyoxylate-condensing enzymes. The fact that the glyoxylate-condensing enzymes exhibit differential rates of heat inactivation suggested that the reactions were not catalyzed by a single enzyme. These data, however, do not exclude the possibility that the reactions may be catalyzed by one enzyme possessing multiple sites. If the reactions were catalyzed by a single enzyme possessing a separate active site for each of the fatty acid acyl-CoA substrates, one would expect the ratios of the activities to be constant and to be independent of the growth conditions. Such a constant ratio of enzymatic activity would, of course, not apply under conditions where the accumulation of metabolites from growth substrates might differentially inhibit one or more of the activities. Table 1 shows that the ratios of the activities vary significantly during growth on glucose, glycolate, valerate, propionate, and butyrate. These data support the concept that the various condensations are catalyzed by separate enzymes.

Furthermore, cells can be grown under conditions such that they contain one or more but not all of the enzymes (Table 2). When *E. coli* is grown in a Trypticase Soy Broth medium, the cells contain malate synthase, but do not exhibit activity for the other condensing enzymes. These data indicate that malate synthase is a separate enzyme. *E. coli* grown on an acetate mineral salts medium possesses high malate synthase activity, and, in addition, exhibits β-*n*-propylmalate synthase activity comparable to that in butyrate-grown *P. aeruginosa* and propionate-grown *E. coli*. However, these acetate-grown cells do not contain α-hydroxyglutarate synthase or β-ethylmalate synthase. Likewise, propionate-adapted *E. coli* grown in a glucose-citrate anaerobic medium contain neither α-hydroxyglutarate synthase nor β-ethylmalate synthase, but do exhibit low β-*n*-propylmalate synthase activity. It appears, therefore, that *E. coli* is capable of synthesizing malate synthase under growth conditions where the activities of the other condensing enzymes cannot be demonstrated. Likewise, during growth on acetate, *E. coli* apparently synthesizes β-*n*-propylmalate synthase but not α-hydroxyglutarate nor β-ethylmalate synthase.

Using this criterion of enzyme heterogeneity, in addition to differential heat inactivation of the various glyoxylate-condensing enzymes, we propose that malate synthase and β-*n*-propylmalate synthase are different enzymes. Whereas the activities of α-hydroxyglutarate synthase and β-ethylmalate synthase can be differentiated by thermal inactivation, it has thus far not been possible to demonstrate the heterogeneity of these two enzymes by differential enzyme synthesis. It has not yet been possible to provide growth conditions which would permit the formation of one, but not the other, of these latter two enzymes. However, the fact that these activities do not exhibit a constant ratio of activity with respect to each other under varying growth conditions (Table 1) suggests that they are catalyzed by different enzymes.

DISCUSSION

The results presented in this report suggest the involvement of different enzymes in the condensation reactions of glyoxylate with a series of short-chain fatty acid acyl-CoA esters. The fact that these condensation reactions are catalyzed by different enzymes rather than by a single enzyme with broad specificity may permit the cell to regulate the synthesis of these enzymes more efficiently.

The mechanisms and factors determining the

formation of these enzymes are not yet clear. It had been considered previously that perhaps the fatty acid growth substrates induced the formation of the respective condensing enzymes. This is apparently not the case. It would be difficult to conceive how growth on propionate, for example, would lead to inducing concentrations of butyric or valeric acids.

It was also considered that glyoxylate might act as the inducer for these enzymes. This appeared reasonable in light of the fact that growth on these short-chain fatty acids resulted in the formation of high levels of isocitratase. The functioning of this enzyme might be considered to result in the accumulation of inducing concentrations of glyoxylate. Further, growth on glycolate, which in *E. coli* is metabolized to glyoxylate, resulted in the formation of low levels of all these condensing enzymes. However, data obtained when *E. coli* was grown in an acetate medium rule out the action of glyoxylate as the inducer, since growth on acetate resulted in the formation of isocitratase, and, hence, in the formation of glyoxylate, but did not result in the formation of α-hydroxyglutarate nor β-ethylmalate synthase.

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