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Factors Affecting the Level of Alanine Racemase in *Escherichia coli*[1]

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Alanine racemase occupies a key position in the alanine branch of peptidoglycan biosynthesis. The level of this enzyme in *Escherichia coli* W is a function of the carbon source. For example, growth on L-alanine causes a 25-fold higher level of alanine racemase when compared with growth on glucose. When potential inducers of this enzyme are added to either a glucose or succinate medium, a low specificity is observed with those compounds that cause higher levels of enzyme. Growth of *E. coli* W on either pyruvate, D-alanine, or L-alanine resulted in lower levels of L- and D-alanine in the internal pool. With each of these carbon sources, the level of alanine racemase was markedly elevated when compared to glucose-grown cells; thus, with single carbon sources, the concentration of alanine in the pool is inversely related to the specific activity of alanine racemase. These observations support derepression as a possible mechanism that gives rise to higher levels of alanine racemase. Since multiple forms of the alanine racemase were not detected in extracts from *E. coli* W grown on various carbon sources, it would appear that this type of heterogeneity is not a consideration in interpreting the above results.

Alanine racemase is the initial enzyme in the alanine branch of the pathway for the biosynthesis of uridine diphosphate (UDP)-*N*-acetyl-muramyl (MurNAc)-pentapeptide. This nucleotide and UDP-*N*-acetylglucosamine are precursors of peptidoglycan, the structural heteropolymer found in bacterial cell walls. In the alanine branch, the incorporation of D-alanine from L-alanine into the nucleotide precursor is catalyzed via the sequential action of alanine racemase (EC 5.1.1.1), D-alanine: D-alanine ligase (ADP) (EC 6.3.2.4), and UDP-MurNAc-L-ala-D-glu-L-lys: D-ala-D-ala ligase (ADP) (10). The key position of the racemase suggests that it might constitute a sensitive control point in the biosynthesis of UDP-MurNAc-pentapeptide.

Thornton and Johnson (Fed. Proc. **26**:843, 1967) and Rosso et al. (13) observed an increase in the specific activity of alanine racemase in *Bacillus subtilis* and *Pseudomonas putida* when cells grown on alanine as the sole carbon source were compared with cells grown on glucose. The increase in specific activity was attributed to induction.

In the present work, a 25-fold increase in the specific activity of alanine racemase in *Escherichia coli* was observed when alanine-grown cells were compared with glucose-grown cells. Our experiments with this organism implicate repression-derepression as a possible mechanism that gives rise to higher levels of alanine racemase. This paper presents two lines of evidence that support this proposal: (i) the low specificity with those compounds that cause high levels of alanine racemase and (ii) the low concentration of free alanine in cells with high levels of enzyme grown on either L-alanine, D-alanine, or pyruvate.

MATERIALS AND METHODS

Materials. L-[*1*-^{14}C]alanine (13.2 μCi/μmole) and D-[*1*-^{14}C]alanine (13.5 μCi/μmole) were products from the New England Nuclear Corp. L-Alanine and D-alanine were purchased from Cyclo Chemical Corp. Glutamic-pyruvic transaminase (EC 2.6.1.2, 27 units/mg) was the product of the Boehringer Mannheim Corp. D-Amino acid oxidase (EC 1.4.3.3, 2.3 units/mg) and catalase (EC 1.11.1.6, 7.1 $\times$ 10^4 units/mg) were purchased from Worthington Biochemical Corp. All other chemicals were reagent grade.

Growth of bacteria. *E. coli* W (ATCC 9637) was maintained in vacuo at −20 C. Bacteria were grown

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on the minimal salts medium described by Wargel et al. (17). Carbon sources were either 2×10^{-2} M glucose, 2×10^{-2} M sodium succinate, 2×10^{-2} M sodium pyruvate, 2×10^{-2} M glycerol, 5×10^{-3} M L-alanine, 5×10^{-3} M D-alanine, or 5×10^{-3} M DL-alanine. In several experiments, 10^{-3} M amino acid was added in addition to one of the above carbon sources. The cultures were grown at 37 C on a New Brunswick gyratory shaker until late-log phase was attained. The cells (~1.5 g) were harvested, washed, and suspended in 10 ml of 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 8.0 (4 C). Three drops of antifoam were added, and the cells were disrupted by shaking for 5 min with 9 g of washed glass beads (0.11 μm, Will Corp.) in a Bronwill mechanical homogenizer (Braun model, MSK, 4,000 cycles/min). The beads were removed by filtration, and the suspension was centrifuged at 14,000 × *g* for 10 min to remove unbroken cells and cell walls. The turbid supernatant fraction was stored at 4 C until assayed for activity and protein.

Measurement of pool sizes. *E. coli* W was grown on minimal medium with the indicated carbon source, harvested at late-log phase by centrifugation at 8,000 × *g* for 20 min, and washed with 0.02 M Tris-hydrochloride, *p*H 8.0. The cells (~0.1 g dry weight) were suspended in 6.0 ml of the above buffer, and a sample (0.50 ml) was taken for determination of dry weight. The remaining cells were extracted at 100 C for 10 min. The extracted cells were washed two times with 2 ml of boiling water. An equal volume of acetone was added to the extract; after 1 hr at 37 C, the precipitated material was removed. The acetone-treated extract was evaporated to dryness and redissolved in water. The amino acids in the extract were isolated by passage of the sample over a column of Dowex 50 (H^+). After the column was washed with 0.1 N HCl, the amino acids were eluted with concentrated NH_4OH. The solution was evaporated to dryness and dissolved in 0.2 N sodium citrate, *p*H 2.2. The amino acid profile was determined on a Beckman model 120 amino acid analyzer by the method of Spackman et al. (14). The above procedure was used to determine total alanine in the sample.

To determine L-alanine, a portion of the sample was treated with D-amino acid oxidase. The remaining alanine was separated from pyruvate by adsorption on Dowex 50 (H^+). The sample (1.0 ml) was incubated with a D-amino acid oxidase solution (40 μliters) containing 9.2 units of D-amino acid oxidase, 54 units of catalase, 0.4 nmole of flavine adenine dinucleotide and 0.4 μmole of sodium pyrophosphate buffer, *p*H 8.3. After 2 hr at 37 C, the reaction was terminated by addition of 0.5 ml of 0.2 N sodium citrate. The reaction mixture was then applied to the Dowex 50 (H^+) column and treated in the same manner as the sample used for total alanine determination. The difference in total alanine and in L-alanine was taken to be D-alanine.

Measurement of enzymatic activity. Alanine racemase was assayed in both directions by converting the product of the reaction to pyruvate. In the forward direction (L-alanine to D-alanine assay), D-alanine was deaminated with D-amino acid oxidase, and in the reverse direction (D-alanine to L-alanine assay), L-alanine was deaminated with glutamic-pyruvic acid transaminase. The ^{14}C-pyruvate was separated from the substrate by ion-exchange chromatography. The assays described by Lynch and Neuhaus (6) were used at 25 C. Both assays were linear with respect to time and enzyme concentration. A unit of enzyme is defined as that amount which will catalyze the formation of 1 μmole of product in 1 hr at 25 C.

Analytical methods. Protein was determined by the method of Lowry et al. (5). Measurements of radioactivity were made in polyethylene vials by use of a Tri-Carb liquid scintillation spectrometer (model 314-EX; Packard Instrument Co., Inc.). The scintillation fluid used was Triton X-100-toluene (1:2, v/v), as described by Patterson and Green (11) and evaluated by Benson (1).

RESULTS

Production of the enzyme. When *E. coli* W was grown on L-, D-, or DL-alanine as a carbon source, the specific activity of alanine racemase was higher than that in cells grown on glucose (Fig. 1). Growth of *E. coli* on L-alanine resulted in a higher specific activity than growth on either D-alanine or DL-alanine. The

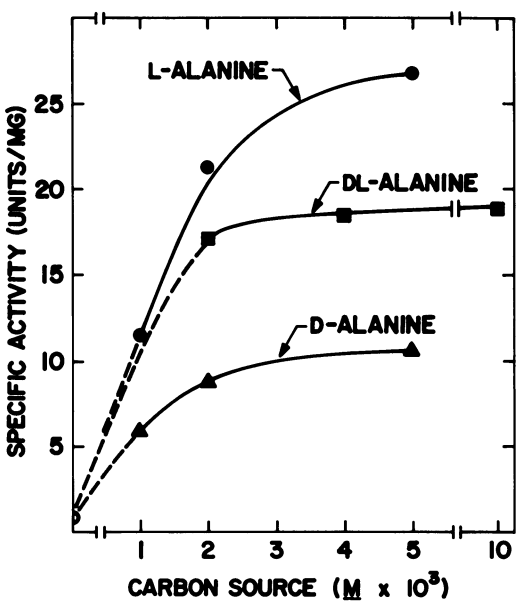

FIG. 1. *Effect of carbon source on the specific activity of alanine racemase. Bacteria were grown to late-log phase on the indicated amino acid: L-alanine (●), DL-alanine (■), and D-alanine (▲). The specific activities were determined as described in Materials and Methods. The specific activity of alanine racemase in cells grown on 2×10^{-2} M glucose is presented on the ordinate for comparison (○).*

effect of other compounds used as sole carbon sources is shown in Table 1. The level observed with pyruvate is in agreement with a similar observation made by Thornton (Ph.D Dissertation, University of Nebraska, Lincoln, 1967). Addition of L-alanine to the growth medium containing either glucose, pyruvate, glycerol, succinate, or aspartate caused a further increase in the specific activity of alanine racemase (Table 1). In the case of glycerol plus L-alanine, the specific activity was eight times as high as that with glycerol as a carbon source.

According to the model of Jacob and Monod (4), both induction and repression result from the specific interaction of compounds (agents) with either an aporepressor or a repressor. To establish specificity, compounds that might function as possible inducers were added to either a glucose or succinate medium, and the specific activity of alanine racemase from cells grown on that medium was measured. As illustrated in Table 2, many compounds cause a higher specific activity of enzyme when compared with cells grown on either succinate or glucose alone. For example, either L-α-amino-n-butyric acid or D-serine produces a four- to fivefold higher level of alanine racemase. The addition of D-alanine, however, results in only a two- to threefold higher level.

If alanine acts as an inducer, the intracellular concentration of this amino acid would be expected to increase when induction occurred. This proposal may be examined by measuring the free alanine pool under a variety of growth conditions. *E. coli* W was grown with the indicated compounds as the sole carbon source (Table 3). The cells were extracted with boiling water, and the amounts of D- and L-alanine in the extracts were determined. The selected carbon sources provided a wide range of specific activities of the racemase. As illustrated in Table 3, growth on either pyruvate, D-alanine, or L-alanine results in lower levels of L-alanine and D-alanine than does growth on glucose. For comparison, the specific activity of alanine racemase derived from growth on each carbon source is also listed in Table 3. From these data, it is concluded that the concentration of alanine in the pool is inversely related to the specific activity of alanine racemase.

TABLE 1. *Alanine racemase as a function of carbon source*[a]

Carbon source	Specific activity (units/mg)	
	A	B
Glucose	0.9	1.9
Pyruvate	3.3	6.7
Aspartate	1.1	5.0
Glycerol	1.6	12.4
Succinate	0.8	4.6

[a] In A, *E. coli* W was grown to late-log phase on the indicated carbon source (2×10^{-2} M). In B, 10^{-3} M L-alanine was added to the carbon source. The cells were harvested, and the specific activity of alanine racemase in cell-free extracts was determined. The "L-alanine to D-alanine assay" was used to measure activity.

TABLE 2. *Specificity requirements for the increased levels of alanine racemase*

Additions (10^{-3} M)[a]	Specific activity (units/mg)[b]	
	Glucose	Succinate
None	0.7	0.8
D-Alanine	1.3	2.6
D-α-Amino-n-butyric acid	1.2	3.9
D-Norvaline	0.6	0.7
D-Serine	3.3	3.6
D-Threonine	1.1	1.3
D-Cysteine	2.1	0.7
L-Alanine	1.9	4.6
L-α-Amino-n-butyric acid	3.3	4.2
L-Norvaline	1.2	3.7
L-Serine	1.2	2.3
L-Threonine	0.7	0.8
L-Cysteine	0.4	1.7

[a] Additions were made to cultures of minimal salts medium (1 liter) which were then inoculated with *E. coli* W grown on the specified carbon source (2×10^{-2} M). The cultures were harvested at late-log phase, and the specific activity was determined.

[b] Values are the average of at least two separate experiments except for D-cysteine, D-threonine, and L-threonine.

TABLE 3. *Concentration of free alanine in Escherichia coli W*

Carbon source	Total alanine[a] (nmoles/mg)	L-Alanine[b] (nmoles/mg)	D-Alanine (nmoles/mg)	Alanine racemase specific activity (units/mg)
Glucose	38.5	23.3	15.2	0.9
Pyruvate	17.6	13.9	3.7	3.3
D-Alanine	11.7	10.2	1.5	~10
L-Alanine	8.6	6.7	1.9	~26

[a] Determined from amino acid analysis.

[b] Determined from amino acid analysis after incubating the amino acid mixture with D-amino acid oxidase.

Thus, the following results are not consistent with induction as a mechanism of regulation: (i) the lack of specificity in the compounds causing higher levels of alanine racemase and (ii) the observation that the alanine concentration is significantly lower in cells with a high specific activity of alanine racemase than in cells with a low specific activity.

Investigation of multiple enzymes. Certain bacteria are known to produce multiple enzymes that catalyze the same reaction but differ in their regulatory properties. These enzymes are often elaborated at key control sites when a given enzymatic reaction is required for more than one metabolic pathway. Thus, it was considered essential to establish whether multiple forms of alanine racemase exist in *E. coli* and whether the proportion of these forms varied with different growth conditions.

Bacteria were grown on minimal salts medium with either glucose, D-, or L-alanine as the carbon source. Cell-free extracts were prepared, and selected physical properties of alanine racemase were compared. One of the physical parameters selected for study was the sedimentation of the enzyme in a sucrose density gradient. Alanine racemase from cells grown on each carbon source sedimented as a single band with identical velocities (Fig. 2).

The heat inactivation of alanine racemase from cells grown on glucose, D-alanine, or L-alanine was determined under a variety of conditions. Without dialysis, a wide variation in the rate of inactivation was observed. For example, the $t_{1/2}$ for the inactivation of alanine racemase from L-alanine-grown cells was 37.2 min, whereas the $t_{1/2}$ for alanine racemase from glucose-grown cells was 15.8 min, and from D-alanine-grown cells, 6.4 min. After dialysis of the extracts, however, there was no significant difference in the heat inactivation of the racemase in the three extracts. Over 90% of the activity of dialyzed extracts was lost upon exposure to 55 C for 5 min, whereas a maximum of 40% of the activity of undialyzed extracts was lost upon exposure to the same conditions. These observations suggested that dialysis removed a stabilizing agent for the racemase. The addition of either L- or D-alanine to the dialyzed extract protected the racemase from heat inactivation at 55 C. To standardize the conditions for heat inactivation, L-alanine was added to dialyzed extracts prepared from cells grown on different carbon sources to a final concentration of 10^{-2} M. As shown in Fig. 3, no significant difference in the rate of heat inactivation of alanine racemase was observed among the three extracts.

The third physical parameter to be compared for the alanine racemase from each carbon source was the mobility of the enzyme on polyacrylamide disc gel electrophoresis. No significant differences in the mobilities of alanine racemase from cells grown on different carbon sources were observed.

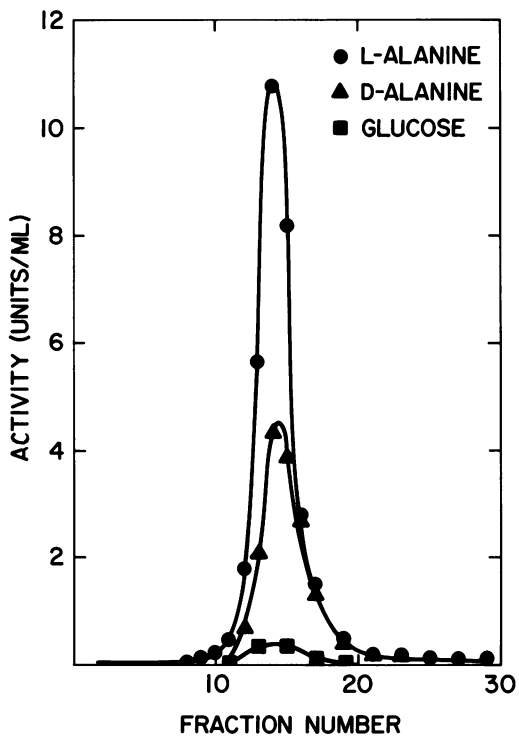

FIG. 2. *Sedimentation of alanine racemase in a sucrose density gradient. Samples (1.5 mg) of extracts prepared from* L*-alanine-grown cells* (●), D*-alanine-grown cells* (▲), *and glucose-grown cells* (■) *were centrifuged in separate gradients (5 to 40% sucrose) for 12 hr at 100,000 × g. Fractions (0.2 ml) were assayed for activity in the "* L*-alanine to* D*-alanine assay."*

DISCUSSION

In previous work on the regulation of alanine racemase in *B. subtilis* (M. P. Thornton and R. B. Johnston, Fed. Proc. **26**:843, 1967) and *P. putida* (13), the increase in the specific activity of the enzyme upon transfer to alanine as a carbon source was attributed to induction. The following observations are not consistent with induction as a mechanism of regulating the level of alanine racemase in *E. coli:* (i) there is a low specificity in those compounds that cause high levels of enzyme; (ii) the intracellular concentration of alanine is greatly lowered in cells with high levels of enzyme grown on either L-alanine, D-alanine, or pyruvate.

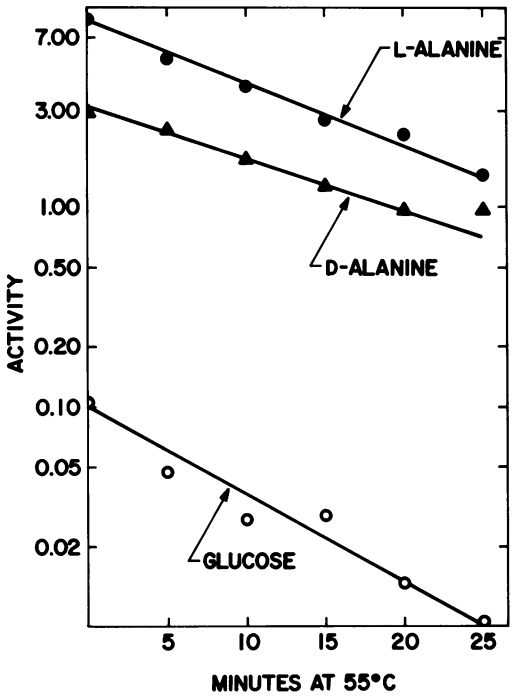

FIG. 3. *Heat inactivation of alanine racemase from various sources in the presence of* L-*alanine. Samples of extract from cells grown on the indicated carbon source were diluted to a final protein concentration of 1.0 mg/ml in 0.02* M *Tris (pH 8.0) and* 10^{-2} M L-*alanine was added. The diluted extracts were maintained at 55 C; samples were taken at the indicated times and assayed for activity with the "*L-*alanine to* D-*alanine assay."*

Three additional regulatory mechanisms that could result in elevated levels of alanine racemase are: repression-derepression (4); induction by a common inducer derived from multiple secondary inducers (2); and release from catabolite repression (7).

The first mechanism, repression-derepression with alanine as the co-repressor, is more consistent with our data. In this mechanism, repressor is synthesized as aporepressor which is activated by combination with the co-repressor alanine. *E. coli* grown on alanine has higher levels of racemase and lower intracellular concentrations of alanine than it has when grown on glucose. Therefore, the concentration of active repressor is low because the level of alanine is low; low levels of repressor result in the high levels of the racemase. An example of induction by a common inducer derived from multiple secondary inducers is the case of alanine dehydrogenase in *B. subtilis* (2). In this organism, induction by a broad spectrum of inducers was shown to be the result of conversion of many compounds to a common inducer, D-alanine. Since both D- and L-alanine decrease in *E. coli* with elevated levels of alanine racemase, it would appear that alanine cannot function as a common inducer. The possibility of pyruvate or another compound being a common inducer has not been eliminated.

In analyzing our data, the role of catabolite repression in the control of alanine racemase must be assessed. With those enzymes that are subject to catabolite repression, e.g., phosphoenolpyruvate carboxykinase, isocitratase, acid phosphatase, amylomaltase, and β-galactoside permease, Hsie and Rickenberg (3) observed a 10-fold higher specific activity of these enzymes when *E. coli* K-12 was grown on succinate instead of glucose as the carbon source. In the case of alanine racemase, no significant difference was observed with these carbon sources in the absence of L-alanine. However, when 10^{-3} M L-alanine is added to a glycerol medium, a higher level of racemase is observed when compared with cells grown on 10^{-3} M L-alanine and glucose. On the basis of the latter comparison, release from catabolite repression may be one factor in interpreting the enhanced levels of racemase.

In our interpretation, it is assumed that the observed changes in the alanine pool size reflect those changes at the site where alanine acts in controlling the level of alanine racemase. Compartmentation of alanine (two pools) has been described and the differences between alanine-grown cells and glucose-grown cells have been presented (17). The ratio of alanine in the two pools changes markedly when cells are grown on different carbon sources. The size of the alanine pool in glucose-grown cells is similar to the values reported by Mandelstam (8) and Raunio and Rosenqvist (12).

Although our results would seem to indicate derepression, we have not demonstrated repression of enzyme synthesis. This failure results from our inability to control the alanine level in *E. coli*. Attempts were made to alter the intracellular alanine level by growing *E. coli* on glycerol plus L-alanine. The concentration of alanine in these cells was approximately the same as in cells grown on glycerol (Lambert and Neuhaus, *unpublished data*). It appears that any lowering of the alanine level at the control site, as predicted by the increase in specific activity (Table 1), is masked by the high level of alanine in the cell. Thus, although we have correlated our results with control by repression-derepression, other control mechanisms may be operative.

In *B. subtilis*, Berberich et al. (2) proposed

that D-alanine does not regulate its pathway from L-alanine by repressing alanine racemase but, rather, by inducing L-alanine dehydrogenase. The increased level of L-alanine dehydrogenase converts the excess L-alanine to pyruvate. In this organism, the specific activity of alanine racemase is not affected by exogenous L- or D-alanine (2). In *Pseudomonas aeruginosa*, either D-alanine, D-valine, D-methionine, D-phenylalanine, or D-histidine induces the formation of a D-amino acid dehydrogenase (9). In contrast to *B. subtilis*, the level of D-alanine in *P. aeruginosa* is regulated by the induction of a dehydrogenase for the D-amino acid. In *E. coli*, we have not succeeded in demonstrating the presence of either L-alanine dehydrogenase or D-alanine dehydrogenase. Thus, the control mechanisms for alanine metabolism in these two organisms are clearly different from those that function in *E. coli*.

The production of multiple enzymes for the same reaction in the regulation of pathways has been reviewed by Stadtman (15). The possibility of regulatory isozymes of alanine racemase is of consequence because the racemase can be thought of as a key enzyme in both anabolic and catabolic pathways. The anabolic pathway is involved in the formation of D-alanine necessary for the biosynthesis of peptidoglycan. The catabolic pathway is involved in the conversion of D-alanine to L-alanine for the production of pyruvate. As in the case of threonine deaminase (16), there may be an enzyme specific for each pathway. Our observations on alanine racemase in extracts prepared from cells grown on different carbon sources do not indicate the existence of multiple forms.

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