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D-Alanine Oxidase from *Escherichia coli*: Localization and Induction by L-Alanine

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Dialyzed membranes of *Escherichia coli* prepared by an ethylenediaminetetraacetic acid-lysozyme method catalyze the oxidation of both L-alanine and D-alanine. The specific activities for the oxidations of both D-alanine and L-alanine are increased fivefold when the cells are grown in the presence of either L-alanine or DL-alanine, but are increased only slightly when grown in the presence of D-alanine. In the DL-alanine-induced system, the specific activities for the oxidations of some other D-amino acids are also raised. DL-alanine also induces two other alanine catabolizing enzymes, alanine dehydrogenase and alanine-glutamate aminotransferase which are found in the "soluble" fraction of lysozyme-treated cells. The oxidations of both L-alanine and D-alanine were associated with the membranes of induced cells. After the membranes were disintegrated by sonic treatment, both L-alanine and D-alanine oxidation catalysts sedimented in a sucrose density gradient together with D-lactate and L-lactate dehydrogenases, apparently as a single multienzyme complex.

Stephenson and Gale pioneered in studying factors which influence bacterial deaminations including amino acid oxidation. They claimed that in *Escherichia coli*, alanine deaminase was repressed by glucose and that the cell content was not affected by the presence of amino acids or by the age of the cell culture (23). Later it was shown in *Pseudomonas aeruginosa* that D-alanine oxidase was membrane bound and that it was an inducible enzyme with DL-alanine and DL-valine acting as inducers. The oxidation of D-alanine was linked to oxygen through a cytochrome system (17).

It has also been reported that other microbial enzymes metabolizing alanine are inducible. The nicotinamide adenine dinucleotide-dependent L-alanine dehydrogenase of *Bacillus subtilis* is induced by L-alanine. D-alanine and 11 other D-amino acids were also found to be effective inducers of L-alanine dehydrogenase when the cells had a racemase capable of converting the D-amino acids into their L isomers (3). Alanine racemase (EC 5.1.1.1.) was found to be an inducible enzyme in *Pseudomonas putida* and *E. coli* W when grown with L-alanine, D-alanine or DL-alanine (14, 21; M. P. Thornton and R. B. Johnston, Fed. Proc., p. 843, 1967).

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Although *E. coli* cell wall-membrane preparations are known to catalyze many oxidative processes, such as the oxidation of L-lactate and D-lactate, the amino acid oxidases do not appear to have been localized. In other organisms, discrete peroxisomes or microbodies have been found which, typically, contain catalase and a variety of hydrogen peroxide-producing flavoprotein oxidases including both amino acid and hydroxy acid oxidases (5, 10).

MATERIALS AND METHODS

Bacteria. *E. coli* strains B, B/r, ML30, K-12, and W were stock cultures obtained from the Department of Microbiology, Indiana University, Bloomington. In each instance, cells were transferred from a slant into a medium containing 30 mM dipotassium phosphate, 15 mM monopotassium phosphate, 0.5% tryptone, 0.5% yeast extract, and 2% glucose. After incubation at 37 C, cells were harvested by centrifugation and washed twice with 0.9% sodium chloride-saline solution. This inoculum was then transferred to a minimal medium in which the yeast extract and tryptone were replaced by 0.4 mM magnesium sulfate together with 0.1% ammonium sulfate. In various experiments, other compounds were added in concentrations up to 1% and glucose was replaced by 2% glycerol.

The cells were shaken in a Brunswick gyratory water bath shaker (model G76) until growth reached the stationary phase. The volume of medium was normally 20% of the total volume of the Erlenmeyer flask. The cells were harvested by centrifugation for

15 min at 12,000 × *g* and then were washed twice with saline.

Preparation of the membrane fraction (12). The cell mass obtained from *E. coli* grown in 50 ml of minimal medium was suspended in 2.0 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0, and then 7.5 μmol of Na_2-ethylenediaminetetraacetic acid and 80 μg of egg white lysozyme were added. The suspension was incubated at room temperature for 1 h and was then frozen and thawed twice to lyse the cells. The viscous solution was liquified by the addition of $MgCl_2$ (15 μmol) and deoxyribonuclease (DNase; 2 mg) followed by an additional 2 h of incubation at room temperature. This suspension was centrifuged for 30 min at 15,000 × *g*, and the membrane pellet, together with the supernatant solution if needed, then was dialyzed against 50 mM Tris-hydrochloride buffer, pH 8 (400 vol).

In some experiments, the pellet was resuspended in Tris-hydrochloride buffer and washed twice with buffer by centrifugation.

Enzymatic assays. D-alanine and L-alanine oxidases in these washed membrane fractions were assayed by measuring the pyruvate formed from 50 mM D-alanine or L-alanine in a 2.5-ml reaction medium. Results are given in terms of international enzyme units (U). The assay tubes were shaken intermittently during the reaction to ensure adequate aeration. The pyruvate was measured directly by the salicylaldehyde method (11).

L-alanine dehydrogenase was assayed by the procedure of Yoshida and Freese (24). L-alanine-L-glutamate aminotransferase activity was assayed by the method of Jenkins and Saier (11). D-lactate and L-lactate oxidase activities were assayed by the procedure of Barnes and Kaback (2) in which the anaerobic reduction of 2,6-dichlorophenolindophenol is measured spectrophotometrically at 620 nm.

Oxygen uptake was measured at 30 C with a Clark electrode in a YSI (model 53) oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). The overall reaction stoichiometry is reported in another paper (19).

Alanine uptake was measured by the method of Raunio, Elfving, and Lilius (18). Protein was measured by a modified biuret method with crystalline bovine serum albumin as a standard (15). In some cases, protein concentrations were estimated from the absorbancy at 280 nm. Linear 5 to 30% sucrose density gradients were made in 50 mM Tris-hydrochloride buffer, pH 8.3, and were allowed to equilibrate in a cold room before use. Centrifugation was subsequently carried out in a Spinco L3-50 ultracentrifuge at 4 C.

L-alanine, D-alanine, and DNase from bovine liver (300 Kunitz units/mg solid) were purchased from Sigma Chemical Co. D[−] lactic acid and L[+] lactic acid were obtained from Calbiochem as their calcium salts.

RESULTS

D-alanine oxidase activities in different E. coli strains. All *E. coli* strains when harvested in the late exponential growth phase on 0.5% DL-alanine in a minimal medium containing 2% glycerol as carbon source showed D-alanine oxidase activity in the cell-free membrane fraction. The specific activity values (expressed as units per gram of protein) were found to be 8.5 (strain B), 5.6 (strain B/r), 4.7 (strain K-12), 2.2 (strain ML30), and 2.0 (strain W). *E. coli* strain B was therefore chosen for all subsequent experiments.

Variations in oxidase activity during growth. Some enzymatic activities vary considerably during the varying bacterial growth phases. Figure 1 shows the specific activities for D-alanine oxidase during the growth of *E. coli* strain B on a medium containing 2% glycerol and 0.5% DL-alanine. Although there was a lag in enzyme production, relative to growth measured by the optical density at 660 nm, there was no marked change in the specific activity.

Induction of alanine oxidases. Different additions were made to cultures in minimal media, and the oxidation rates for both D-alanine and L-alanine were subsequently assayed (Table 1, Fig. 2).

L-alanine, but not D-alanine, increased the rates of oxidation of both D-alanine and L-alanine isomers (Table 1). The effects of both glucose and aeration appeared to be slight. The amount of D-alanine oxidase was maximal with 0.5 to 1% L-alanine; the degree of discrimination between inductions by D-alanine and L-ala-

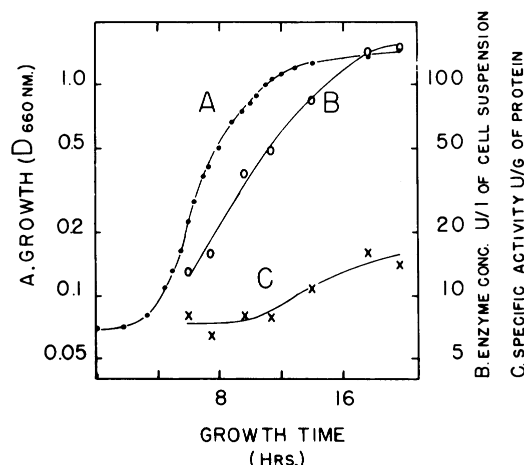

FIG. 1. *D-Alanine oxidase activity during the different growth phases of Escherichia coli B. The cells were grown in 1 liter of the minimal medium containing 2% glycerol as a carbon source and 0.5% DL-alanine. The culture was shaken in a gyratory water bath shaker at 37 C, and 10-ml samples were withdrawn at certain intervals in order to analyze D-alanine oxidase content in the cell-free membrane preparation. The inoculum cells had been grown in the same medium.*

TABLE 1. *The effects of different growth conditions upon the induction of L-alanine and D-alanine oxidases of Escherichia coli B*[a]

Additions and carbon source	Specific activity (U/g of protein)	
	D-alanine	L-alanine
Experiment 1		
Glucose (2%)	0.9	0.3
Glucose (2%) plus L-alanine (1%)	4.6	1.0
Glucose (2%) plus D-alanine (1%)	0.6	0.4
Glycerol (2%)	1.2	0.5
Glycerol (2%) plus L-alanine (1%)	4.2	0.7
DL-alanine (2%)	3.2	0.3
Experiment 2		
Glucose (2%)	0.7	0.2
Glucose (0.2%)	0.7	0.2
Glucose (0.1%)	0.5	0.2
Glucose (0.02%)	0.5	0.1
Glucose (2%), no shaking	0.3	0.1
Glucose (2%), plus L-alanine (0.5%)	7.3	2.3
Glucose (2%), plus Na-pyruvate (0.5%)	1.4	0.3
Glucose (2%), plus DL-alanine (0.5%)	5.2	1.1

[a] The cells were grown in minimal medium containing glycerol, glucose, or DL-alanine as a carbon source. The additions were made before inoculation of cells from yeast-extract-tryptone medium to 50 ml of the minimal medium. The flasks were shaken in a water bath at 37 C, and the cells were harvested at the end of exponential-growth phase. Dialyzed membrane fractions were analyzed for their alanine oxidase content.

nine was also then most marked (Fig. 2). This difference did not seem to be caused by permeability barriers, for several reports have shown that D-amino acids are transported into bacterial cells, including *E. coli* (13, 16). We measured the uptake of L-alanine and D-alanine cells of *E. coli* from cultures in the late exponential phase and found that the rate of uptake of D-alanine was 50% that of L-alanine. This observation, together with the fact that *E. coli* B grows better on D-alanine than on L-alanine, indicates that low permeability is not the cause of the relative ineffectiveness of D-alanine to act as an inducer. It is still possible, however, that the intracellular pool of D-alanine is compartmentalized, for one must postulate that D-alanine does not have free access to alanine racemase in order to explain the experimental observation that D-alanine is not as effective an inducer as is L-alanine.

Cells grown on DL-alanine, as the only carbon source, had a lower specific activity for amino acid oxidation than did cells grown with a combination of glucose with L-alanine (Table 1).

Burton proposed that there might be a correlation between high amino acid oxidase activities and a slow growth rate in *Neurospora* (4). We did not confirm this suggestion in *E. coli* when growth was restricted by reducing the glucose concentration (Table 1) or alternatively by raising the pH in the growth medium. Gale had proposed earlier that the formation of oxidases might be a detoxification mechanism at high pH values (9). However, the specific activity did not rise markedly during growth at high pH values (Fig. 3).

Induction of alanine oxidases by different L-amino acids. It was of interest to see which L-amino acids, other than L-alanine, show an inducing effect on D-alanine and L-alanine oxidases, for it has been shown that DL-valine induces D-alanine oxidase in *P. aeruginosa* (17). The results of this experiment (Table 2) showed that L-valine, L-isoleucine, and L-serine induced both L-alanine and D-alanine oxidases.

Specificity of the D-alanine oxidase. Because a variety of L-amino acids act as inducers, it was of interest to study the substrate specificity requirements for the D-amino acid oxidase. Table 3 shows that various other amino acids are oxidized besides D-alanine. There is no correlation shown, however, with the inducers

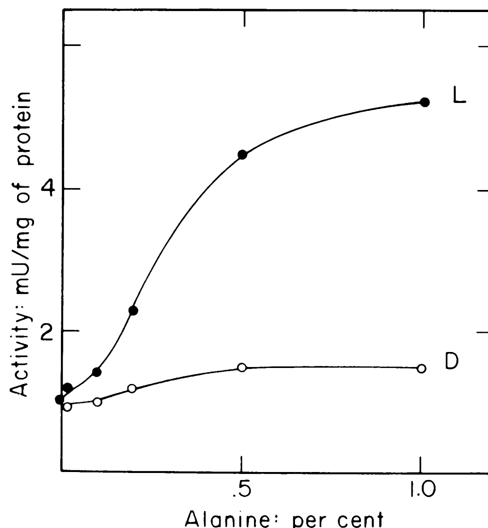

FIG. 2. *Effect of L- and D-alanine on the induction of D-alanine oxidase. The experimental conditions are the same as described in Table 1. L, L-alanine in the growth medium; D, D-alanine in the growth medium.*

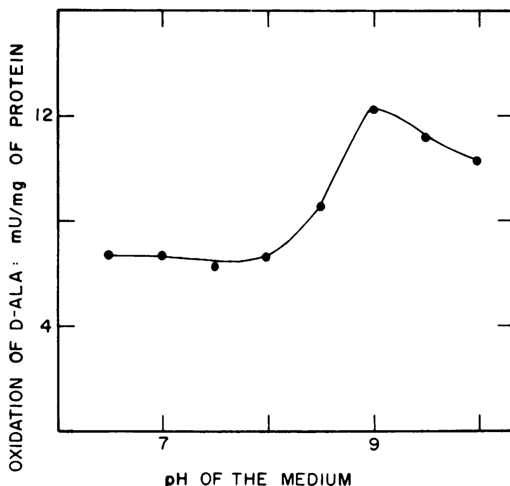

FIG. 3. *Variation of D-alanine oxidase activity in Escherichia coli B grown in media of different pH. The pH was kept constant by addition of KOH during the growth. The growth rate was lower above pH 9.*

shown in Table 2. Additional knowledge of the specificity requirements of the D-amino acid oxidase will depend upon a rigorous purification of the enzyme.

Induction of other alanine metabolizing enzymes. In order to understand the physiological significance of the changes in the activities of D-alanine and L-alanine oxidations by the membranes, it was necessary to measure the activities of two other enzymes capable of deaminating L-alanine: alanine-glutamate aminotransferase (together with glutamic dehydrogenase) and L-alanine dehydrogenase. No aminotransferase or dehydrogenase activities were found in the membrane fractions in *E. coli* B, but the activities were found in the supernatant fraction when the procedure outlined in the Materials and Methods section was followed. The specific-activity values presented in Table 4 show that only the L enantiomer of alanine induces the soluble L-alanine dehydrogenase.

Localization of alanine oxidases after disintegration of the membranes. When the membrane fraction was prepared from *E. coli* B by the EDTA-lysozyme procedure described earlier, usually more than 90% of the total activity was recovered in the membranes. Additional purification by disintegration of the membranes was successfully achieved only by using a 3-min sonic treatment at 0 C. About 80% of the total activities for both enantiomers was in fact recovered from the readily sedimentable membranes after such treatment.

The 15,000 $\times$ g supernatant solution, after disintegration of the membranes by a 3-min sonic treatment, was fractioned in a sucrose density gradient (5 to 30%) in Tris-hydrochloride buffer, pH 8.3. Because the activities were associated with hydroxy acid oxidases in other organisms, the fractions were assayed for both amino acid and hydroxy acids oxidases. The results (Fig. 4) show that L-alanine and D-alanine oxidases occur in the same fractions in the sucrose density gradient as do the enzymes which oxidize L-lactate and D-lactate. L-lactate and D-lactate, however, do not inhibit the oxidation of D-alanine so apparently distinct enzymes are involved. Furthermore, because the relative activities do not vary from fraction to fraction, the presence of a discrete multienzyme complex is indicated. The activities found in the bottom fractions were probably due to residual membranes, for if the membrane fraction was not sonically treated, all of the en-

TABLE 2. *Oxidation of D-alanine and L-alanine by cell membranes of Escherichia coli B grown in the presence of a single L-amino acid supplement to the minimal medium*[a]

Amino acid in the medium	Specific activity (mU/mg of protein)	
	D-alanine	L-alanine
Experiment 1		
None	0.6	0.2
L-Alanine	6.3	1.5
L-Valine	5.4	1.2
L-Isoleucine	2.1	0.6
L-Serine	1.1	0.3
L-Threonine	0.9	0.3
Glycine	0.4	0.1
L-Leucine	0.6	0.2
Experiment 2		
None	0.6	0.2
L-Glutamate	0.3	0.1
L-Methionine	0.7	0.2
L-Histidine	0.7	0.2
L-Aspartate	0.7	0.2
L-Phenylalanine	0.8	0.2
L-Tyrosine	0.4	0.1
L-Proline	0.9	0.3
Experiment 3		
None	0.7	0.2
L-Cysteine	1.0	0.3
L-Cystine	0.4	0.1
L-Lysine	0.6	0.2

[a] The minimal medium containing glucose as a carbon source and L-amino acid at 0.5 percent concentration was inoculated with the cells of *E. coli* B grown in yeast extract and tryptone medium. The oxidation of D-alanine and L-alanine was assayed from washed membrane fractions obtained after lysozyme-EDTA treatment from cells harvested in the retardation phase.

TABLE 3. *Oxidation of* D*-amino acids by cell membranes of Escherichia coli B grown in the presence or absence of 0.5%* DL*-alanine supplement to the minimal medium*[a]

Substrate[b]	Specific activity	
	Cells grown without inducer	Cells grown with 0.5% DL-alanine
D-Alanine	16	82
D-Asparagine	6	30
D-Aspartate	4	12
D-Methionine	3	21
D-Phenylalanine	19	35
D-Tyrosine	2	12

[a] Oxygen uptake was measured by using a Clark-type oxygen electrode, and the reaction mixture contained 20 mM D-amino acid in 50 mM Tris-hydrochloride buffer, pH 8.3, and 3 to 15 mg of membrane protein of *E. coli* B. The total reaction mixture was 3.0 ml, and the temperature was 30 C. The results are expressed as μliters of O_2 used per hour per milligram of protein.

[b] The activity of the following D-amino acids was below 5% compared with the oxidation rate of D-alanine: D-lysine, D-leucine, D-glutamate, D-glutamine, D-threonine, D-serine, D-isoleucine, D-cystine, D-valine, D-tryptophan, and glycine. The activity was tested from the cells grown on DL-alanine.

TABLE 4. *The effects of alanine additions to a minimal media containing 1% glucose or 2% glycerol on the* L*-alanine-glutamate aminotransferase and* L*-alanine dehydrogenase activities in Escherichia coli B*

Additions	L-Ala-glu aminotransferase[a]	L-ala dehydrogenase[a]
None, glucose as C-source	58	0
As above plus 1% L-alanine	143	9
As above plus 1% D-alanine	132	0
None, glycerol as C-source	292	0
As above plus L-alanine (1%)	332	17
As above plus D-alanine (1%)	266	0

[a] Results are expressed as milliunits per milligram of protein.

zymatic activities were found in the bottom fractions after only 2 h of centrifugation at 25,000 rpm with a Spinco SW-27 rotor which gave an average centrifugal field of 90,000 $\times$ *g*.

Catalase, which was present in the original membrane preparation, was liberated into the supernatant solution by sonic treatment, and the activity was found in the top fractions in the sucrose density gradient.

DISCUSSION

The original aim of this work was to investigate the possible connection between the oxidation of L-alanine and the oxidation of D-alanine. In mammalian, plant, and yeast cells two separate oxidases are involved, but they are associated with a single cytoplasmic organelle called a peroxisome or microbody (5, 10). Two approaches were therefore used. In one we studied the effects of changing the growth condition on the specific activities of the two oxidations and in the other we sought to localize the activities to a single multienzyme complex by selective disintegration of the cell membrane. Because the membrane fractions catalyzed the oxidation of L-alanine to pyruvate and ammonia, but did not contain significant amounts of either the alanine dehydrogenase or the alanine aminotransferase, one of three alternative pathways of oxidation seemed likely: (i) a racemase coupled to the D-alanine oxidase; (ii) an oxidase which was not stereospecific and which would oxidize both enantiomers, albeit preferring the D-isomer; and (iii) two separate amino acid oxidases which are known to occur in mammalian peroxisomes. All three alternatives are consistent with our finding that whenever the D-alanine oxidase activity is changed by induction (Tables 1 and 2), differential extraction, or by enzymic distributions in a sucrose density gradient (Fig. 4), the L-alanine

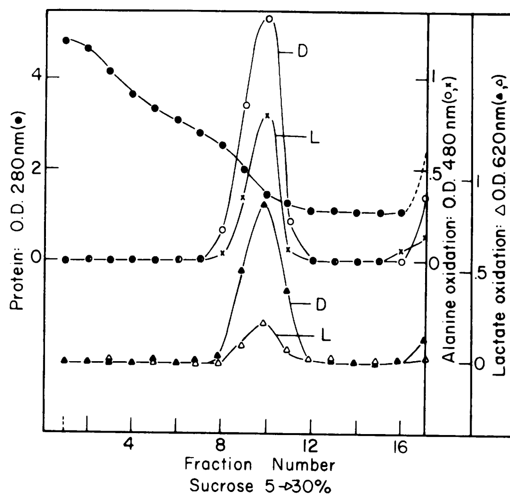

FIG. 4. *Localization of* D*-alanine,* L*-alanine,* L*-lactate, and* D*-lactate oxidase activities in a 5 to 30% sucrose gradient in 50-mM Tris-hydrochloride buffer, pH 8.3. One ml of the supernatant solution containing 3.2 mg of protein after sonic treatment of washed membranes of Escherichia coli B was layered on the top of the sucrose gradient and centrifuged at 4 C for 2 h at 25,000 rpm. Fractions (2 ml) were collected and analyzed for their enzyme and protein content. D,* D *isomer; L,* L *isomer.*

oxidase activity was of a comparable but lesser magnitude. Study of inhibitors and the reaction kinetics, however, indicates that only the first alternative is correct (19).

The most interesting feature of the induction experiments was the finding that D-alanine oxidase was induced by L-alanine but not by D-alanine. The induction by L-alanine is understandable, teleologically, if the D-alanine oxidase is involved in the oxidation of L-alanine. We are however, intrigued by the evolutionary and ecological significances of this finding that L-alanine controlled the rate of decomposition of its own enantiomer. It will be interesting to find out how general this behavior proves to be in other organisms.

The fact that L-alanine induces several enzymes which appear to be closely associated suggests that all of the enzymes of some specific organelle are being produced in a coordinated fashion as in yeast mitochondriogenesis (20).

Although in vivo studies showed that other L-amino acids besides L-alanine were effective as inducers of D-alanine oxidase, it is not presently possible to decide whether this is an indirect or a direct effect. For example, L-valine may itself be active or, alternatively, it may be acting indirectly by increasing the intracellular concentration of L-alanine by means of the valine-alanine aminotransferase known to be present in *E. coli* (1, 22).

We did not exclude the possibility that the increased enzymic activity might be due to a reduced rate of enzyme degradation, as suggested by Lambert and Neuhaus for the racemase (14), but we think that it is primarily the rate of enzyme synthesis which is being affected, for protein turnover is minimal in *E. coli* growing logarithmically.

The amino acid specificity of the D-alanine oxidase is interesting for two reasons. Firstly, it appears to be comparable to that of other *E. coli* enzymes acting on L-alanine, such as the transaminase B of Rudman and Meister for example (22). Another point of interest is that some of the best substrates have enantiomers which are ineffective as inducers (Tables 2 and 3).

The fact that sonic treatment was a successful method for the disintegration of the membranes, whereas other methods such as deoxycholate treatment only caused inactivation, suggests that the activities are not an integral part of the whole membrane matrix, but instead reside in a discrete particle or organelle loosely associated with the membrane. DeLey arrived at similar conclusions, isolating what he termed "oxidosomes" from *Acetobacter peroxydans* (6-8).

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