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D-Alanine Oxidase from *Escherichia coli*: Participation in the Oxidation of L-Alanine

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Cell wall-membrane preparations of *Escherichia coli*, prepared by the ethylenediaminetetraacetic acid-lysozyme method, contain enzymes which catalyze the oxidation of D-alanine and, to a lesser extent, L-alanine into pyruvate and ammonia without the formation of hydrogen peroxide. The kinetic parameters were (i) pH optima of 8.3 to 8.4 for L- and D-alanine and (ii) a K_m value of 6.6 ± 0.2 mM for D-alanine. Several coenzymes were without effect when added to the reaction mixture. The participation of D-alanine oxidase in the oxidation of L-alanine was demonstrated. The evidence is based on (i) results of cellular fractionation; (ii) labeling experiments; (iii) inhibition studies with aminooxyacetate and cycloserine; (iv) denaturation experiments; and (v) demonstration of the presence of an active racemase.

The physiological significance of the high concentrations of D-amino acid oxidase present in some mammalian cells has been an enigma, for the cells do not contain D-amino acids (5, 15). Bacteria, however, do contain D-amino acids as components of their cell walls (18). To make these amino acids, bacteria employ racemases and aminotransferases which are specific for D-amino acids (12, 23, 25). It is unlikely, however, that they use the reverse of these synthetic routes for the degradation of D-amino acids, for the cells contain a D-amino acid oxidase (7, 20, 21). Although it has long been assumed that D-amino acids are degraded directly, rather than by conversion to their L enantiomers, only recently has it been suggested that some L-amino acids in bacteria might be degraded indirectly by conversion first to their D enantiomers (1, 6, 19, 24).

In our previous paper characterizing the D-amino acid oxidase of *Escherichia coli* B we found that the enzyme was associated with a discrete membrane particle which would also, albeit less rapidly, degrade L-alanine (17). Because other enzymes, capable of degrading L-alanine, were absent from these membrane and particle preparations, we were able to investigate the possible participation of D-amino acid oxidase in the oxidation of L-alanine unequivocally.

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MATERIALS AND METHODS

Preparation of cell membranes. *E. coli* B was grown in a medium containing 0.5% DL-alanine as inducer (17). Washed cell membranes were made by using the lysozyme-ethylenediaminetetraacetic (EDTA) method of Kaback (4, 17). Usually more than 90% of the L-alanine and D-alanine oxidation activities were recovered in the membranes prepared by this method. No marked changes in the enzymatic activities were detected in these frozen membrane preparations during storage for several months.

Assay of enzyme activities. Four types of enzymatic assays were used for the oxidation of L- and D-alanine: measurement of oxygen uptake, pyruvate or ammonia production, and dye reduction. Oxygen uptake was measured with a Clark electrode in a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). When the reaction stoichiometry was investigated, the reaction mixture was aerated just before the assay, and the needle of the oxygen monitor was then adjusted to 100%. The concentration of dissolved oxygen in this reaction mixture was estimated to be 0.27 μmol per ml at room temperature (20 to 22 C).

Pyruvate was determined by using salicylaldehyde or 3-methyl-2-benzothiazolone hydrazone reagents (17, 22). In the isotopic assay radioactive pyruvate, formed from radioactive alanine in the deamination reaction, was separated from the excess substrate by using small columns of Dowex cation exchanger (200 to 400 mesh, AG50W-X16, 4.9 kg/g, dry weight, Bio-Rad Laboratories, Richmond, Calif.). Details of this method are given elsewhere (9). In the ammonia assay, ammonium ions were converted to ammonia gas by alkalinization in a diffusion bottle, and ammonia was then determined by using Nessler's solution (2).

In the dye reduction assay, 2,6-dichlorophenolindophenol was employed as an electron acceptor. The assay described by Norton, Bulmer, and Sokatch was followed (16).

The reaction mixture for the assay of alanine oxidase contained 50 mM L- or D-alanine, 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.35, and washed or dialyzed membrane preparation. The reaction was started by addition of enzyme solution, and perchloric acid or 10 N sulfuric acid in the isotopic assay (10%, vol/vol) were used to stop the reaction. The solutions were shaken during the reaction in order to provide oxygen for the oxidation. Alanine was replaced by water in a control incubation.

Protein was determined by the method of Mokrasch and McGilvery (14).

Materials. Aminooxyacetic acid hydrochloride was purchased from K and K Laboratories; sodium benzoate and sodium azide were from Matheson, Coleman, and Bell; L- and D-alanine, hog kidney, and D-amino acid oxidase (obtained as a crystalline suspension which oxidized 10 μmol of D-alanine per min per mg of protein in the presence of catalase, pH 8.3, at 25 C) were from Sigma Chemical Company; and D-cycloserine was from California Corp. for Biochemical Research. Radioactive L-alanine-^{14}C (U) and D-alanine-1-^{14}C were purchased from New England Nuclear Corp. All other materials were reagent grade obtained from commercial sources.

RESULTS

Dependence of alanine oxidation of pH. The pH optima for the oxidations of both D-alanine and L-alanine are shown in Fig. 1. Maximal activities were at pH 8.3 to 8.4 for both enantiomers; however, the ratio between D-alanine and L-alanine oxidation rates varied in the pH region tested. At the low pH values, the oxidation rate for L-alanine decreased relatively more than that for D-alanine.

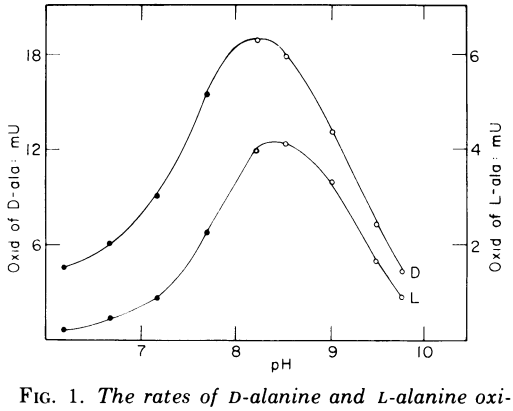

FIG. 1. *The rates of D-alanine and L-alanine oxidations at different pH values by using a dialyzed membrane preparation of Escherichia coli. The reaction mixture contained 25 mM L- or D-alanine in 25 mM sodium phosphate (●) or sodium borate (○) buffers, pH 6.1 to 9.8, and 4.5 mg of dialyzed membrane preparation. Pyruvate was assayed by the salicylaldehyde method. D, D-alanine as as substrate; L, L-alanine as substrate. The total reaction volume was 2.5 ml.*

Reaction rates. Pyruvate production from D-alanine and L-alanine during the initial reaction period with low substrate levels produced the results shown in Fig. 2.

A small lag was observed in the oxidation of L-alanine that usually lasted fewer than 10 min under these conditions. To test the hypothesis that this lag in L-alanine oxidation was due to racemase action, an experiment was carried out to see whether D-alanine was formed when L-alanine was incubated together with the membrane fraction in the absence of oxygen (Fig. 3).

This experiment showed the presence of an active racemase in the washed membrane preparation of *E. coli* B.

Effect of substrate concentration on

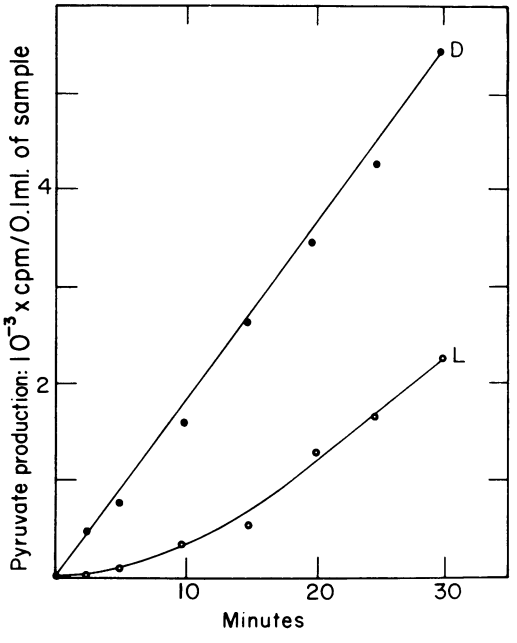

FIG. 2. *The production of pyruvate from D-alanine or L-alanine as a function of time. The reaction mixture contained 4.7 mM unlabeled D-alanine or unlabeled L-alanine, 4.9 μg of D-alanine-1-^{14}C (17.9 mCi/mmol), or 0.73 μg of L-alanine-^{14}C (U) (122 mCi/mmol) and 2.9 mg of washed membrane preparation from Escherichia coli B in 50 mM Tris-hydrochloride buffer, pH 8.35. The total reaction volume was 2.11 ml, and 0.1 ml-samples were withdrawn at certain intervals. The reaction mixtures were shaken manually during the reaction at 30 C. D, D-alanine as substrate; L, L-alanine as substrate.*

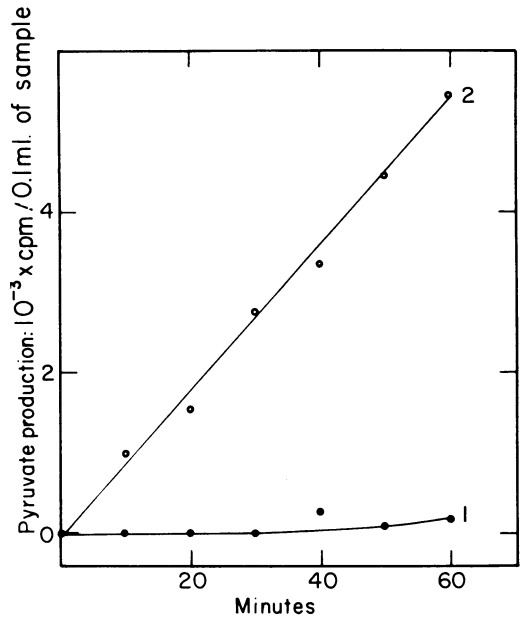

FIG. 3. *The conversion of* L-*alanine to* D-*alanine in the absence of air by an Escherichia coli B membrane preparation. The reaction mixture and the assays are the same as described in Fig. 2. The reaction mixture, containing* L-*alanine as a substrate, was deaerated by bubbling nitrogen gas through the mixture before the addition of the enzyme preparation as well as during the reaction. Two 0.1-ml samples were withdrawn at certain times. One was passed through an ion exchange column immediately, and the other was treated with 25 μg (5 μliters) of hog kidney* D-*amino acid oxidase preparation for 10 min before pyruvate assay. 1, Pyruvate production in anaerobic reaction mixture; 2, pyruvate production after treatment with an excess of kidney* D-*amino acid oxidase.*

activity. The reaction rates for the oxidations of D-alanine and L-alanine, measured after the initial lag, were determined at various substrate concentrations at pH 8.35 (50 mM Tris-hydrochloride buffer) (Fig. 4).

A Michaelis constant value of about 6.6 mM was computed for D-alanine. The maximum initial rate of D-alanine oxidation was about five times higher than the maximum rate for L-alanine; the racemase must therefore be the rate-limiting enzyme for the oxidation of L-alanine under these conditions.

No activation or inhibition of oxidation was found with either D-alanine or L-alanine as substrates when β-nicotinamide adenine dinucleotide (NAD), reduced form β-NAD, flavine-adenine dinucleotide, flavine mononucleotide, or pyridoxal 5′-phosphate in concentrations of 10^{-4} M were added to the reaction mixture before the start of the incubation. Activities were measured by either the salicylaldehyde assay or by measuring the conversion of radioactive alanine into pyruvate (pyridoxal-5′-phosphate and reduced from NAD experiments).

Cell membranes of *Pseudomonas aeruginosa*, prepared by the lysozyme-EDTA method, were reported also to catalyze the oxidation of D-alanine. The involvement of a cytochrome system was indicated by cyanide sensitivity, stoichiometry studies, and the appearance of reduced cytochrome bands in the isolated particles (16). In our dye reduction assay of L-alanine and D-alanine oxidases, cyanide appeared to increase the reduction rate. This apparent increase in the reduction rate, however, was an artifact due to the participation of a cyanide-sensitive cytochrome system in the aerobic reoxidation of the dye by the *E. coli* membranes. In the absence of redox dye, the oxidations of both D-alanine and L-alanine were, as shown later, notably cyanide resistant.

Stoichiometry of the reactions. Information as to the number of oxygen molecules consumed and pyruvate and ammonia molecules produced

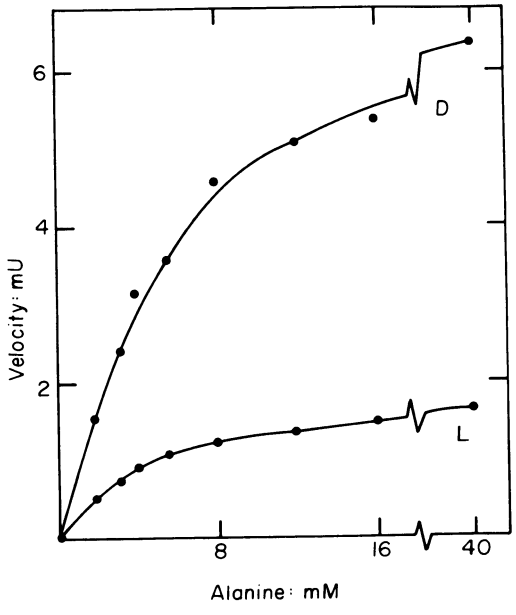

FIG. 4. *Effect of* D-*alanine and* L-*alanine on the velocities of* D-*alanine and* L-*alanine oxidations. The velocities were measured by the formation of pyruvate by using the salicylaldehyde assay. Alanine was added at a concentration of 2 to 40 mM to 2 ml of 50 mM sodium borate buffer, pH 8.4, and the reaction was started by addition of 3.0 mg of Escherichia coli B washed membrane preparation. The reaction time was 1 h at 30 C. D,* D-*alanine as a substrate; L,* L-*alanine as a substrate.*

during the reactions is of interest in elucidating further the nature of the oxidation and possible differences between L-alanine and D-alanine oxidations. Our results (Table 1) show that 1 mol of both pyruvate and ammonia was produced for each atom of oxygen utilized; the data were similar for D-alanine and L-alanine as substrates. Additions of catalase or catalase plus ethanol did not affect the stoichiometry. The lack of doubling of oxygen uptake upon the addition of ethanol indicates that H_2O_2 was not produced (10). Thus, if the enzyme is a flavoprotein, as is suggested by the dye reduction, it is not apparently autooxidizable to form H_2O_2. The membrane preparation did not degrade pyruvate.

Inhibition studies. In an attempt to find specific inhibitors for L-alanine and D-alanine oxidations, several known inhibitors of racemases and D-alanine oxidase were tested. In our system, by use of a washed membrane preparation from *E. coli* B, sodium benzoate, sodium azide, sodium borohydride, and potassium cyanide (at a concentration of 10 mM) were very weak inhibitors; the inhibition was less than 15%. The effect of these compounds was the same for both D-alanine and L-alanine oxidations.

The inhibitions caused by aminooxyacetate and the cycloserine enantiomers, which were more extensive, were studied more closely. The effects of these compounds on the oxidations of D-alanine and L-alanine are presented in Fig. 5.

Marked differences in the inhibitions of alanine isomer oxidations were obtained especially with aminooxyacetate and D-cycloserine (curves A to D). For L-alanine, 50% inhibition was obtained (curve B) with 2.2×10^{-6} M aminooxyacetate, whereas there was no inhibition of the oxidation of the D-isomer (curve A). When D-cycloserine inhibited the reactions, the oxidation of L-alanine was an order of magnitude (7×10^{-4} M versus 1.1×10^{-2} M) more sensitive to inhibition than was the D-isomer. L-Cycloserine was a weaker inhibitor than was D-cycloserine; values of 5×10^{-3} M (L-alanine) and 1.8×10^{-2} M (D-alanine) gave 50% inhibition.

When aminooxyacetate was added to reaction

TABLE 1. *Stoichiometry of oxygen uptake and pyruvate and ammonia production from D- and L-alanine by Escherichia coli B membrane preparation*[a]

Additions	Oxygen uptake[b]	Pyruvate production[c]	Ammonia production
D-Alanine	0.8	1.4	1.4
D-Alanine + 5 μg of catalase	0.7	1.3	1.3
As above + 0.1 ml of absolute ethanol	0.7	1.3	1.3
L-Alanine	0.6	1.0	1.2

[a] Values are expressed as micromoles per reaction mixture.

[b] The reaction took place in a sample chamber of YSI model 53 biological oxygen monitor at room temperature (20 to 22 C). The reaction mixture in a volume of 3.35 ml contained 50 mM D- or L-alanine in 50 mM Tris-hydrochloride buffer, pH 8.35, plus 5.3 mg of *E. coli* B membrane protein. The mixture in the chamber was aerated as described, and after consumption of about 70 to 90% of the oxygen, pyruvate and ammonia were assayed.

[c] Pyruvate was assayed by the 3-methyl-2-benzothiazolone hydrazone method (22).

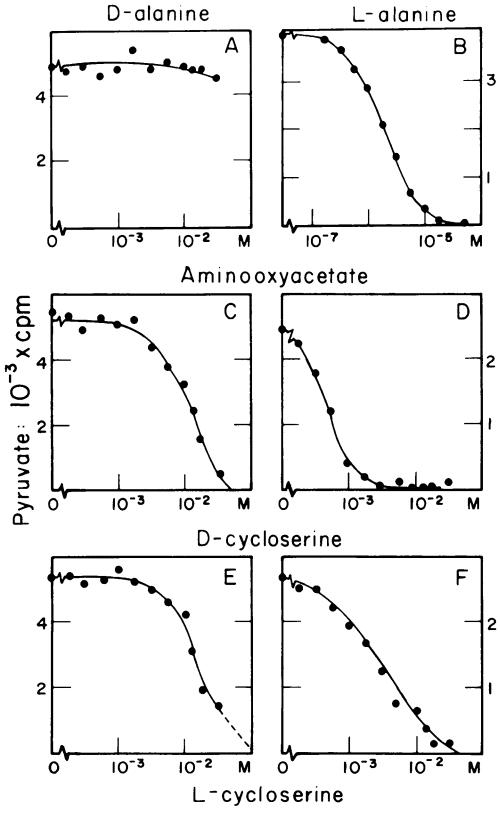

FIG. 5. *Inhibition of the oxidation of D-alanine and L-alanine catalyzed by Escherichia coli B membrane preparation by aminooxyacetate, D-cycloserine, or L-cycloserine. The reaction mixture in a volume of 0.5 ml contained 5 mM L- or D-alanine containing radioactive alanine, inhibitor, and 4.4 mg of washed membrane protein in 50 mM Tris-hydrochloride buffer, pH 8.35. After incubation at 30 C for 30 min (D-alanine) and for 60 min (L-alanine), the reaction mixture was analyzed for radioactive pyruvate. The addition of membrane preparation started the reaction. A, C, and E, D-alanine as a substrate; B, D, and F, L-alanine as a substrate.*

mixtures containing L-alanine as substrate, the progress curves showed that inhibition was not caused by an increased lag (cf. Fig. 2). Results confirmed that the racemase, which is very sensitive to aminooxyacetate (11), limited the oxidation rate of L-alanine (Fig. 6).

Competition between L-alanine and D-alanine. Isotope dilution in a metabolite derived from some radioactive precursor, upon the addition of an unlabeled hypothetical intermediate, helps to distinguish between alternate possible metabolic pathways. Figure 7 shows the results when the flow of carbon 14 from D-alanine and L-alanine to pyruvate was measured in the presence of unlabeled alanine enantiomer. It can be seen that the reduction of the radioactivity appearing in pyruvate from radioactive L-alanine is much higher than from radioactive D-alanine. This striking difference in the rates of appearance in pyruvate supports the hypothesis that L-alanine is oxidized via D-alanine; the reduced incorporation into pyruvate from D-alanine upon the addition of unlabeled L-alanine is probably due to the production of unlabeled D-alanine by the racemase.

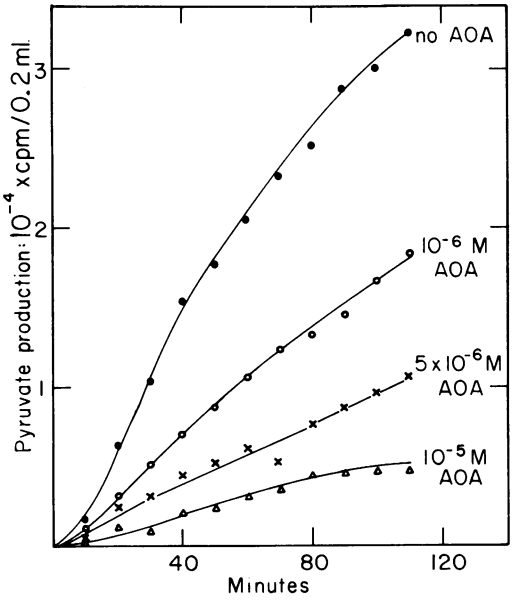

FIG. 6. *Effect of different concentrations of aminooxyacetate on the pyruvate production from* L-*alanine during the course of the reaction. The reaction mixture contained 5 mM radioactive* L-*alanine, aminooxyacetate (AOA) at concentrations of 0, 10^{-6}, 5 × 10^{-6}, and 10^{-5} M, and 5.2 mg of protein of washed Escherichia coli B membranes in 50 mM Tris-hydrochloride buffer, pH 8.35. Samples (0.2 ml) were withdrawn at certain intervals and analyzed for pyruvate content after excess amino acid was removed by cation exchange resin. The reaction temperature was 30 C.*

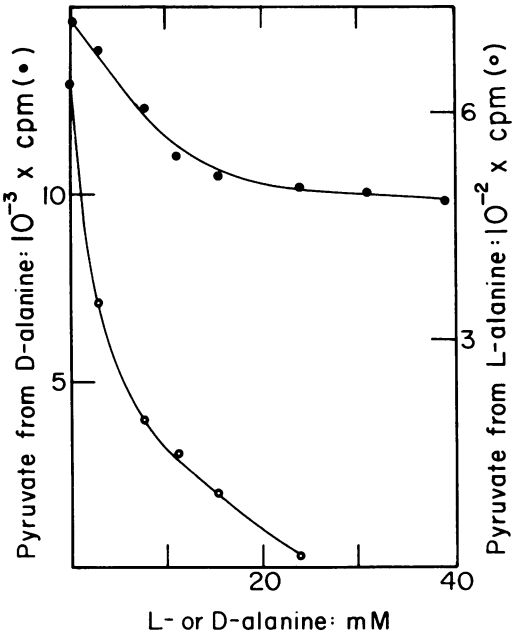

FIG. 7. *Effect of unlabeled* L- *or* D-*alanine on the formation of radioactive pyruvate from radioactive* D- *or* L-*alanine. The reaction mixture contained 6.2 mM cold* D- *or* L-*alanine, 55 nmol of* D-*alanine-1-^{14}C, specific activity 18 mCi/mmol, or 8.2 nmol of* L-*alanine-^{14}C (U), specific activity 122 mCi/mmol, in 50 mM Tris-hydrochloride buffer, pH 8.35, cold enantiomer of the substate, alanine, and 0.6 mg of Escherichia coli B membrane preparation. The total reaction volume was 0.26 ml, and the production of labeled pyruvate in that volume was estimated by using cation exchange column assay. The reaction at 30 C was started by addition of enzyme preparation and stopped after 30 min.*

Heat denaturation studies. Heat denaturation studies were carried out in the absence of substrate or any additional cofactors. The activity for L-alanine oxidation is slightly more heat sensitive than is the oxidation of D-alanine (Fig. 8).

Inactivation by deoxycholate treatment. The activities for L-alanine and D-alanine oxidations decreased similarly when the concentration of deoxycholate was increased (Fig. 9). This activity could not be recovered by dialysis.

DISCUSSION

Five different lines of evidence thus show that the oxidation of L-alanine by *E. coli* membranes proceeds with the participation of D-amino acid oxidase. (i) The L-alanine deaminase is associated with a discrete particle which contains both alanine racemase and D-amino acid oxidase. (ii) There is a short lag in the formation of pyruvate from L-alanine which is not observed

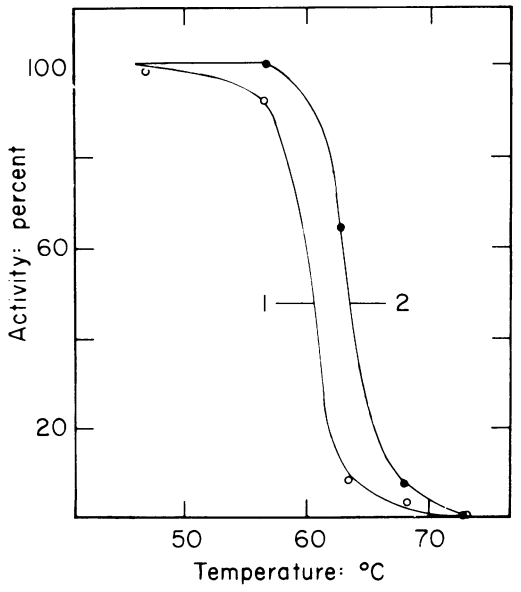

FIG. 8. *Heat inactivation of activities of* D-*alanine and* L-*alanine oxidations catalyzed by washed Escherichia coli B membrane preparation. The membrane preparation was divided into several samples in 50 mM Tris-hydrochloride buffer, pH 8.35, and they were incubated at different temperatures for 10 min and cooled in ice, and the rates of oxidation of* D-*alanine and* L-*alanine were measured by using salicylaldehyde. 1,* L-*Alanine as a substrate; 2,* D-*alanine as a substrate. The activities are expressed as percentage from the nontreated sample activity.*

when there is a preliminary anaerobic incubation to permit racemization. (iii) Certain inhibitors, especially known inhibitors of alanine racemase such as aminoxyacetate and D-cycloserine, specifically inhibit the oxidation of L-alanine without affecting the activity of the D-alanine oxidase. (iv) Addition of an excess of D-alanine reduces the conversion of radioactive L-alanine into pyruvate essentially to zero, whereas the addition of L-alanine reduces the conversion of radioactive D-alanine into pyruvate only to a limited level. (v) During induction or selective inactivation by either heating or deoxycholate treatment or simply by changing the pH, the L-alanine oxidation rate is always less than that of D-alanine.

Other pieces of evidence also indicate that the racemase is the rate-limiting enzyme in the oxidation of L-alanine by these membrane preparations. For example the rate of L-alanine oxidation was not increased by the addition of commercial pig kidney D-amino acid oxidase.

Sodium benzoate, sodium azide, sodium borohydride, and potassium cyanide, all known to be powerful inhibitors of the mammalian D-amino acid oxidase, were practically without effect on the oxidations of L-alanine and D-alanine by the *E. coli* membranes. There are reports in the literature showing similar results with other bacterial D-amino acid oxidases (7, 13, 16).

Aminooxyacetate and D-cycloserine inhibited the oxidation of L-alanine much more effectively than they inhibited the oxidation of D-alanine. Similar results were obtained with whole cells by Vyshepan, Ivanova, and Ledneva (24) and by Rosso, Takashima, and Adams (19) with whole cells and homogenates. It was these findings, primarily, which led them to suggest that L-alanine might be metabolized by conversion first into D-alanine. The purified alanine racemase from *E. coli W* showed, quantitatively, a similar response to inhibitors: aminooxyacetate was a very potent inhibitor, and D-cycloserine was slightly more effective than L-cycloserine (11). The D-alanine aminotransferase of *Bacillus subtilis* is most sensitive to D-cycloserine, whereas the L-alanine aminotransferase of pig heart is inhibited primarily by the L enantiomer (12).

Although there are many similarities between the particles we have isolated from *E. coli* cell membranes (17) and peroxisomes or micro-

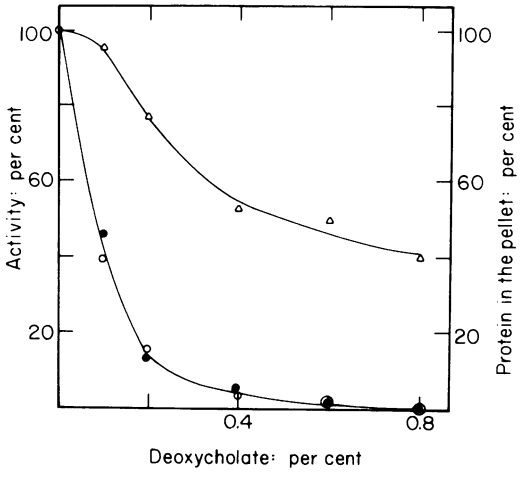

FIG. 9. *Treatment of washed cell membranes of Escherichia coli B with sodium deoxycholate. Samples of washed membranes in 50 mM Tris-hydrochloride buffer, pH 8.3, were incubated in the presence of 0.1 to 0.8% sodium deoxycholate, pH 8.3, for 30 min at room temperature. After incubation, the suspension was centrifuged at 15,000 × g for 30 min, and the supernatant solution was assayed for protein and alanine oxidase activity after dialysis against 4 liters of 50 mM Tris-hydrochloride buffer, pH 8.3, in a cold room.* ●, D-*Alanine as a substrate;* ○, L-*alanine as a substate;* △, *protein.*

bodies of other cells, there appear also to be some significant differences. Peroxisomes do not contain an amino acid racemase, but instead possess two distinct enzymes for the oxidation of L-amino acids and D-amino acids, respectively (3, 8). Furthermore, the peroxisome D-amino acid oxidase characteristically produces hydrogen peroxide which is then decomposed back to oxygen by the catalase associated with the particle. The bacterial particles appear instead to have an autoxidizable, cyanide-insensitive component coupled to a flavoprotein oxidase. The flavoprotein moiety can utilize redox dyes as acceptors but, unlike its mammalian counterpart, it is not apparently autoxidizable to yield hydrogen peroxide. Although it is present in the original membrane preparation, catalase was solubilized by the sonic treatment of the membranes used to prepare the oxidase particle. It is known, however, that catalase is readily removed from mammalian peroxisomes (3, 8). It was mentioned previously that the bacterial and mammalian oxidases appear to differ, especially when their sensitivity to inhibitors such as benzoate, cyanide, and azide is studied.

Our results and results published elsewhere (6, 19, 24) indicate that L-alanine is oxidized principally by conversion first into D-alanine. We consider it likely that this mechanism, involving a racemase and D-amino acid oxidase is also a general one for the oxidation of other amino acids. The metabolic pathway L-amino acid $\rightleftarrows$ D-amino acid $\rightarrow$ keto acid + NH_3 may serve also as a regulator to maintain intracellular pools of D-amino acids for cell wall biosynthesis (18) or conceivably may be involved in amino acid uptake, for it is associated with the particle-oxidizing lactate (4).

ACKNOWLEDGMENTS

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