Purified D-amino acid transaminase from *Bacillus sphaericus* catalyzes an α,β elimination from the D isomer of β-chloroalanine to yield equivalent amounts of pyruvate, chloride, and ammonia; the L isomer of chloroalanine is not a substrate for this transaminase. During the β elimination there is a synchronous loss in enzyme activity; the *K*<sub>inact</sub> for β-chloroalanine was estimated to be about 10 μM. The α-aminoacyl-Schiff base intermediate formed after β elimination of chloride ion is probably the key intermediate that partitions between one inactivation event for every 1500 turnovers. In the presence of D-alanine and α-ketoglutarate, which are good substrates for the transaminase activity of this enzyme, β-chloroalanine is a potent, competitive inhibitor (*K*<sub>i</sub> = 10 μM) with D-alanine and a weak, uncompetitive inhibitor with α-ketoglutarate.

Bacterial D-amino acid transaminase possesses strict stereochemical specificity but has a broad range in its side chain specificity for various n-amino acids (1-3). Thus, it is a major catalyst in the synthesis of bacterial n-amino acids and hence a potential target for the design of antimicrobial agents. Recently, we reported that the D isomer of β-chloroalanine was effective in inhibiting bacterial growth (4). We found that the pyridoxal phosphate enzymes, alanine racemase and n-amino acid transaminase, were almost completely inactive in extracts prepared from cells that had been treated with the chloroamino acid. We could demonstrate that β-chloroalanine was incorporated into the pool of free intracellular amino acids and that the amounts of the other free amino acids examined were not significantly affected. However, the free intracellular alanine in the treated cultures was predominantly of the L configuration, whereas the untreated cells contained almost exclusively the D isomer. These results were consistent with the specific inactivation of the enzymes that catalyzed the synthesis of alanine. However, it is possible that β-chloroalanine is first converted to highly reactive chloropyruvate and that this latter compound is the actual inactivating species (5, 6). Accordingly, it is desirable to study the reactions of β-chloroalanine with the purified alanine racemase and D-amino acid transaminase. Recently, Henderson and Johnston (7) reported that the D isomer of β-chloroalanine was a potent competitive inhibitor of purified alanine racemase from *Bacillus subtilis*. In the present study we report a modified procedure for the preparation of bacterial n-amino acid transaminase and the effects of β-chloro-D-alanine upon this enzyme.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—CM- and DEAE-cellulose (CM52 and DE52) were purchased from Whatman and were washed and equilibrated according to the directions of the manufacturer. The *L. lactis* (Bio-Gel HTP) was from Bio-Rad. Methanesulfonic acid (4 M) was obtained from Pierce. Lactic dehydrogenase, α-ketoglutarate, and pyridoxal 5'-phosphate were purchased from Sigma; D-alanine was obtained from Calbiochem. β-Chloro-D-alanine, which was from Fox-Vega, eluted just before aspartic acid on amino acid analysis at 34℃; no other amino acids were present. The color yield of chloroalanine with ninhydrin is 80% of that for leucine. Elemental analysis of chloroalanine hydrochloride gave:

C₄H₆ClNO₂·HCl

Calculated: C 22.50, H 4.49, N 8.76
Found: C 22.08, H 4.52, N 9.08

All other chemicals were reagent grade.

**Enzyme Assays**—For routine assay of D-amino acid transaminase activity during purification, the method described by Martinez-Carion and Jenkins (1), which measures the production of pyruvate with salicylaldehyde, was used with minor modifications. We used 0.3 M potassium phosphate, pH 8.5, as a buffer with 1 mM pyridoxal phosphate and 1 mM dithiothreitol. The concentrations of the substrates, D-alanine and α-ketoglutarate, were increased to 100 mM.

For kinetic studies, n-amino acid transaminase activity was measured by determination of pyruvate with lactate dehydrogenase and NADH. The reaction mixture contained 25 mM D-alanine, 25 mM α-ketoglutarate, 0.1 mM NADH, 70 μg/ml of lactate dehydrogenase, and about 0.05 unit of the transaminase in 0.3 M potassium phosphate, pH 8.5. The decrease in absorbance at 338 nm was recorded on a Beckman Gilford model 2220 spectrophotometer with the cell compartment thermostated at 37℃.

For some experiments, transamination was measured by the amount of 14C-glutamic acid formed from α-keto[14C]glutarate and amino acid substrate as described above. After the reaction, the 14C keto acid (New England Nuclear; 26,000 dpm/mmol) was separated from any formed 14C-glutamate on an amino acid analyzer equipped with a slow cell oscillation counter (Nucleo-Chicago). Protein concentrations for the purification procedure were determined by the absorbance at 280 nm; an extinction coefficient of 12.6 (A°<sub>280</sub>) was used as determined by Yanaka et al. (3). One unit of enzyme catalyzes the formation of 1 μmol of product per min at 37℃.

**Enzyme Purification**—D-Amino acid transaminase from *B. sphaericus* was isolated by modification of the method of Soda et al. (8). All gel filtration and chromatographic steps were performed at 4℃. When necessary the enzyme was concentrated by ultrafiltration at 4℃ in an Amicon cell with a UM-10 membrane.

Large batches (500 liters) of cells were grown at the New England Enzyme Center under the careful supervision of Dr. Stanley Charm and Mr. Henry Blair. We are grateful to Dr. Emil Gotlieb of *The Rockefeller University, New York, New York 10021*.

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Rockefeller University for growing 50-liter cultures of cells; the culture medium described by Soda et al. (8) was used for the preparations. The cells, which were suspended in 10 mM potassium phosphate, pH 8.5, and 1 mM dithiothreitol, were broken in a pressure cell. After treatment with protamine sulfate and ammonium sulfate as described by Soda et al. (8), a gel filtration step on Sephadex G-100 (5 x 75 cm) was introduced in the purification procedure. The enzyme was eluted between 650 and 800 ml of buffer on the descending limb of a large peak of protein from the breakthrough volume of the column (300 ml).

Chromatography on DEAE cellulose was performed on a column (2 x 30 cm) of the resin equilibrated with 10 mM potassium phosphate, pH 8.5, 1 mM dithiothreitol. The column was developed with a linear gradient from 10 to 200 mM dithiothreitol with a total volume of 600 ml; the enzyme was eluted between 250 and 300 ml of buffer just ahead of a protein contaminant. In order to preclude any of this impurity in the enzyme preparations that were used for structural studies, we divided the enzyme into two pools. Fraction A had a specific activity of 160; Fraction B had a specific activity of 110. Our final yield of pure enzyme (11%) was about the same as in the procedure of Soda et al. (8).

Gel Electrophoresis - Electrophoresis in 10% cross-linked gels in the presence of sodium dodecyl sulfate was performed by the method of Weber and Osborn (9). Gels (6 x 7 cm) that contained about 100 µg of protein were subjected to electrophoresis for 5 to 6 h at 8 mA/gel until the tracking dye (bromphenol blue) was about 1 cm from the end of the gel. Staining of the protein bands with Coomassie blue and removal of excess dye from the gel were carried out as described by Weber and Osborn (9).

Amino Acid Analysis - Hydrolysis in 6 N HCl was carried out in vacuo at 110° for periods of 20 and 72 h (10). Hydrolysis in methanesulfonic acid was performed in vacuo for 20 h at 110° (11). The amino acid composition of each hydrolysate was determined on an analyzer with a 0.28-cm diameter column as described by Liao et al. (12). Determination of half-cystine as cysteic acid was accomplished by the procedure of Moore (13).

Other Assays - Keto acids were identified as their 2,4-dinitrophenylhydrazones (14); spectra were recorded on an Amino DW-2 spectrophotometer. Analysis for chloroacetic acid was performed by the Volhard method (15) after removal of proteins with 5% trichloroacetic acid. The concentration of ammonia was determined with Nessler's reagent after neutralization of the deproteinized solution with NaOH.

RESULTS

Some Properties of D-Amino Acid Transaminase from Bacillus sphaericus - Fraction A of the preparation from B. sphaericus is about 95% homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1) and has a subunit molecular weight of about 30,000; the native enzyme is dimeric (3). The specific activity of Fraction A is somewhat lower than the value of 110 reported for the crystalline enzyme from the same organism by Soda et al. (8), perhaps because we used higher substrate concentrations that were saturating for the duration of the assay. The amino acid composition of the enzyme (see below) was performed on Fraction A. Fraction B, which is about 70% pure, was used for the kinetic experiments with β-chloro-L-alanine.

The amino acid composition of the transaminase from Fraction A is given in Table I. The use of methanesulfonic acid as described by Simpson et al. (11) has permitted the determination of tryptophan in the sample. The value of 8 for the half-cystine residues in the molecule was found both after oxidation to cysteic acid (13) and after derivatization to S-sulfoxycysteine (11). Yonaha et al. (28) found that this transaminase had four sulfhydryl groups that were titratable by 5,5'-dithiobis(2-nitrobenzoic acid). The other four sulfhydryl groups must either be present as disulfide bridges or be inaccessible to the titrant.

Reaction of β-Chloro-L-alanine with D-Amino Acid Transaminase - The isomer of β-chloro-L-alanine is a substrate for the transaminase in the absence of a co-substrate α-keto acid...
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These properties are absent in the derivative formed with the keto acid product produced from β-chloro-D-alanine. A similar calculation for transamination between D-alanine and α-ketoglutarate shows that about 1350 nmol min⁻¹ of product are formed. Therefore, the transaminase is about 1 order of magnitude less efficient in catalyzing β elimination than it is in catalyzing transamination. A similar ratio of catalytic efficiency has been found previously for β-chloro-L-glutamate (19) and β-chloro-L-alanine (18) with glutamate-aspartate transaminase and for β-chloro-L-alanine and aspartic acid β-decarboxylase (17).

From the data in Fig. 4, it is also possible to calculate the number of turnovers of β elimination per molecule of enzyme inactivated. At 50% inactivation, about 35 nmol of keto acid have been formed. Since there were 42 pmol of active enzyme in this experiment, we calculate that about 1500 turnovers of β elimination take place for each inactivation event.

![Absorption spectra of the 2,4-dinitrophenylhydrazone derivatives of the keto acid formed as a function of the time of incubation with D-amino acid transaminase. The samples for derivatization were taken from the experiment described in Fig. 2. ---, spectrum of the derivative of authentic pyruvate.](image)

**TABLE II**

Lack of transamination of β-chloro-D-alanine with D-amino acid transaminase

Each reaction mixture contained 1 mM α-keto[¹⁴C]glutarate (36,000 dpm/mmol), 0.1 mM NADH, lactate dehydrogenase (100 μg/ml), and D-amino acid transaminase (3 μg/ml) in 0.1 M potassium phosphate, pH 8.5. Separate incubations were carried out for the designated times with β-chloro-D-alanine (0.75 mM) and D-alanine (2.5 mM) at 25°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incubation time</th>
<th>[¹⁴C]Glutamate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Chloro-D-alanine</td>
<td>30 min</td>
<td>0 dpm</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>3 min</td>
<td>68,000 dpm</td>
</tr>
</tbody>
</table>

* If ketoglutarate had a role in the inactivation by chloroalanine as it does with vinylglycine (20), the theoretical amount of [¹⁴C]glutamate would be about 14,000 dpm. This value is based on the fact that the V_max for β elimination is one-tenth that of normal transamination and that the enzyme is about 50% inactivated after a 3-min exposure to chloroalanine (Fig. 4). Since we could have detected 700 dpm of [¹⁴C]glutamate, we can set the upper limit of any transamination of β-chloroalanine at about 5%.

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Fig. 2. Substrate utilization and product formation from β-chloro-D-alanine catalyzed by D-amino acid transaminase. At the indicated times during the reaction, samples were removed and assayed for chloroalanine (○) by amino acid analysis, keto acid (●) as the 2,4-dinitrophenylhydrazone derivative, chloride (□) by the Volhard method, and ammonia (■) with Nessler's reagent. At 15 min the enzyme was 96% inactivated.
Kinetics of the Inactivation of D-Amino Acid Transaminase by β-Chloro-D-alanine—The profile of the inactivation of the D-amino acid transaminase by β-chloro-D-alanine fits a pseudo-first order plot (Fig. 5, open circles); dialysis of the inactive enzyme does not restore activity. D-Alanine affords almost complete protection against inactivation (open triangles), whereas the L isomer of alanine offers little protection (closed triangles). The L isomer of β-chloroalanine does not inhibit D-amino acid transaminase. In contrast to the potentiality by D-ketogluutarate of the inactivation of the D-amino acid transaminase by vinylglycine (20), this keto acid is not necessary for the inactivation of the enzyme by β-chloro-D-alanine (Fig. 5, closed circles).

The pseudo-first order kinetics of inactivation shown in Fig. 5 suggest that the inactivation proceeds via a preformed enzyme-inhibitor complex.

$$E + I \rightarrow E + I_{\text{free}} \rightarrow E_{\text{inact}}$$

Preliminary experiments to determine the inactivation rate constant for β-chloro D-alanine indicated that this value was below 0.1 mM. Since the ratio of turnover to inactivation is high (approximately 1500), studies with these concentrations of inhibitor are not experimentally feasible because there would be only a small fraction of the enzyme inactivated with consumption of a major portion of the chloroamino acid. Therefore, we carried out the experiments in the presence of concentrations of D-alanine in the range of its K_m value to ensure that pseudo-first order kinetics for the inactivation by β-chloro-D-alanine (Fig. 5, closed circles).

The double reciprocal plot of the pseudo-first order rate constant for the inactivation of D-amino acid transaminase as a function of β-chloro D-alanine concentration. The experiments were carried out in the presence of either 1 mM (C) or 4 mM (○) D-alanine in 0.3 M potassium phosphate, pH 8.5, bovine serum albumin (0.1 mg/ml), and the indicated concentrations of β-chloro-D-alanine at 25°C; at time zero the transaminase (3.0 µg/ml) was added. At convenient intervals during the first 4 min, the reaction was terminated by addition of portions (100 µl) to a standard mixture for kinetic assays (2.7 ml) described in the text but without D-ketogluutarate or lactic dehydrogenase; the saturating concentrations of D-alanine served to stop the β elimination reaction until all of the aliquots had been removed. The residual activity was then determined immediately after addition of 0.1 M each of ketogluutarate (750 mM) and lactic dehydrogenase (2.5 mg/ml) and assayed as described under "Experimental Procedures."
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**Discussion**

The β-chloro derivatives of L-glutamic acid, L-α-aminobutyric acid, and L-alanine are substrates for the respective pyridoxal phosphate enzymes that carry out catalysis with the parent L-amino acids (17–19, 23). However, the β-chloro compounds direct the reaction specificity of the enzyme (19), since they are converted catalytically to chloride, ammonia, and the corresponding α-keto acid. With the 4- and 5-carbon substrates and L-glutamate-L-aspartate transaminase, the reactions go to completion and the enzymes are completely active subsequently (19, 23). However, for the 3-carbon substrates with good leaving groups at C-3 such as β-chloroalanine (17, 18) and serine O-sulfate (24), the enzyme is slowly inactivated during the process of α, β elimination. There is evidence that the enzyme-bound aminoacrylic acid formed after β elimination of the anion from C-3 inactivates the enzyme by alkylation of an essential active site residue (25, 26). This type of behavior in substrates that can be converted catalytically to a product that inactivates the enzyme has been termed "suicide substrate" for the flavin enzymes (27). The scheme shown in Fig. 9, which is similar to that proposed for the L isomer of β-chloroalanine and other pyridoxal phosphate enzymes (25, 26), is a possible mechanism for the inactivation of the amino acid transaminase by β-chloro-D-alanine that is consistent with the data in the present communication.

The β-chloroalanine-Schiff base (I) first loses the α-proton to form the ketoamine form of the coenzyme (II). Although we cannot at this time rigorously exclude intermediate II as the species that alkylates the enzyme, it seems more likely to us that the aminoacrylate-Schiff base (III) is the key intermediate because of the linear relationship between released keto acid, remaining active enzyme (IV), and inactive enzyme (V). When the keto acid product of chloroalanine is released, the enzyme is in the pyridoxal form (IV), for immediate reaction with more chloroalanine, consistent with the observation that α-keto acids are not required for inactivation. The identification of the group on the enzyme that is alkylated is the subject of current investigation.

The results described in this communication indicate that β-chloro-D-alanine can act upon isolated D-amino acid transaminase in two ways. At high concentrations of enzyme and low amounts of D-alanine, the enzyme is irreversibly inactivated. At low concentrations of the enzyme and high amounts of D-alanine, the chloroalanine is a potent competitive inhibitor. However, since the milieu within the intact microorganism may differ from our experimental conditions, it remains to be determined which of these modes contributes to the antimicrobial action of β-chloro-D-alanine.

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