β-Elimination of β-Halo Substrates by D-Amino Acid Transaminase Associated with Inactivation of the Enzyme. Trapping of a Key Intermediate in the Reaction†

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ABSTRACT: D-Amino acid transaminase from *Bacillus sphaericus* catalyzes a β-elimination reaction with either β-bromo-D-alanine or β-bromopyruvate; a D-amino acid cosubstrate is required only with the latter compound. During the course of the reaction, the enzyme is gradually inactivated in proportion to the amount of each substrate processed. The turnover/inactivation ratio is about 350 for β-bromo-D-alanine at pH 7.0 and about 600 for β-bromopyruvate at pH 8.5. When small thiols such as β-mercaptoethylamine are present the enzyme is protected from inactivation, substrate is still consumed, but pyruvate is not produced. The major product of this trapping reaction with this thiol is *S*-(β-aminoethyl)cysteine and a minor product is Δ^1-thiomorpholine-2-carboxylic acid. The former derivative was found to be the D isomer—a result which indicates that the trapping is stereoselective and occurs at the active site of the transaminase. These results, taken together with the finding that large thiols do not protect the enzyme from inactivation, probably because they cannot penetrate the active site, provide direct evidence for an α-aminoacrylate–Schiff base intermediate in the β-elimination reaction.

Initial studies on the antibacterial properties of β-chloro-D-alanine suggested that it acts by inactivating the enzymes that catalyze the formation of D-amino acids that are essential for bacterial growth (Manning et al., 1974). The finding that the inhibition of bacterial growth by the D isomer of β-chloroalanine could be completely reversed by D-alanine supported this idea and further suggested that the D isomer of β-chloroalanine had a limited number of target enzymes. Conversely, we found that the antibacterial effect of the L isomer of β-chloroalanine could be only partially reversed by high concentrations of L-alanine, and Arfin & Koziell (1971) found that inhibition was reversed completely by L-isoleucine and L-valine. These results indicated that some of the enzyme systems for L-amino acids had been inactivated by β-chloro-L-alanine (Relyea et al., 1974; Morino & Tanase, 1978).

Recent studies on D-amino acid transaminase (Soper et al., 1977b) and alanine racemase (Henderson & Johnston, 1976; Wang & Walsh, 1978), purified from bacterial sources, have provided some insight into the mechanism of inactivation of these enzymes by β-chloro-D-alanine. With purified D-amino acid transaminase, β-chloro-D-alanine undergoes β elimination to yield pyruvate, ammonia, and chloride; one enzyme molecule is inactivated for every 1500 turnovers of substrate at pH 8.5, presumably by an α-aminoacrylate–Schiff base intermediate (Soper et al., 1977b; cf. Figure 7). However, an alternative explanation for the inactivation was by alkylation of the enzyme by undetectable amounts of β-chloropyruvate formed during the reaction; such a mechanism would not require alkylation by the putative α-aminoacrylate–Schiff base intermediate. In the present communication we test both of these possibilities by studies with the potent alkylating agent, β-bromopyruvate, and by experiments with an added scavenger, β-mercaptoethylamine, in attempts to trap the putative pyridoxyl–aminoacrylate intermediate.

Experimental Procedures

Enzymes and Reagents. D-Amino acid transminase was purified from *Bacillus sphaericus* ATCC 14577 as described previously (Soper et al., 1977b). The pure enzyme has a specific activity of 150 μmol of pyruvate per min per mg of protein at 37 °C when assayed by the method of Martinez-Carrion & Jenkins (1965). Lactate dehydrogenase, malate dehydrogenase, NADH, α-ketoglutarate, β-bromopyruvate, β-hydroxypyruvate, sodium borohydride, mercaptoethylamine, and D-amino acid oxidase were purchased from Sigma; D-alanine and *S*-(aminoethyl)-L-cysteine were obtained from Calbiochem. The isomers of β-chloroalanine were from Vega/Fox. L-Amino acid oxidase was from Worthington. β-Bromoethylamine was purchased from Eastman. L-Leucine *N*-carboxyanhydride was obtained from Miles/Yeda and from Vega/Fox. β-Bromo-α-ketobutyrate was purchased from Adams Chemical Co. All other materials were reagent grade.

Preparation of β-Bromoalanine. β-Bromo-D,L-alanine was prepared from β-chloro-L-alanine by a modification of the procedure of Dang et al. (1976); these workers showed that racemization occurs during the synthesis. β-Chloro-L-alanine (0.6 mmol) was dissolved in 2 mL of 30% (w/w) anhydrous HBr in acetic acid and incubated in a sealed tube for 5 h in a boiling water bath; nearly equivalent amounts of β-bromo- and β-chloroalanine were present at this time. The yields were decreased by heating for longer periods. The solvent was removed by evaporation at 40 °C and the brownish residue was extracted with ethyl acetate. The remaining white solid was dissolved in 0.2 N sodium citrate, pH 2.2, and β-bromoalanine, which eluted at 68 mL with pH 3.25 buffer, was separated from unreacted β-chloroalanine, which eluted at 60 mL, in two applications to the 0.9 × 60 cm column of the amino acid analyzer (Spackman et al., 1958). The β-bromo-D,L-alanine thus isolated (0.1 mmol) was >95% free of other amino acids. Attempts at chromatographic desalting on Dowex 2 to remove sodium citrate led to decomposition of β-bromoalanine. Solutions of this haloamino acid in citrate were adjusted to the desired pH before use.

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Enzyme Assays. D-Amino acid transaminase was assayed by coupling the keto acid product to the appropriate dehydrogenase as described previously (Soper et al., 1977b). Since β-bromopyruvate is a substrate for lactate dehydrogenase, we employed malate dehydrogenase to measure the production of oxaloacetate from aspartate in the presence of β-bromopyruvate.

Synthesis of the D Isomer of S-(β-Aminoethyl)cysteine. S-(β-Aminoethyl)cysteine was synthesized by a modification of the procedure of Cavallini et al. (1955). D-Cysteine (1 g) was dissolved in 10 mL of water and the solution was bubbled with nitrogen for 1 h. NaOH (1.2 g) in 0.2 mL of H_2O saturated with nitrogen and then 1.4 g of β-bromoethylamine was added; the solution was stirred at room temperature for 3 h. The solution was desalted on a Dowex 2-X8 column by the procedure of Drèze et al. (1954). The ninhydrin-positive material was eluted with 1 N acetic acid and concentrated to dryness on a rotary evaporator. The yellow-orange oil was dissolved in water and the solution was adjusted to pH 2. The sample was then applied to a Dowex 50-X8 column (9 × 30 cm) and was eluted with a linear gradient of from 1 to 4 N HCl. The total volume of the gradient was 800 mL. S-(β-Aminoethyl)-D-cysteine was eluted at ca. 2.8 N HCl. The ninhydrin-positive material was pooled and concentrated to a yellow-orange oil by rotary evaporation. The oil was dissolved in hot ethanol and twice crystallized from ethanol/ethyl acetate (1/4). Amino acid analysis showed one peak of ninhydrin-positive material that coincided in position with the authentic L isomer at 43 mL when eluted from a 0.6 × 20 cm column of AA-27 resin with pH 5.26 sodium citrate.

Determination of the Optical Configuration of S-(β-Aminoethyl)cysteine. The D and L isomers of S-(β-aminoethyl)cysteine were separated as their diastereoisomeric peptides on the amino acid analyzer (Manning & Moore, 1968); the basic amino acids were separated as the dileucyl dipeptides as suggested by Mitchell et al. (1978). A 10-fold excess of L-leucine N-carboxyanhydride was used and the peptides were eluted from the 0.6 × 12 cm column of the amino acid analyzer with 0.2 N sodium citrate, pH 5.00, at 66 °C. Under these conditions, N-(α,ε-dileucyl)-L-aminoethylcysteine is eluted at 83 mL and N-(α,ε-dileucyl)-D-aminoethylcysteine is eluted at 110 mL; the color constants for the N-(α,ε-diglutamyl) derivatives of lysine (Manning & Moore, 1968) were used for calculations.

Synthesis of Thiomorpholine-2-carboxylic Acid (TMC).[1] In a typical synthesis, 250 mg (2.2 mmol) of β-mercaptoethylamine hydrochloride was dissolved in 1 mL of water in an 18 × 150 mm test tube wrapped with aluminum foil to exclude light. β-Bromopyruvate, 450 mg (2.7 mmol) in 1.5 mL of water, was added to the solution of β-mercaptoethylamine and the mixture was kept for 30 min at room temperature. Sodium borohydride, 450 mg (11.9 mmol), was then added to the tube with 1 drop of 2-octanol to minimize foaming. When gas evolution had ceased (in approximately 30 min), 2.5 mL of concentrated HCl was added to decompose residual borohydride. The solution was then evaporated to dryness and the white solid material was dissolved in 6 mL of water. Amino acid analysis (Spackman et al., 1958) of the products of the reaction indicated the presence of two major ninhydrin-positive products; one compound was eluted just after alanine and a second component was eluted just after leucine. This mixture was desalted on a Dowex 2-X8 column (2 × 32 cm) by the procedure of Drèze et al. (1954). The ninhydrin-positive material that was eluted with 1 N acetic acid was evaporated to dryness and the brownish residue was dissolved in 2 mL of water. The pH was adjusted to about 2 and the sample was applied to a column of Dowex 50-X8 (1 × 26 cm). The column was washed with 25 mL of water and the eluent was changed to 1 N HCl. Two peaks of ninhydrin-positive material were obtained. The second compound, which elutes just after alanine on the amino acid analyzer, was isolated after removal of the HCl by evaporation. After two recrystallizations from ethanol/ethyl acetate (1/4), this compound gave the correct elemental analysis for the hydrochloride of thiomorpholine-2-carboxylic acid. Anal. Calcd for $C_5H_9NO_2S$·HCl (mol wt 183): C, 32.69; H, 5.48; N, 7.62. Found: C, 32.84; H, 5.40; N, 7.56.

TMC is weakly reactive with ninhydrin with a color constant 8% that of leucine on the amino acid analyzer and an A_{440}/A_{570} ratio of 0.57. The initial yield of product in the reaction mixture was 27% and the amount of pure compound isolated (50 mg) represents a 12% overall yield.

The first peak eluted from the Dowex 50-X8 column is S-(β-lactyl)cysteamine which results from the reduction of the uncyclized adduct (unpublished observations). Its properties were not determined since it is not a product of the enzyme catalyzed reaction with chloroalanine.

Thiomorpholine-2-carboxylic acid was reported to be found in extracts of the brown algae *Undaria pinnatifida* (Oka et al., 1960). Later it was shown that the naturally occurring product was the S-oxide of TMC and that during the isolation the sulfur was reduced (Tominaga & Oka, 1963). Thiomorpholine-2-carboxylic acid was isolated as a minor product (3% yield) from the reaction of 1,2-dibromoethane with cysteine (Däbritz & Virtanen, 1965).

Synthesis of 3-[^{14}C]-β-Chloro-D-alanine. The procedure of Walsh et al. (1971) was used in a scaled down synthesis and, as suggested by Dr. C. T. Walsh, we synthesized the propyl ester of D-serine rather than the methyl ester since the former derivative is more soluble in chloroform than the latter compound. All intermediate crystallization steps were omitted to improve the overall yield.

In a typical synthesis, 100 μCi of 3-[^{14}C]-D-serine was added to 50 mg of D-serine in 5 mL of propanol that had been dried with Linde 4A molecular sieves. HCl gas, which had been dried by passing it through concentrated sulfuric acid, was bubbled through the propanol suspension for 30 min and the solution was refluxed for 30 min. After the solution was cooled, it was concentrated to dryness on a rotary evaporator and the solid material was washed twice with dry benzene and dried overnight in a vacuum desiccator over paraffin and NaOH. The solid material, dissolved in 3 mL of dry, ethanol-free chloroform, was added slowly to phosphorus pentachloride (75 mg in 5 mL of chloroform) as described by Walsh et al. (1971). The solution was concentrated to dryness, dissolved in 5 mL of 6 N HCl, and refluxed for 1 h. This material showed three major radioactive compounds on an amino acid analyzer equipped with a scintillation flow cell (Nuclear-Chicago Chroma cell)—an unidentified acidic impurity that was eluted at the void volume of the column, β-chloroalanine, and unreacted serine. After adjustment to pH 2, the solution was applied to a column of Dowex 50-X8 (0.9 × 30 cm). The acidic impurity was eluted with 25 mL of water and the eluent was then changed to 1 N HCl. β-Chloroalanine and serine were recovered as two separate ninhydrin-positive peaks. The isolated 3-[^{14}C]-β-chloro-D-alanine had a specific radioactivity of 130 nCi/μmol. The overall yield was 12%. The radioactive chloroalanine had only trace impurities and they were not altered by treatment with D-amino acid transminase.

[1] Abbreviation used: TMC, thiomorpholine-2-carboxylic acid.

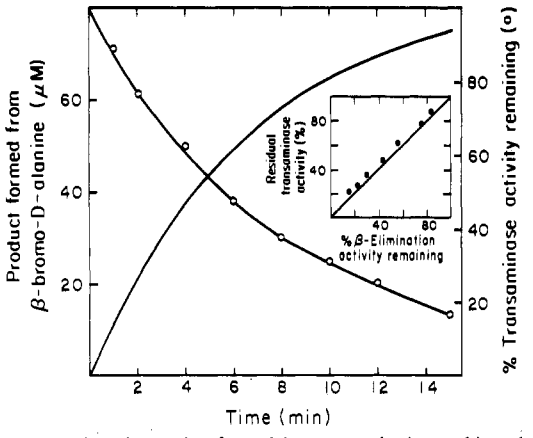

FIGURE 1: Product formation from β-bromo-D-alanine and its relationship to the inactivation of D-amino acid transaminase. Turnover of β-bromoalanine was measured by incubating pure D-amino acid transaminase (15 μg/mL; specific activity, 148), β-bromo-D,L-alanine (0.66 mM), lactate dehydrogenase (70 μg/mL), NADH (0.1 mM) in 0.33 M potassium phosphate, pH 7.0 at 25 °C. The production of α-keto acid was measured at 338 nm (the solid line). From a parallel incubation, portions (100 μL) were removed at the designated times (○) and the β-elimination reaction was quenched by dilution into 2.8 mL of 25 mM D-alanine, 0.1 mM NADH, 70 μg/mL lactate dehydrogenase in 0.33 M potassium phosphate, pH 8.5. The residual transaminase activity was measured by addition of 0.1 mL of α-ketoglutarate (750 mM). The insert shows the correlation between the inactivation of the transaminase and the decreased rate of product formation from β-bromoalanine as determined by the tangent to the solid line.

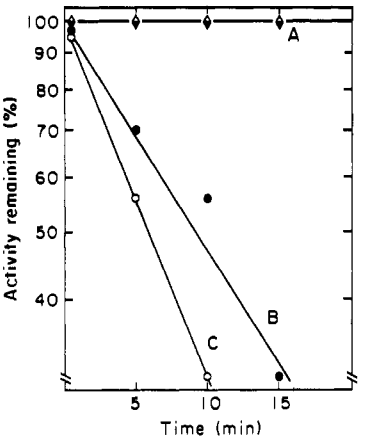

FIGURE 2: Effect of other compounds on the inactivation of D-amino acid transaminase by β-bromo-D-alanine. Pure D-amino acid transaminase (15 μg/mL; specific activity, 148) was incubated with lactate dehydrogenase (70 μg/mL) in 0.33 M potassium phosphate, pH 7.0, with: (A) No β-bromoalanine (△) or β-bromoalanine (0.66 mM) plus 27 mM D-alanine (▼); (B) β-bromoalanine (0.66 mM) plus 27 mM L-alanine (●); (C) β-bromoalanine (0.66 mM) alone (○). At the indicated times, aliquots (100 μL) were removed and the residual transamination activity was measured as described for Figure 1.

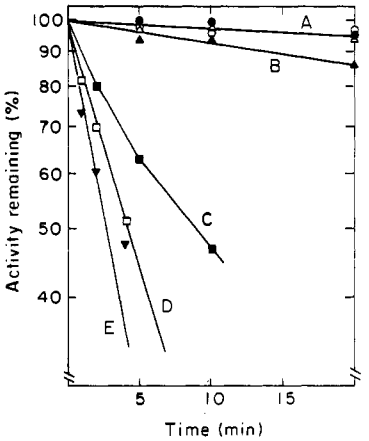

FIGURE 3: Effect of substrates on the inactivation of D-amino acid transaminase by β-bromopyruvate. D-Amino acid transaminase (10 μg/mL; specific activity, 112) was incubated in 0.33 M potassium phosphate, pH 8.5, plus lactate dehydrogenase (70 μg/mL) and (A) no β-bromopyruvate (△), β-bromopyruvate (1 mM) alone (○) or β-bromopyruvate plus 25 mM L-alanine (●); (B) β-bromopyruvate (1 mM) plus α-ketoglutarate (25 mM) (▲); (C) β-bromopyruvate (1 mM) plus α-ketoglutarate (25 mM) and D-glutamate (25 mM) (■); (D) β-bromopyruvate (1 mM) and D-glutamate (25 mM) (□); and (E) β-bromopyruvate (1 mM) and D-alanine (25 mM) (▼). At the indicated times, aliquots (100 μL) were removed and the residual transamination activity was measured as described for Figure 4.

Results

Inactivation of D-Amino Acid Transaminase by β-Bromoalanine. In the absence of cosubstrate, β-bromoalanine is converted into a keto acid by pure D-amino acid transaminase at pH 7.0; spontaneous decomposition of bromoalanine precluded studies at pH 8.5. By analogy with the findings for β-chloro-D-alanine (Soper et al., 1977b), we assume that the products of this reaction are pyruvate, bromide, and ammonia. The decrease in the rate of keto acid production (Figure 1) suggested that the transaminase was being inactivated during the reaction. Indeed, direct measurement of transaminase activity with D-alanine and α-ketoglutarate as cosubstrates confirmed this supposition. The insert of Figure 1 shows a direct correlation between the decreased rate of keto acid formation from bromoalanine and the residual transminase activity. This behavior is identical with that observed in our earlier experiments with β-chloro-D-alanine.

From the data in Figure 1 we calculate that 47 nmol of product are produced when one-half of the enzyme (0.126 nmol) is inactivated, or that there are 370 turnovers per inactivation event. When an experiment similar to that described in Figure 1 was performed with β-chloro-D-alanine at pH 7.0, the turnover/inactivation ratio was found to be 340, a value experimentally indistinguishable from that for bromoalanine. By contrast, the turnover/inactivation ratio at pH 8.5 found with β-chloro-D-alanine is 1500 (Soper et al., 1977b). In addition, from the data in Figure 1 the relative velocities for the β-elimination and transamination activities can be calculated. The initial velocity of the β-elimination reaction at pH 7.0 is 5% that of the transamination rate at pH 8.5. The pH dependence of the β-elimination reaction with β-chloroalanine exhibits a broad pH optimum with a maximum from pH 8.5 to 9; the ratio of the β-elimination activity at pH 8.5 to pH 7.0 is two. Thus, at pH 8.5 the β-elimination activity would be 10% of the transamination activity, a value similar to that calculated for chloroalanine with this enzyme (Soper et al., 1977b).

The inactivation by β-bromoalanine is irreversible since extensive dialysis does not result in recovery of activity. The inactivation follows pseudo-first-order kinetics (Figure 2, line C), a result which indicates that the inactivation proceeds from a preformed enzyme·inhibitor complex. The inactivation rate is considerably reduced by addition of D-alanine (line A) but not by addition of L-alanine (line B). A preliminary estimate of the K_{inact} for β-bromo-D-alanine is 35 μM, a value very similar to that found for chloroalanine. A more quantitative comparison must await resolution of the racemic bromoalanine.

Effect of β-Bromopyruvate on D-Amino Acid Transaminase. Incubation of D-amino acid transaminase with β-bromopyruvate results in no detectable inactivation (Figure 3, line

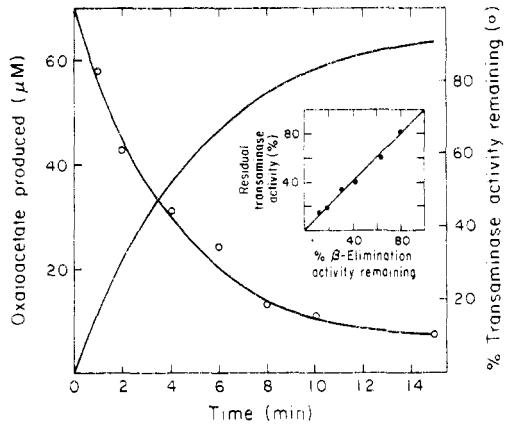

FIGURE 4: Turnover of β-bromopyruvate and D-aspartate and the inactivation of D-amino acid transaminase. Turnover (solid line) was measured by coupling the production of oxaloacetate from D-aspartate to malate dehydrogenase. The incubation mixture contained β-bromopyruvate (1 mM), D-aspartate (25 mM), malate dehydrogenase (33 μg/mL), NADH (0.1 mM), and D-amino acid transminase (10 μg/mL; specific activity, 112) in 0.33 M potassium phosphate, pH 8.5. From a parallel incubation mixture, portions (100 μL) were removed at designated times (○) and diluted into 2.8 mL of 25 mM α-ketoglutarate, 0.1 mM NADH, lactate dehydrogenase (70 μg/mL) in 0.33 M potassium phosphate to quench the reaction; residual transaminase activity was measured by addition of 0.1 mL of D-alanine (750 mM) as described in the text.

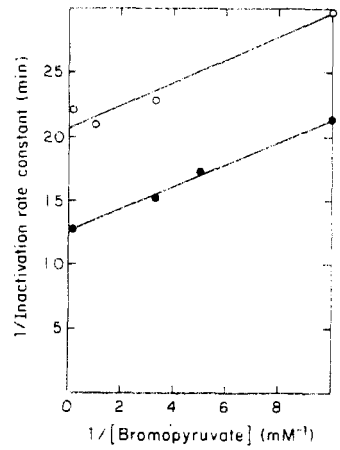

FIGURE 5: Double-reciprocal plot of the pseudo-first-order rate constant for the inactivation of D-amino acid transaminase as a function of β-bromopyruvate concentration. The experiments were carried out in the presence of either 5 mM (○) or 25 mM (●) D-aspartate in 0.33 M potassium phosphate, pH 8.5, the indicated concentrations of β-bromopyruvate at 25 °C, malate dehydrogenase (33 μg/mL), NADH (0.1 mM), and D-amino acid transaminase (26 μg/mL; specific activity, 36). The rate of production of oxaloacetate constantly decreased as in Figure 4. The residual rate of production of oxaloacetate was determined as the tangent to the curve at any given time point. This rate was then plotted as a semilogarithmic function vs. time and the slope of this plot yielded the pseudo-first-order rate constant for inactivation at a given concentration of β-bromopyruvate and D-aspartate.

A) during the time period in which the enzyme is significantly inactivated by β-bromo-D-alanine (Figure 2, line C). However, if D-amino acid substrates such as D-alanine or D-glutamate are included (Figure 3, lines E and D), the transaminase is inactivated. Neither L-alanine nor α-ketoglutarate (Figure 3, lines A and B, respectively) is effective in promoting the inactivation by bromopyruvate. However, α-ketoglutarate is capable of partially overcoming the effect of D-glutamate (Figure 3, line C).

The D-amino acid required for inactivation in the presence of bromopyruvate undergoes turnover to a keto acid as shown in Figure 4. D-Aspartate is converted to oxaloacetate at a constantly decreasing rate in the presence of β-bromopyruvate. The rate at which this activity decreases is directly proportional to the amount of transaminase inactivated (Figure 4, insert). Amino acid analysis did not reveal the presence of any β-bromoalanine; i.e., there is no transamination between β-bromopyruvate and the D-amino acid cosubstrate. When 0.062 nmol of transminase was inactivated, there was 35 nmol of oxaloacetate produced. Therefore, the enzyme processed 560 molecules of substrate for each inactivation event at pH 8.5. When the experiment was repeated with 10 mM β-bromopyruvate, the ratio was found to be 620. The initial maximum rate of product formation with 25 mM D-aspartate and 1 mM β-bromopyruvate was 6% that of the V_{max} for transmination with D-alanine and α-ketoglutarate. With 10 mM β-bromopyruvate this value increased slightly to 8% of the V_{max} for transamination.

β-Bromo-α-ketobutyrate behaves similarly to β-bromopyruvate. When D-amino acid transaminase was incubated with 10 mM β-bromo-α-ketobutyrate and 25 mM D-aspartate, oxaloacetate was produced at a steadily decreasing rate similar to the profile shown in Figure 4 for bromopyruvate and D-aspartate. The initial rate of oxaloacetate formation from aspartate in the presence of β-bromo-α-ketobutyrate was 6% that of the rate with β-bromopyruvate. The half-time for inactivation of the enzyme was 6.8 min with β-bromo-α-ketobutyrate and D-aspartate, and 1 min with β-bromopyruvate and D-aspartate under identical conditions.

The experiments with β-bromopyruvate described above were performed at pH 8.5, the pH optimum for enzyme activity. Bromopyruvate is not completely stable at this pH and is converted to β-hydroxypyruvate. Thus, Sprinson & Chargaff (1946) prepared β-hydroxypyruvate from β-bromopyruvate at pH 8.5. However, there are conflicting data on the rate of conversion of β-bromopyruvate to β-hydroxypyruvate. Meloche (1967) indicates that at pH 8 the reaction is completed in a matter of minutes but Roche et al. (1971) showed that a 4 M solution of β-bromopyruvate at pH 7.7 has a half-life of 160 min. Could the inactivation observed with β-bromopyruvate described in the present communication be due to its prior nonenzymic conversion to β-hydroxypyruvate? The following results indicate that such is not the case. There is little, if any, inactivation of the transaminase with authentic β-hydroxypyruvate under conditions where β-bromopyruvate inactivated the enzyme. Secondly, we find that hydroxypyruvate is slowly transaminated to serine, but no serine was ever observed in any of our experiments with bromopyruvate. Thus, we believe that, even if a small amount of hydroxypyruvate was formed during the experiments outlined in Figures 3 and 4, its presence would not have influenced the results.

The data in Figure 3 show that in the presence of a D-amino acid cosubstrate the transminase is inactivated by β-bromopyruvate according to pseudo-first-order kinetics. This would indicate that the inactivation occurs from a preformed enzyme–bromopyruvate complex. The data in Figure 5 show the rate of inactivation of the transaminase with varying concentrations of β-bromopyruvate at two concentrations of D-aspartate. The parallel lines in the double-reciprocal plot indicate that the two substrates interact with two distinct forms of the enzyme; i.e., they show a "ping-pong" kinetic behavior. D-Aspartate would bind to the pyridoxal form of the transaminase generating the pyridoxamine form and free oxaloacetate. β-bromopyruvate would then bind to the pyridoxamine form and, instead of participating in transamination, it would undergo α,β-elimination to form an α-aminoacrylate–Schiff's base with the coenzyme. Hydrolysis of the α-aminoacrylate

TABLE I: Products Formed during the β-Elimination of β-Chloro-D-alanine in the Presence of β-Mercaptoethylamine.

expt	time (h)	substrate consumed (dpm/μL) chloro-Ala	products formed (dpm/μL) aminoethyl-Cys	thiomorpholine-2-carboxylic acid	Ala
1[a]	0.03	38	10	3	17
	0.13	174	78	20	28
	0.22	246	118	19	41
	0.30	289	133	15	69
2[b]	2.0	321	165	42	89
3[c]	3.0	884	500	56	252

[a] β-[^{14}C]Chloro-D-alanine (4.5 μmol) was incubated with D-amino acid transaminase (14.8 units) and 10 mM β-mercaptoethylamine in 1 mL of 0.1 M potassium phosphate buffer, pH 7.0. At the indicated times 100 μL aliquots were removed and mixed with 50 μL of ice-cold 1 M sodium borohydride in 0.1 M NaOH. The final pH was about 8. After 30 min at room temperature, 10 μL of concentrated HCl was added to decompose residual borohydride and the sample was diluted to 2 mL with 0.2 N sodium citrate (pH 2.2) buffer. The reaction products were analyzed on an amino acid analyzer of the design of Spackman et al. (1958) equipped with a Nuclear-Chicago Chroma cell scintillation counter. [b] The experimental conditions were the same as those described in experiment 1 except that 2.4 units of transaminase were incubated with 0.09 μmol of [^{14}C]chloroalanine and 0.4 μmol of β-mercaptoethylamine in 0.4 mL of the same buffer. [c] The experimental conditions were the same as those described in experiment 2 except that 4.8 units of transaminase and 0.27 μmol of [^{14}C]chloroalanine were used.

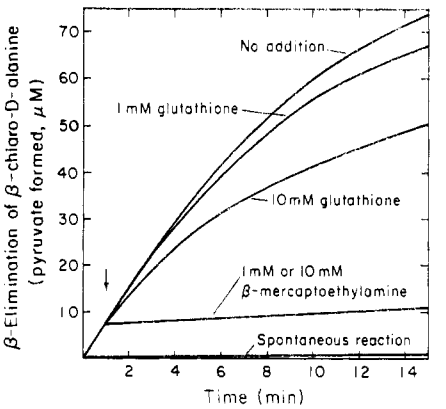

FIGURE 6: Effect of addition of thiol reagents on the β-elimination of β-chloro-D-alanine catalyzed by D-amino acid transaminase. The production of pyruvate from chloroalanine was monitored at 338 nm in a solution containing D-amino acid transaminase (10 μg/mL; specific activity, 116), β-chloro-D-alanine (1 mM), lactate dehydrogenase (70 μg/mL), NADH (0.1 mM) in 0.33 M potassium phosphate, pH 7.0 at 25 °C. The indicated compounds were added to the reaction after 1 min (the arrow). The spontaneous rate of β-elimination of β-chloroalanine represents the rate of pyruvate production in the absence of enzyme.

intermediate would yield pyruvate, ammonia, and the transaminase in the pyridoxal form for the next catalytic cycle. The α-aminoacrylate-pyridoxal-Schiff's base intermediate is postulated as the inactivating species, as proposed for β-chloro-D-alanine (Soper et al., 1977b).

Trapping of an α-Aminoacrylate Intermediate in the β-Elimination of β-Chloro-D-Alanine. Evidence for the existence of an α-aminoacrylate intermediate for pyridoxal phosphate enzymes that catalyze β-elimination reactions with L-amino acids has been obtained previously. This intermediate was trapped with *N*-ethylmaleimide (Flavin & Slaughter, 1969) and with 2-mercaptoethanol (Miles et al., 1968) for threonine dehydrase and the β subunit of tryptophan synthetase, respectively. Friedman et al. (1965) showed that sulfhydryl groups add to α,β-unsaturated compounds much faster than do amino groups, and Snow et al. (1976) demonstrated that thiol reagents readily add to the unsaturated double bond of *N*-acetyl-α-aminoacrylate, a stable analogue of the proposed intermediate.

When β-mercaptoethylamine was added to a reaction mixture during active β-elimination of β-chloroalanine, the enzymic production of pyruvate ceased within a few seconds (Figure 6). Amino acid analysis indicated that β-chloroalanine continued to be consumed after addition of β-mercaptoethylamine but that there was no further production of keto acid that was reactive with lactate dehydrogenase or with 2,4-dinitrophenylhydrazine. Similar effects were found for dithiothreitol, cysteine, and 2-mercaptoethanol, but there was very little effect of reduced glutathione. Snow et al. (1976) showed that the reactivity of glutathione with *N*-acetylaminoacrylate was intermediate between that of mercaptoethanol and dithiothreitol in model reactions. The fact that glutathione does not participate in the β-elimination suggests that the addition to the α-aminoacrylate intermediate by thiols occurs in a sterically hindered environment, such as the active site of the transaminase. Small thiols can freely diffuse into the active site to add to the α-aminoacrylate intermediate but the larger thiol, glutathione, apparently cannot penetrate. Addition of β-mercaptoethylamine to the putative intermediate should yield *S*-(β-aminoethyl)cysteine. The results in Table I indicate that *S*-(β-aminoethyl)cysteine is indeed the major product of this trapping reaction, when the thiol is present at the inception of the reaction. These experiments, which were performed with [^{14}C]-β-chloro-D-alanine, indicate that three additional minor radioactive products were formed: alanine, a compound that eluted just behind alanine on the amino acid analyzer, and an unknown compound that eluted at the void volume of the column. This latter material is probably pyruvate formed from the spontaneous decomposition of chloroalanine as well as from the hydrolysis of any of the aminoacrylate intermediate that escaped trapping by the thiol. The alanine probably arises from transamination of pyruvate by the pathway discussed below. The third product elutes in the same position as authentic thiomorpholine-2-carboxylic acid.

The initial adduct in the trapping reaction would be *S*-(β-aminoethyl)cysteine in aldimine linkage with the coenzyme (see Figure 7, VI). Solvolysis of this intermediate would generate the major product, *S*-(β-aminoethyl)cysteine. However, if the aldimine linkage rearranged to a ketimine linkage (Figure 7, VII) solvolysis would generate *S*-(β-pyruvyl)cysteamine, which would spontaneously cyclize to form Δ^1-thiomorpholine-2-carboxylic acid. Reduction with sodium borohydride would yield TMC. Alternatively, the cyclic product formed in the trapping reaction during β-elimination could arise as a secondary product by transamination of the initially trapped *S*-(β-aminoethyl)cysteine. Indeed, we could demonstrate that *S*-(β-aminoethyl)cysteine is a substrate for D-amino acid transaminase (Table II), although it turns over at a rate which is about 3% that of D-alanine. Transamination between *S*-(β-aminoethyl)cysteine and α-ketoglutarate yielded (after reduction) thiomorpholine-2-carboxylate and glutamate. The scheme in Figure 7 would predict that the production of TMC should be equivalent to the amount of glutamate formed. Since only about half of reduced Δ^1-thiomorpholine-2-carboxylic acid was recovered in this experiment, it is conceivable that during the 3-h incubation and before reduction of the reaction products, some of the Δ^1-morpholine-2-carboxylic acid was

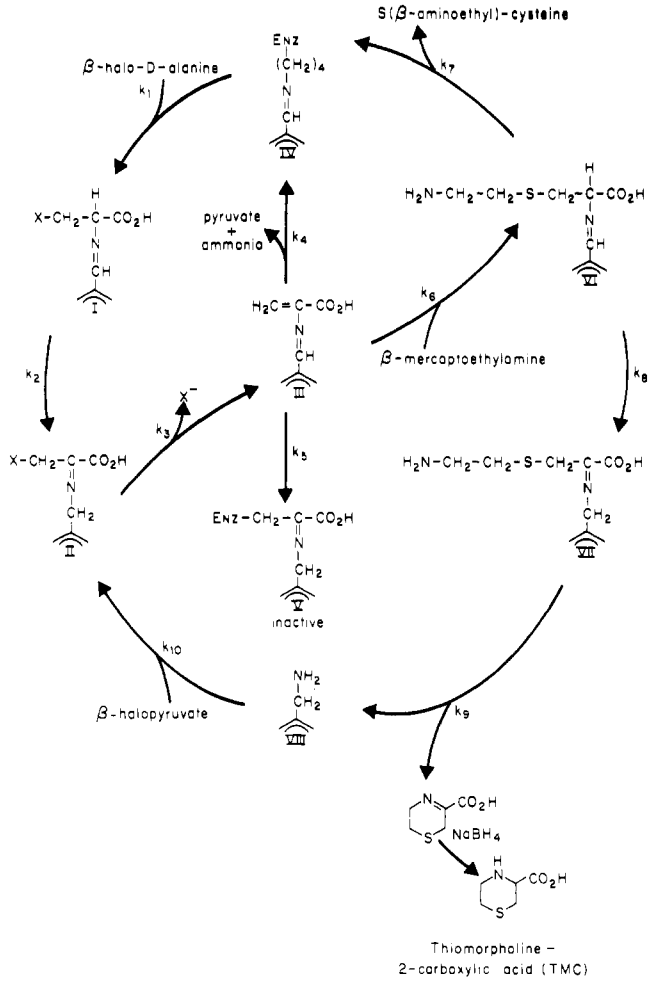

FIGURE 7: Model of the β-elimination scheme and the trapping of an intermediate during reactions catalyzed by D-amino acid transaminase. Only a portion of the enzyme-bound pyridoxal phosphate is shown.

TABLE II: Transamination between *S*-(β-Aminoethyl)-D-cysteine and α-Ketoglutarate.[a]

time (h)	products formed (mM) aminoethyl-Cys	Glu	thiomorpholine-2-carboxylic acid
0	20.6	0	0
3	11.5	9.7	4.5

[a] D-Amino acid transaminase (73 μg/mL) was incubated with the indicated concentration of *S*-(β-aminoethyl)-D-cysteine and 200 mM α-ketoglutarate in 0.4 mL of 0.1 M potassium phosphate, pH 7.0. After 3 h at 24 °C, 0.1 mL was treated with $NaBH_4$ and subjected to amino acid analysis as described in the footnote to Table I.

TABLE III: Configuration of *S*-(Aminoethyl)cysteine Formed during Enzymic β-Elimination of β-Chloro-D-alanine in the Presence of β-Mercaptoethylamine.

method of analysis	aminoethyl-Cys (nmol) amount analyzed	D isomer	L isomer
L-amino acid oxidase[a]	58	57	<1
diastereoisomeric peptide[b]	85	29	<3

[a] A portion of the reaction mixture (0.5 mL) containing *S*-(β-aminoethyl)cysteine was treated with L-amino acid oxidase (0.65 mg) and FAD (5 μg) in 0.1 M potassium phosphate, pH 7.0. After 24 h, the sample was diluted into pH 2.2 sodium citrate and analyzed on the 0.6 × 20 cm column of the amino acid analyzer as described in Experimental Procedure. [b] From another portion of the reaction mixture, *S*-(β-aminoethyl)cysteine was isolated on the 0.6 × 12 cm column of the amino acid analyzer with 0.35 N sodium citrate as the eluent at 52 °C. After desalting by the method of Drèze et al. (1954), the product (310 nmol) was coupled with L-leucine *N*-carboxyanhydride as described in Experimental Procedure.

hydrolyzed. Indeed, the finding that the concentration of TMC does not increase as a function of time and, in fact, decreases (Table I) lends support to this idea.

The configuration of the *S*-(β-aminoethyl)cysteine that was formed was determined by two different methods. β-Chloro-D-alanine (10 μmol) and β-mercaptoethylamine (20 μmol) were incubated with D-amino acid transaminase (5 units) at pH 7.0 for 48 h at 24 °C. The reaction mixture was divided into two parts. In one portion the *S*-(β-aminoethyl)cysteine was found to be refractory to the action of L-amino acid oxidase (Table III) under conditions where the authentic L isomer was completely oxidized and the synthetic D isomer was unchanged. The D isomer of *S*-(β-aminoethyl)cysteine is not a substrate for D-amino acid oxidase. For the second portion of the reaction mixture the *S*-(β-aminoethyl)cysteine formed was isolated and was coupled with L-leucine *N*-carboxyanhydride. Amino acid analysis of the diastereoisomeric peptides indicated that the *S*-(β-aminoethyl)cysteine was the D isomer (Table III). The low yield of desired tripeptide product may have been due to significant amounts of the monoleucyl derivatives. These results indicate that the addition of β-mercaptoethylamine to the planar α-aminoacrylate–coenzyme intermediate yields at least 90% of the D isomer of aminoethylcysteine and, taken together with the selective effects of thiols described above, the data suggest that the trapping of intermediate III by thiols takes place in stereospecific fashion at the active site of the transaminase.

Discussion

To date we have described four inhibitors of D-amino acid transaminase which act by three distinct catalytic pathways: (A) a unimolecular β-elimination reaction characteristic of β-bromo- and β-chloroalanine; (B) a bimolecular "ping-pong" β-elimination reaction with β-bromopyruvate which requires a D-amino acid cosubstrate; and (C) a bimolecular "ping-pong" transamination between D-vinylglycine and an α-keto acid cosubstrate that also leads to inactivation of the enzyme (Soper et al., 1977a). Figure 7 portrays a composite scheme of the catalytic inactivation of D-amino acid transaminase by the class A and class B inhibitors; some of the intermediates are common to those for normal transamination and also for the class C inhibitor. Also shown in the scheme is the trapping of the putative α-aminoacrylate intermediate by β-mercaptoethylamine. The scheme is an expansion of our previous model that depicted the inactivation of D-amino acid transaminase by β-chloro-D-alanine alone (Soper et al., 1977b). In the present model all of the reaction steps are shown as being unidirectional even though most of the reactions are undoubtedly microscopically reversible, although some are probably macroscopically irreversible, i.e., steps k_3, k_4, and k_5.

The class A inactivators, β-bromo- and β-chloro-D-alanine, act by pathway k_1, k_2, k_3, k_4/k_5. Upon Schiff base formation with pyridoxal phosphate (k_1), the α proton and β-halide ion are rapidly eliminated (k_2 and k_3) to yield intermediate III, an α-aminoacrylate Schiff base with the coenzyme. Solvolysis

of this intermediate yields pyruvate, ammonia, and free active enzyme in the aldimine form (IV). Nucleophilic attack at the β carbon of intermediate III would produce an irreversibly alkylated enzyme molecule (V). No kinetic difference was observed between the two class A inactivators studied here; this finding suggests that the nature of the leaving group on the β carbon of these inactivators is relatively unimportant; i.e., step k_3 is probably not the rate-limiting step in this reaction. These results do not support an S_N2 type nucleophilic attack at the β carbon of intermediate I or II to produce inactivation of the transaminase.

The V_{max} for the β-elimination of haloamino acid substrates is about 10% of the V_{max} of transamination with D-alanine and α-ketoglutarate as substrates, whereas the K_m for substrates that undergo β-elimination is one to two orders of magnitude lower than the K_m for transaminase substrates. These results suggest that the rate-limiting step in the β-elimination reaction is not k_2, as it is for substrates that undergo transamination (Banks et al., 1968). Trapping of intermediate III with β-mercaptoethylamine (pathway k_6, k_7), which occurs at a rate approaching that of the overall β-elimination reaction, indicates that the enzyme must pass through intermediate III during the β-elimination of β-halo derivatives of D-alanine. It is possible that the decomposition of the α-aminoacrylate–coenzyme intermediate, k_4, is the rate-limiting step in this reaction; studies on the deuterium isotope effect of the transaminase reaction compared with the β-elimination reaction will be a test for this proposal.

The class B inactivator, β-bromopyruvate (and probably β-chloropyruvate), does not interact with the pyridoxal phosphate form of the transaminase. A D-amino acid cosubstrate is required to generate the pyridoxamine form of the coenzyme (VIII) to permit such interaction. β-Bromopyruvate would form a Schiff base with VIII to form intermediate II which would undergo β-elimination to form the α-aminoacrylate coenzyme intermediate (III). Thus, the class B inactivators (k_{10}, k_3, k_4/k_5) share a portion of the class A pathway (k_1, k_2, k_3, k_4/k_5). It is important to note that the possible pathway k_1, k_2, k_{10} would represent the reversible transamination of β-halo-D-alanine to β-halopyruvate. All of the individual steps in the reaction scheme should be theoretically at least partially reversible as discussed above. However, we have been unable to detect any transamination of β-chloroalanine, β-bromoalanine, or β-bromopyruvate even by very sensitive isotopic methods. Perhaps once intermediate II is formed, regardless by which pathway, it is quantitatively converted into intermediate III via k_3. This step, which is functionally irreversible, yields a product whose decomposition proceeds via two other functionally irreversible steps (k_4 or k_5) and these processes rapidly deplete the concentration of species II. Whereas steps k_2 and k_{10} are theoretically reversible, they thus become functionally irreversible, and no transamination of these β-halo substrates can be demonstrated.

The proposed pathway for inactivation by β-bromopyruvate is not the only possible mechanism for its mode of action. It should be noted that, whereas β-bromopyruvate was originally proposed to inactivate glutamate–aspartate transaminase by this active site directed pathway (Okamoto & Morino, 1973), inactivation by a syncatalytic mechanism was later demonstrated in which a cysteine residue adjacent to the active site was exposed and alkylated by bromopyruvate (Birchmeier & Christen, 1974). From our data it is not possible to determine whether the inactivation of D-amino acid transaminase by bromopyruvate occurs by an affinity labeling mechanism or by a syncatalytic mechanism. Since α-keto acids partially prevent the inhibition and "ping-pong" kinetics are observed (Figure 5), the affinity labeling mechanism may be operative but we cannot rule out syncatalysis nor can we exclude a combination of both pathways. However, in the context of this communication, the important point is that inactivation of the transaminase by β-bromopyruvate has an absolute requirement for a D-amino acid cosubstrate. This finding excludes the possibility that a trace amount of β-halopyruvate, undetectable in our original analysis (Soper et al., 1977b), is involved in the mechanism of action of the class A inactivators, β-bromo- and β-chloro-D-alanine.

The class C inactivator, D-vinylglycine, was initially studied as a stable analogue of α-aminoacrylate. We anticipated that an intermediate, which would be homologous with intermediate III, would be formed between vinylglycine and the pyridoxal form of the enzyme. However, we found that vinylglycine underwent transamination and that an α-keto acid cosubstrate was required for inactivation (Soper et al., 1977a). With vinylglycine it is not clear, at this time, whether the inactivation occurs in aldimine linkage (analogous to I of Figure 7) or in ketimine linkage (analogous to II of Figure 7) (Soper et al., 1977a).

Our interest in inactivators of D-amino acid transaminase is twofold: to provide information on the mode of action of this important bacterial enzyme and to determine whether these compounds have potential pharmacological value since, by inhibition of this enzyme, the bacteria would be deprived of a major source of D-glutamate, a necessary constituent of bacterial cell walls. Thus, inactivators of this enzyme should be potential antibacterial agents. Indeed, the class A inactivator, β-chloro-D-alanine, has been shown to have antibacterial action (Manning et al., 1974). A potential problem with the use of β-chloroalanine is that this compound is a substrate for renal D-amino acid oxidase (Walsh et al., 1971). The product of this reaction is either pyruvate or β-chloropyruvate depending on the oxygen tension. β-Chloropyruvate would be expected to inactivate many enzymes just as β-bromopyruvate does and would be an undesirable side product. Recently, it was reported that β-bromo-D-alanine is converted by renal D-amino acid oxidase only into pyruvate and not into β-bromopyruvate (Dang et al., 1976). Thus, β-bromo-D-alanine may be preferable to β-chloro-D-alanine as an antibacterial agent. Testing of the antibacterial action of β-bromo-D-alanine must await resolution of the racemic compound since the L isomer will undoubtedly inactivate other enzyme systems as does β-chloro-L-alanine. The class B inactivator β-bromopyruvate reacts with several enzymes (Meloche, 1967; Rashed & Rabin, 1968; Roche et al., 1971; Fonda, 1976) and is therefore without pharmacological interest.

Rando (1974) found that thiols prevented the inhibition of alcohol dehydrogenase by allyl alcohol. He concluded that the product of the enzyme reaction, acrolein, accumulated free in solution, later diffused back to the enzyme and inactivated it; added thiol would react with free acrolein and prevent the inhibition. For other enzymes, lack of protection by thiols has been taken as evidence that the inactivation takes place directly at the active site of the enzyme. In the present study added thiol protects the enzyme against inactivation by stereospecific addition to a transaminase intermediate. This finding indicates that the ability of a thiol to penetrate the active site of a given enzyme should also be taken into consideration when such effects of thiols are interpreted.

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