Studies on the Elimination Reaction of D-Amino Acid Oxidase with α-Amino-β-chlorobutyrate

FURTHER EVIDENCE FOR ABSTRACTION OF SUBSTRATE α-HYDROGEN AS A PROTON

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SUMMARY

D-Amino acid oxidase catalyzes the nonoxidative conversion of α-amino-β-chlorobutyrate to α-ketobutyrate, but does not catalyze its oxidation to β-chloroketobutyrate. The elimination reaction requires the oxidized flavoprotein, i.e. it does not occur with the apoenzyme or when the enzyme-bound flavin is reduced. The occurrence of this elimination reaction supports the proposal that removal of the substrate α proton is an early step in reactions catalyzed by D-amino acid oxidase.

Addition of α-amino-β-chlorobutyrate to D-amino acid oxidase leads to the sequential formation of three spectroscopically recognizable complexes (I, II, and III). All of these complexes have the spectra of oxidized flavin and in addition have long wave length absorbance extending to 800 nm. They are formed with the following rate constants: \( k_1, 2000 \text{ min}^{-1} \); \( k_2, 40 \); and \( k_3, -1 \). (\( k_t \) is dependent on substrate concentration. The value given is the limiting value at infinite substrate concentration.) When an α-deuterated substrate is used, \( k_t \) shows a deuterium isotope effect of about 5. Therefore, formation of Complex I must involve breaking of the substrate α C—H bond. Complex I could be a complex between flavin and a substrate-derived carbanion, or, if the elimination of HCl is complete at this stage, a complex between substrate-derived enamine and flavin. Formation of Complex I cannot be rate-determining, since the turnover number for the catalytic process is 6 to 9.5 min\(^{-1}\). From experiments carried out with α-amino-β-chloro[α-\(^2\)H]butyrate and in \( H_2O \), it is concluded that the action of D-amino acid oxidase on the substrate results in the formation of an enzyme-bound enamine which then undergoes two competing reactions: (a) It is released to the medium and spontaneously ketonizes to form ketobutyrate. (b) The β position of the enamine is protonated on the enzyme to form α-iminobutyric acid, which is then released and hydrolyzes to α-ketobutyrate. The proton which is added to the β position is originally derived from the α position of the substrate. The enzyme thus has the capability of sequestering the α proton of the substrate so that it does not exchange with the solvent. The relative extent to which these two processes (enamine reprotonation versus release) occur is temperature-dependent.

In a previous communication we have described an unusual reaction of β-chloroalanine with D-amino acid oxidase from hog kidney (1). With that substrate the enzyme catalyzes an elimination reaction, under anaerobic conditions, which yields pyruvate. Under aerobic conditions, chloropyruvate, the expected oxidation product, as well as pyruvate, are formed. The ratio of products formed depends on oxygen concentration, although the total amount of product, i.e. chloropyruvate + pyruvate, is independent of oxygen concentration. We proposed that the occurrence of this elimination reaction establishes the ability of the enzyme to abstract protons from the α position of the substrate and concluded that abstraction of a proton from the substrate is an early step in the normal oxidative process (1).

We have now extended these investigations to another substrate, α-amino-β-chlorobutyric acid. This substrate enabled us to test the proposal made previously, that the immediate product of the enzyme-catalyzed elimination reaction is the enamine, as shown in Scheme 1.

\[
\begin{align*}
R-\text{C}&-\text{C}-\text{CO}_2^- \quad \text{D-amino} \\
\text{Cl}^{\text{NH}_2^+} \quad \text{acid} \\
\quad \text{oxidase} \\
\quad \text{HCl} \\
\quad \text{enamine} \\
\quad \text{imine} \\
\end{align*}
\]

SCHEME 1

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According to this reaction sequence the reaction product should acquire 1 deuterium atom per product molecule when the reaction is carried out in $\text{D}_2\text{O}$. Furthermore, when $R = \text{CH}_3$, the resulting $\beta$ deuteroketobutyrate should be racemic. In the course of this investigation it was also found that $\alpha$-amino-$\beta$-chlorobutyric acid differs from chloroalanine in that it is not oxidized to chloroketo acid and only undergoes the elimination reaction to ketobutyrate. This fact greatly facilitated a kinetic analysis of the reaction.

**EXPERIMENTAL PROCEDURE**

**Materials**

D-Amino acid oxidase was purified from pig kidney and crystallized according to the procedure of Brumby and Massev (2) in the form of the benzotriazole complex. The enzyme was stored as this complex in 50% saturated ammonium sulfate at 4°C. Benzotriazole-free enzyme and apoenzyme were generated as described (2) and stored at $-20^\circ$C. Lactate dehydrogenase was purchased from Boehringer. Allo-free nL-threonine and nL-threonine methyl ester-HCI. a-Ketobutyric acid was purchased from Sigma. Crystalline nL-serine dehydrogenase was a gift of Dr. R. Labow, University of Michigan. 2-Chloropropionic acid was obtained from Eastman. DL-[1-3H]chloroalanine-HCl and DL-[2-3H]serine-HCl were synthesized as described (1).

**Methods**

**Chemical Syntheses**

DL-erythro-\(\beta\)-chloro-$\alpha$-aminobutyrate-HCl—The synthesis of DL-erythro-$\beta$-chloro-$\alpha$-aminobutyrate-HCl from DL-threonine was adapted from that of Plattner et al. (3). DL-threonine methyl ester-HCl (m.p. 125°C) was purchased as indicated or prepared from threonine as described for serine (1). Typically, 2.0 g of the ester-HCl was dried in vacuo over P$_2$O$_5$ for 48 hours and then suspended in 70 ml of chloroform freshly dried by passage through an alumina column. The suspension was stirred irreversibly while 3 g of solid PCl$_3$ were added in aliquots over 40 min at room temperature to the tightly stoppered flask. The suspension became floculent within 1 hour. After continued stirring for 12 hours, the white solid was filtered off and dried in a vacuum. The recovered weight of methyl ester was 670 mg. The solid melted at 168-170°C (literature value 172°C (4)). If the suspension was chilled before filtration, the product was contaminated with starting material. The ester was hydrolyzed by refluxing for 4 hours in 10 ml of 6 N HCl. The solution was then taken to dryness on a rotary evaporator, taken up in a minimal volume of dry methanol, and crystallized by the addition of dry ether. The final yield was 665 mg of DL-erythro-$\beta$-chloro-$\alpha$-aminobutyrate-HCl (m.p. 195°C; literature value 196-197°C (4)). This product displayed one ninhydrin-positive spot on high voltage paper electrophoresis at pH 1.9 (1). Under these conditions, threonine migrated 10 cm toward the negative electrode while the chloroaminobutyrate migrated 7.5 cm.

The paper chromatographic system of Shaw and Fox (5) was utilized as a diagnostic test for diastereomeric purity for both the starting material (threonine) and the product. In this system, threonine (three-$\beta$-hydroxy-$\alpha$-aminobutyrate) has an $R_f$ of 0.20, while allothreonine (erythro-$\beta$-hydroxy-$\alpha$-aminobutyrate) has an $R_f$ of 0.13. Erythro-$\beta$-chloro-$\alpha$-aminobutyrate has an $R_f$ of 0.11, while three-$\beta$-chloro-$\alpha$-aminobutyrate (see below) has an $R_f$ of 0.22. The $\alpha$ isomer of erythro-$\beta$-chloro-$\alpha$-aminobutyrate was prepared as described for the racemic mixture except that allo-free nL-threonine served as starting material.

**DL-three-$\beta$-chloro-$\alpha$-aminobutyrate-HCl—The three form of DL-chloroaminobutyrate was synthesized by chlorination of DL-allothreonine by the above procedure. The final yield was 25 to 30%, and the product was free of erythro diastereomers.**

Nuclear magnetic resonance spectroscopy of these compounds was performed in $\text{D}_2\text{O}$ against an external trimethylsilyl signal as previously described (1). Threonine and allothreonine gave the following signals with the assignments designated: doublet at 1.35 ppm (3H, methyl); doublet at 3.84 ppm (1H; $\alpha$ proton); multiplet at 4.6 ppm (1H, $\beta$ proton). The chloroaminobutyrates had signals shifted downfield by substitution of the $\beta$ hydroxyl by a chlorine: doublet at 1.67 ppm (3H, methyl); doublet at 4.26 ppm (1H, $\alpha$ proton); and a multiplet centered around 4.90 ppm and partly obscured by the water signal from the $\text{D}_2\text{O}$ impurity in the solvent $\text{D}_2\text{O}$.

**2- and 3-$\beta$-chloro-$\alpha$-aminobutyrate—**Threonine was specifically deuterated in the $\alpha$ position by a modification of the procedure described by Elliot (6) for interconverting the four diastereomic $\beta$-hydroxy-$\alpha$-aminobutyric acids. In essence the modified method started with the racemic nL-threonine and first involved inversion of the $\beta$ carbon to give the erythro series. Then mutarotation was allowed to proceed at the $\alpha$ carbon under conditions such that all of the solvent hydrogen was deuterium in the form of C$_2$D$_5$OD (absolute, from Merck). Because the initial cis-oxazoline ester formed, starting from DL-threonine, is thermodynamically much less stable than the trans-oxazoline ester (6), mutarotation produces greater than 95% of the trans isomer at equilibrium and greater than 95% incorporation of deuterium at the $\alpha$ carbon.

DL-threonine (allo-free) (10 g; 84 mmoles) was converted into the N-benzoyl compound with benzoyl chloride (12 ml, 100 mmole) in 252 ml of 1x NaOH under Schotten-Baumann conditions (7). After 4 hours at ice bath temperature, the solution was acidified to pH 1, allowed to stand in ice for 20 min, and filtered. The filtrate was concentrated, chilled overnight, and filtered. The combined solids were recrystallized from a minimal volume of hot ethyl acetate. Contaminating benzooic acid was removed by digestion on a hot plate for 15 min with boiling carbon tetrachloride. The solid was filtered and dried. The recovered weight of DL-N-benzoyl threonine was 6.3 g (m.p. 142-144°C; literature value 143-144°C (8)). The N-benzoyl threonine (5 g) was converted to the methyl ester with diazomethane. The solid was finely ground and suspended in dry ether. An ethereal solution of diazomethane (1 g) was cautiously added and swirled in a methanol-ice bath for 45 min until all of the solid had dissolved and the yellow color was dissipated. The ethereal solution was then taken to dryness on a rotary evaporator and crystallized by the addition of dry ether. The yellow residue was immediately dissolved in 100 ml of chloroform and poured slowly into 100 ml of 10% sodium carbonate solution with efficient stirring. The chloroform layer was separated, the aqueous phase extracted with another 100 ml of chloroform,
and the combined chloroform layers were washed once with 50 ml of H2O, dried with anhydrous sodium sulfate, filtered, and added to a tared round bottom flask. The chloroform was evaporated and the resultant oil dried in a vacuum over phosphorus pentoxide to give 4.2 g (21 mmoles) of colorless semisolid material, methyl cis-M-2-phenyl-5-methyl-3-2-oxazoline-4-carboxylate. The dry material was dissolved in 25 ml of absolute C2H5OH (99% OH) and kept stopped before immediate addition to a solution of 25 ml of C2H5OH containing 0.54 g of dissolved metallic sodium. There was an instantaneous development of a pale yellow color on mixing. The mix was allowed to stand for 10 min at room temperature before 50 ml of 2H2O were added and the solution refluxed for 15 min to hydrolyze the methyl ester, thereby generating m-trans-2-phenyl-5-methyl-3-2-oxazoline-4(2H)carboxylate. The solution was then evaporated to 20 ml, added to 15 ml of 6 N HCl, and refluxed for 5 hours to hydrolyze the trans-oxazoline acid to mL-[2H]-threonine-HCl. On cooling, large crystalline needles of benzoic acid deposited and were filtered off. The filtrate was extracted twice with 50 ml of ether to remove any remaining benzoic acid; then the aqueous phase was evaporated to dryness, the residue suspended in 20 ml of absolute ethanol, and heated to boiling. The suspension was filtered to remove insoluble salts, and the ethanol was then evaporated to yield a pale oil which resisted attempts at crystallization. The amino acid hydrochloride was then converted to the free base. It was dissolved in 20 ml of hot absolute ethanol and 1.8 ml of redistilled pyridine was added with stirring. The solution was allowed to stand overnight at \(-20°C\) and deposited dense crystals which were filtered, washed with ice-cold ethanol and ether, and dried.

The weight of mL-[2H]-threonine recovered was 1.05 g (10.2 mmoles). It displayed one ninhydrin-positive spot on electrophoresis at pH 1.9 and migrated with authentic threonine. In the paper chromatographic system of Shaw and Fox (5), the deuterated threonine had an RF value equal to authentic threonine and displayed no detectable allothreonine contamination. The deuterium content and location were analyzed by NMR spectroscopy, and the deuterated preparation compared with the same concentration of authentic threonine. The \(\alpha\)-labeled sample had an identical doublet (3H, methyl) at 1.35 ppm and a quartet at 4.6 ppm (IH, \(\beta\) proton), but contained no detectable signal at 3.84 ppm corresponding to the \(\alpha\)-hydrogen. A 5% contamination of \(\alpha\)-hydrogen could easily have been detected in the \(\alpha\) deuterated threonine, from which it is concluded that the sample was greater than 95% deuterated, specifically at the \(\alpha\) carbon.

mL-\(\beta\)-chloro-\(\alpha\)-amino(\(\alpha\)-H)butyrate-HCl—The mL-[\(\alpha\)-2H]-threonine, 750 mg, was converted to the methyl ester-HCl with dry HCl gas in dry methanol as described above and then chlorinated with inversion at the \(\beta\) carbon to yield the erythro series. The final yield of mL-erythro-\(\beta\)-chloro-\(\alpha\)-amino(\(\alpha\)-H)butyrate-HCl was 284 mg. It was free of any contaminating ninhydrin-positive material by the electrophotographic and paper chromatographic criteria applied above and was coincident with authentic erythro-\(\beta\)-chloro-\(\alpha\)-amino-\(\alpha\)-aminobutyrate. There was no detectable \(\alpha\)-H signal in the deuterated product by NMR analysis.

Enzymatic Methods

Enzymatic activity was monitored either by oxygen uptake with a Clark-type oxygen electrode, or more generally by keto acid production. The keto acids were estimated as their respective 2,4-dinitrophenylhydrazone derivatives or as thiosemicarbazones (1). Alanine, chloroalanine, threonine, \(\beta\)-chloro-\(\alpha\)-aminobutyrate, and phenylalanine all yielded keto acid products from reaction with \(\alpha\)-amino acid oxidase which were substrates for lactate dehydrogenase, and these compounds were also assayed by coupled spectrophotometric assay of loss of absorbance at 340 nm due to NADH disappearance. In all incubations except those where changes in spectra of enzyme-bound flavin were analyzed, free FAD was included at a level of \(4 \times 10^{-5}\) M. Anaerobic incubations were performed under nitrogen or argon. In some instances, glucose (20 mM) and glucose oxidase (10+ M) were added to further minimize oxygen contamination. Flavin spectra were recorded on a Cary 14 or 17 spectrophotometer as well as a Unicam sp 800 instrument.

\(\alpha\)-Keto acids were decarboxylated with neutral hydrogen peroxide and the resulting saturated acids were isolated by silicic acid column chromatography as described (1). Acids were dissolved in 0.5 ml of \(2\)H2O for nuclear magnetic resonance spectroscopy. Pyruvate was converted to lactate by reduction with excess borohydride. The lactate was then assayed to a Dowex 1-C1- column (1 × 15 cm) and subsequently eluted with 0.1 N HCl. The lactate fractions were evaporated to dryness, dissolved in water, neutralized, and passed through a column of Dowex 50-H+ (1 × 15 cm) eluted with water. Lactate samples were evaporated to dryness and dissolved in 0.5 ml of \(2\)H2O for nuclear magnetic resonance spectroscopy. Lactate samples were subjected to paper electrophoresis at pH 8.9 in 1% ammonium carbonate for 20 min at 60 volts per cm. Authentic lactate migrates 15 cm to the positive electrode under these conditions.

Stopped flow experiments were performed in a Gibson-Milnes instrument. Anaerobic conditions were obtained by five cycles of alternate evacuation and flushing with nitrogen. Substrate concentrations were varied as indicated. Small amounts of contaminating \(\Delta\)-serine in chloroalanine preparations and \(\Delta\)-threonine contaminations in \(\Delta\)-chloro-\(\alpha\)-aminobutyrate solutions were removed by prior inclusion of \(\Delta\)-serine dehydrogenase in the substrate solutions. With \(\beta\) chloro \(\alpha\) aminobutyrate in stopped flow experiments, identical results were obtained whether the solutions mixed were aerobic or anaerobic.

RESULTS

Elimination Reaction with \(\alpha\)-Amino-\(\beta\)-chlorobutyrate—By analogy with results previously obtained with \(\beta\)-chloroalanine (1), it would be expected that \(\alpha\)-amino acid oxidase should, under anaerobic conditions, catalyze the conversion of \(\alpha\)-amino-\(\beta\)-chlorobutyrate to \(\alpha\)-ketobutyrate. This was found to be the case. When \(\alpha\)-amino-\(\beta\)-chlorobutyrate was added to \(\alpha\)-amino acid oxidase under anaerobic conditions, a keto compound was formed as indicated by a colorimetric phenylhydrazine reaction (1). The spectrum obtained from the enzymatic reaction and reference spectrum of authentic \(\alpha\)-ketobutyrate are shown in Fig. 1. The spectrum is that of an \(\alpha\)-keto acid and is clearly different from that of \(\alpha\) keto acids containing chloro substituents in the \(\beta\) position (1). The formation of ketobutyrate was further confirmed by oxidatively decarboxylating the enzymatically formed keto acid with \(\text{H}_2\text{O}_2\). The resulting product was chromatographed on silicic acid as described previously (1). Its elution volume was identical to authentic propionic acid and was clearly separated from \(\alpha\)-chloropropionic acid, which would have been obtained by decarboxylation of \(\beta\)-chloro-\(\alpha\)-ketobutyric acid.

The effect of \(\text{O}_2\) upon the action of \(\alpha\)-amino acid oxidase on \(\beta\)-chloro-\(\alpha\)-aminobutyrate was examined. No \(\text{O}_2\) uptake was observed when \(\beta\)-chloro-\(\alpha\)-aminobutyrate was added to en-
Evidence for an enamine intermediate in the anaerobic elimination reaction with chloroalanine and chloroaminobutyrate

In Experiments 1 and 2, an aerobic elimination was carried out in *H2O. The amount of deuterium incorporated into pyruvate and *CH3 was calculated as described under "Methods." Incubations 3, 4, and 5 contained, in a final volume of 15.0 ml: 50 mm NaPPi (pH 8.5), 2 mg of catalase, 0.05 mM FAD, 1500 mg of substrate, and d-amino acid oxidase added last in. In Experiments 3 and 4, 24 mg of enzyme were used, while 50 mg of enzyme was used in Experiment 5. The amount of keto acid formed in 4-hour incubations in air at 25° for 3, 4, and 5 was, respectively, 308, 870, and 425 mg.

| Experiment | Substrate | Atmosphere | Medium | Atoms of deuterium in *H position of product | Product optical rotation formed |
|------------|-----------|------------|--------|-----------------------------------------------|--------------------------------|
| 1          | DL-d-chloroalanine | *H2O | 0.5 | No determined |
| 2          | d-alanine | Air | *H2O | 0.0 | |
| 3          | DL-erythro-d-chloro-a-aminobutyrate | Air | *H2O | 0.5 | R,S |
| 4          | DL-a-aminobutyrate | Air | *H2O | 0.0 | |
| 5          | DL-erythro-d-chloro-a-aminobutyrate | Air | *H2O | 0.2 | |

*Product isolated as lactate in Experiments 1 and 2, as propionate in Experiments 3 to 5.*

When DL-threo- or erythro-d-chloro-a-aminobutyrate was employed, only 50% of the substrate reacted. Similarly, the d isomer alone was completely utilized under conditions where it was the limiting component.

The catalytic activity of the apoenzyme was also examined with this substrate. No product could be detected, nor was any O2 consumed. In these experiments sufficient enzyme was used so that 1% of the reaction obtained with the holoenzyme could be detected. Also, it was determined that F-FADH2 generated by anaerobic incubation with either n-alanine or diithonite (1) was inactive in the chloroaminobutyrate reaction. Thus, an oxidized flavin-enzyme complex is required for the elimination reaction, as was true with chloroalanine.

Enamine Formation as a Result of Chloride Elimination—To test the previously proposed hypothesis that the immediate product of the enzymatic elimination is the enamine (see Scheme 1), the conversion of the *d*-chloro-aminobutyric acids to keto acids was carried out in *H2O. The amount of deuterium incorporated into pyruvate and *CH3 was calculated as described. In each case only 0.5 atoms of deuterium were incorporated. For determination of deuterium content, product keto acids were decarboxylated: pyruvate to lactate, and *CH3 to propionate. The optical rotation of the *d*-H.
propionate derived from $\beta$-$^3$H ketobutyrate was found to be zero. Thus, one-half of the product molecules contain deuterium which is incorporated nonenzymatically after release from the enzyme. We conclude from these results that these molecules, about 50% of the product, are released as enamine and then ketonize spontaneously. Two structurally analogous substrates which undergo normal oxidative reactions exclusively, alanine and $\alpha$-aminobutyrate, were converted to product keto acids in $H_2O$, and the resulting keto acids were subjected to the same isolation procedure (oxidative decarboxylation) as that applied to the products derived from chloroalanine and $\alpha$-amino- $\beta$-chlorobutyrate. As shown in Table II no deuterium was incorporated. This indicates that the deuterium found in the keto acid products derived from the $\beta$-chloro-$\alpha$-amino acids was not introduced through nonenzymatic exchange into the imino acid or free keto acid.

**TABLE III**

**Direct evidence for intramolecular transfer of hydrogen from $\alpha$ carbon of chloroalanine to $\beta$ carbon of pyruvate**

The incubation mixtures for the first three lines of the Table contained 110 pmols of dl-$[\alpha-^3$H]chloroalanine (17,000 cpm of $^3$H per pmole), 50 mM NaPP (pH or pH 8.5), 0.05 mM FAD, 100 $\mu$g of catalase, and 2.4 mg of $\alpha$-amino acid oxidase. Incubations were performed anaerobically with aliquots withdrawn to estimate pyruvate formation. After 40 to 50 min, sodium borohydride (4.5 to 9.0 mg) was added to reduce the keto acid to lactate. The solutions were then shell frozen in Dry Ice-acetone mixtures and the water distilled under vacuum and collected. The volatile $^3$H was counted and listed under $^3$H$\_2$O. The residue after distillation was dissolved in 5 ml of $H_2O$ and the lactate was isolated by Dowex column chromatography as described in the legend to Table I.

| Substrate | Medium | Temperature | Atmosphere | $^3$H in H$\_2$O | $^3$H at $\beta$ position of products | Intramolecular transfer |
|-----------|--------|-------------|------------|-----------------|--------------------------------------|------------------------|
| dl-$\beta$-Chloro- $[\alpha-^3$H]alanine . . . . . . . . . . | $H_2O$ | 25° | 100% N$\_2$ | 230,000 | 67,000 | 20 |
| dl-$\beta$-Chloro- $[\alpha-^3$H]alanine . . . . . . . . . . | $H_2O$ | 4° | 100% N$\_2$ | 115,000 | 78,000 | 40 |
| dl-$\beta$-Chloro- $[\alpha-^3$H]alanine . . . . . . . . . . | $H_2O$ | 25° | 100% N$\_2$ | 265,000 | 85,000 | 24 |
| dl-$[\alpha-^3$H] Serine . . . . . . . . . . . . . . . . . . . | $H_2O$ | 25° | 20% O$\_2$ | 320,000 | 410 | 0.1 |

The presence of only 0.5 atoms of deuterium in the $\beta$ position of the product keto acids derived from the $\beta$-chboro-$\alpha$-amino acids suggests that some intramolecular hydrogen transfer has occurred. This point was tested directly with $\alpha$-amino-$\beta$-chloro- $[\alpha-^3$H] lactobutyrate. As shown in Table II, deuterium was found in the $\beta$ position of the enzymatically formed ketobutyrate. This result confirms that intramolecular hydrogen transfer occurs from the $\alpha$ carbon of $\alpha$-amino-$\beta$-chlorobutyrate to the $\beta$ carbon of the product, $\alpha$-ketobutyrate. Data presented below (Table III) show that with $[\alpha-^3$H]chloroalanine, direct $^3$H transfer also occurs to the $\beta$ position of pyruvate.

We conclude from these results that the initial product in the elimination reactions is an enzyme-bound enamine which can react through either of two pathways as shown in Scheme 2:

(a) The enamine is released from the enzyme and then spontaneously converts to the imino acid and finally the keto acid. (b) The enzyme-bound enamine is protonated, with the proton originally removed from the $\alpha$ carbon prior to release.

Through Reaction Path 1 the products acquire deuterium nonstereospecifically from the solvent, while Reaction Path 2 involves intramolecular hydrogen transfer and no deuterium incorporation from the solvent. To test whether two competing pathways actually exist, $[\alpha-^3$H]chloroalanine was converted to pyruvate at two different temperatures. If two pathways exist, they will probably have different temperature dependences, so that the amount of intramolecular $^3$H transfer should vary with temperature (9). The data in Table III confirm this expectation. At 4° there is twice as much intramolecular transfer as at 25°. The intramolecular hydrogen transfer which occurs is presumably mediated by a base at the active site. This base could be a base which, when fully protonated, has 3 hydrogen atoms, such as $-NH_3^+$, or 1 hydrogen atom, such as imidazole-$H^+$ or $-COOH$. Since as much as 40% intramolecular $^3$H transfer occurs with $[\alpha-^3$H]chloroalanine, it is unlikely that it is a 2-hydrogen base. With such a base one would expect no more than 33% $^3$H transfer if there were no isotope effect; with an isotope effect the value would be lower. The data in Table III provide further evidence for a 1-hydrogen base. The same amount of $^3$H transfer occurs in $H_2O$ and $^3$H$\_2$O. If a 3-hydrogen base were involved, in $H_2O$ there would be 2 $^3$H atoms and $^3$H, which should lead to increased percentage of internal $^3$H transfer in $^3$H$\_2$O.

Substrate Kinetic Isotope Effects on Product Formation with $[\alpha-^3$H]Threonine and $[\alpha-^3$H]Chloroaminobutyrate—Our previous studies with chloroalanine indicated a kinetic isotope effect of 1.7 to 1.9 when the $\alpha-^3$H was replaced by $^3$H (1). This isotope effect was constant from 0 to 100% $O_2$, while product was changing from pyruvate to chloropyruvate. These data suggested...
TABLE IV
Substrate kinetic isotope effects on product formation with [α-2H]-threonine and chloroamino[α-2H]butyrate

Each incubation mixture contained, in 1.0 ml: 50 mM Na2PPi (pH 8.5), 50 μg of catalase, 0.05 mM FAD, 30 mM n-substrate, and 600 to 800 μg of n-amino acid oxidase added last to start the incubation. Incubations were in air at 25° in the indicated oxygen atmospheres. Aliquots were removed for keto acid assay at 1-, 2-, 5-, 10-, and 15-min points. The reaction was linear through at least 10 min and the ratio k¹H:k¹H remained constant in each aliquot.

| Substrate | Atmosphere | V₀ | k¹H:k¹H |
|-----------|------------|----|---------|
| DL-[α-2H]Threonine | 20% O₂ | 0.068 | 1.95   |
| DL-[α-2H]Threonine | 20% O₂ | 0.035 |         |
| DL-[α-2H]Threonine | 0% O₂  | 0.000 |         |
| DL-[α-2H]Threonine | 0% O₂  | 0.000 |         |
| DL-erythro-chloroamino[α-2H]butyrate | 20% O₂ | 0.11  | 1.84   |
| DL-erythro-chloroamino[α-2H]butyrate | 20% O₂ | 0.060 |         |
| DL-erythro-chloroamino[α-2H]butyrate | 0% O₂  | 0.12  | 1.97   |
| DL-erythro-chloroamino[α-2H]butyrate | 0% O₂  | 0.061 |         |
| DL-erythro-chloroamino[α-2H]butyrate | 100% O₂| 0.11  | 1.81   |
| DL-erythro-chloroamino[α-2H]butyrate | 100% O₂| 0.061 |         |

that for chloroalanine there existed some slow, common hydrogen transfer step during formation of either keto acid product. To determine if, similarly, some step involving transfer of the substrate-derived α proton might be a slow step during enzymatic conversion of chloroaminobutyrate to α-ketobutyrate, the α-deuterated amino acid was synthesized and incubated with the enzyme. Results from such incubations comparing α-H and α-DH substrate are shown in Table IV. For purposes of comparison, data for [α-2H]threonine are also shown in Table IV. Both the chloroaminobutyrate and threonine show kinetic isotope effects of just under 2, even though one substrate undergoes the O₂-independent elimination while the latter is always oxidized.

Effect of β-Chloro-α-aminobutyrate on the Absorption Spectrum of Enzyme-bound Flavin—When chloroaminobutyrate is added to anaerobic solutions of E-FAD, a visibly green color develops as a flavin spectrum with long wave length absorbance is generated (Fig. 2A). For purposes of comparison the spectra obtained with anthranilate (Fig. 2B), as well as the steady state spectra obtained when n-amino acid oxidase acts upon chloroalanine (2C), are shown. The green spectrum obtained after addition of chloroaminobutyrate persists through the anaerobic catalytic process (the elimination reaction), but returns to the yellow spectrum of E-FAD when the substrate is exhausted. The modified flavin spectrum has long wave length absorbance to 800 nm. Also, there is a hypsochromic shift of the 455-nm peak of FAD to about 435 nm and a similar smaller shift in the 375-nm band. The three and erythro diastereomers generated identical spectra.

This same absorption spectrum of enzyme-bound flavin persisted when chloroaminobutyrate was added to the enzyme in the presence of air and even in solutions equilibrated with 100% O₂. This supports the conclusion reached in earlier sections that oxygen has no effect in the enzymatic elimination reaction with chloroaminobutyrate. When alanine was added to the green solutions of enzyme and chloroaminobutyrate, anaerobically the bleached spectrum of fully reduced enzyme was...
produced, while in the presence of oxygen the yellow color of E-FAD was produced.

\[ E + S^{-} \rightarrow H \xrightarrow{2000 \text{min}} II \xrightarrow{40 \text{min}} III \rightarrow P + E \]

**Scheme 3**

**Stopped Flow Analysis of the Reaction between \( \delta \)-Amino Acid Oxidase and Chloroaminobutyrate**—When \( \delta \)-amino acid oxidase and chloroaminobutyrate were mixed in the stopped flow spectrophotometer, it was found that at least two long wavelength-absorbing species of the enzyme were formed, with distinctively different absorption spectra. The first formed intermediate (Compound I in Scheme 3) is characterized by comparatively weak absorbance at 600 nm and comparatively strong absorbance at around 505 nm. Its rate of formation is dependent on the concentration of chloroaminobutyrate. The second intermediate (II in Scheme 3) has much greater absorbance at 600 nm and lower absorbance at 505 nm than the first intermediate; its rate of formation does not vary with the chloroaminobutyrate concentration. Figure 3 shows kinetic traces of the reaction, recorded at 505 and 600 nm, and recorded on different time scales to emphasize the biphasic nature of the reaction. Under the concentration conditions of Fig. 3, analysis of the data shows the apparent first order rate constants for the formation of the two intermediates to be 1160 to 1100 min\(^{-1}\) and 36 to 37 min\(^{-1}\).

The kinetic measurements at 600 nm were repeated at different substrate concentrations and also with a range of concentrations of \( \delta \)-amino-\( \beta \)-chloro-\( \alpha \)-amino-\( \beta \)-aminobutyrate. The results are summarized in Table V. It is clear that only the rate of the fast phase is substrate-dependent and shows a kinetic isotope effect of 5 (Fig. 5). The slower rate shows no substrate deuterium kinetic isotope effect.

Both the fast rate and the slower rate observed in these stopped flow traces (Table V) exceed the turnover number of the enzyme with this substrate. With different enzyme preparations the turnover number (assuming a molecular weight of 40,000 per active site (10)) varies from 0 to 9.5 per min. So some subsequent reaction after those observed by stopped flow must be slow in conversion of the green complex to observed keto acid product.

**DISCUSSION**

The data reported here show that \( \delta \)-amino acid oxidase catalyzes the nonoxidative, oxygen-independent conversion of \( \alpha \)-amino-\( \beta \)-chlorobutyrate to ketobutyrate + \( \text{NH}_3^+ + \text{Cl}^- \). This type of elimination reaction has now been demonstrated with three substrates with \( \delta \)-amino acid oxidase (chloroalanine (1), \( \alpha \)-acetyl-serine, and chloroaminobutyrate). It has also been shown to occur with \( \delta \)-amino acid oxidase (chloroalanine) subsequent reaction after those observed by stopped flow must be slow in conversion of the green complex to observed keto acid product.

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Fig. 4. Changes in absorbance on reaction of \( \delta \)-amino acid oxidase with \( \beta \)-chloro-\( \alpha \)-aminobutyrate. Enzyme, 2.86 \( \times \) \( 10^{-3} \) M with respect to bound FAD, was mixed with an equal volume of \( \frac{m}{75} \) \( \delta \)-\( \beta \)-chloro-\( \alpha \)-aminobutyrate. The initial and 2-min spectra were recorded with a Cary 17 recording spectrophotometer; the spectra at 175 ms and 8 s were obtained with a Gibson-Milnes stopped flow spectrophotometer. All spectra are displayed relative to the initial enzyme concentration (2.86 \( \times \) \( 10^{-5} \) M). Conditions, in 0.1 M Na pyrophosphate, pH 8.5, 25°. The substrate was preincubated for 20 min before use with 1.1 \( \times \) \( 10^{-4} \) M \( \delta \)-serine dehydrase prepared from Escherichia coli (a gift of Dr. R. Labow) in order to remove any contaminating \( \delta \)-threonine.

**TABLE V**

| Substrate                | \( k_f \) | \( k_{II} \) | \( k_{III} \) |
|--------------------------|-----------|--------------|--------------|
| DL-\( \delta \)-erythro-chloroamino[\( \delta \)-\( H \)]-butyrates | \( \delta \)-Erythro-chloroamino[\( \delta \)-\( H \)]-butyrates | \( \delta \)-Erythro-chloroamino[\( \delta \)-\( H \)]-butyrates | \( \delta \)-Erythro-chloroamino[\( \delta \)-\( H \)]-butyrates |
| \( m/1000 \)            | 220       | 32           | 0.97         |
| \( m/800 \)             | 410       | 33           |             |
| \( m/400 \)             | 712       | 38           |             |
| \( m/60 \)              | 1600      | 38           |             |
| **Infinite**            | 2000      |              |             |
| DL-\( \delta \)-erythro-chloroamino[\( \delta \)-\( H \)]-butyrates | \( \delta \)-Erythro-chloroamino[\( \delta \)-\( H \)]-butyrates | \( \delta \)-Erythro-chloroamino[\( \delta \)-\( H \)]-butyrates | \( \delta \)-Erythro-chloroamino[\( \delta \)-\( H \)]-butyrates |
| \( m/1000 \)            | 122       | 38           |             |
| \( m/400 \)             | 200       | 40           |             |
| \( m/200 \)             | 275       | 42           |             |
| **Infinite**            | 400       |              |             |

* Indicates the concentration of \( \delta \) isomer after mixing.
.......from this group is the proton originally removed from the a-carboxyl group at the enzyme active site. The proton donated to the solvent hydrogen (deuterium) at the p carbon of the product. This involves the specific addition of a proton from a l-proton to the normal catalytic process.

Pathway 1 (responsible for about 50% product formation at 25°C in H2O with a-amino-β-chlorobutyrate) involves direct release of the enamine to the solvent where it is subsequently converted nonenzymatically to the imine product. This pathway results in the observed nonstereospecific incorporation of solvent hydrogen (deuterium) at the β carbon of the product. Pathway 2 involves protonation of the enzyme-bound enamine and conversion to the imine prior to release from the enzyme. This involves the specific addition of a proton from a 1-proton basic group at the enzyme active site. The proton donated from this group is the proton originally removed from the α-carboxyl group of the β-chloroaminobutyrate substrate. Therefore, the substrate α proton, after transfer to the basic group on the enzyme, is not subject to exchange with solvent protons until after product release. The ability of an enzyme to shield a specific proton from exchange with solvent has been observed in other enzymatic reactions (9). The nonexchangeability of the enzyme-held α proton with n-amino acid oxidase may also account for our previous observation that no hydrogen exchange occurs into the α position of unreacted substrate when the reaction is carried out in H2O or D2O (1).

It might be noted that the proton introduced at the β carbon by Pathway 2 in Scheme 2 should be introduced stereospecifically; this point has not been tested.

The catalytic capability detected here, of n-amino acid oxidase to catalyze the shielded transfer of a proton from the α carbon of a substrate to the β carbon of the product molecule, may possibly have relevance to hydrogen transfer mechanisms in other flavoenzymes. Pyridine nucleotide transhydrogenase (13) and cytochrome b5 reductase (14) are two flavoproteins which have been shown to catalyze direct hydrogen transfer; while hydride ion transfer mechanisms are generally invoked, shielded proton transfers remain possible.

The conversion of β-chloro-α-aminobutyrate to α-ketobutyrate differs from the corresponding reaction with chloroalanine in that no oxidative reaction occurs even in an atmosphere of 100% O2. This difference in reaction pattern must be attributed to the presence of an additional —CH2 group in α-amino-β-chlorobutyrate, which somehow greatly enhances the rate of the elimination reaction relative to the oxidative process. In an atmosphere of 100% O2 less than 2% pyruvate is formed from chloroalanine (1). This indicates that the oxidation process is at least 50 times as fast as the elimination reaction under these conditions. With α-amino-β-chlorobutyrate in 100% oxygen, no oxidative product (<0.2%) is detected so that the elimination reaction now proceeds at least 500 times faster than the oxidative process. This relatively large enhancement of the elimination rate relative to the oxidation (500 x 50 = 25,000-fold in changing from chloroalanine to chloroaminobutyrate) cannot be entirely due to an electronic effect of the —CH2 group and must be attributed to steric effects which make it more difficult to achieve the geometry required for electron transfer than for the elimination reaction. Until it is known what, if any, covalent intermediates are required for electron transfer, it is impossible to further define the mechanism of selection against the oxidative reaction.

The long wave length flavin intermediates formed in the chloroaminobutyrate elimination reaction have the spectra of oxidized type flavins, i.e. full absorbance at 450 nm, consistent with the lack of oxygen reactivity during enzymatic conversion of chloroaminobutyrate. A similar long wave length spectrum is seen with chloroalanine during elimination to form pyruvate. With this substrate, however, at high substrate concentrations, the absorbance at 450 nm in the intermediate decreases, indicating formation of reduced flavin. This is consistent with our earlier observation that chloroalanine, unlike chloroaminobutyrate, is subject to normal oxidation as well as elimination.

While the long wave length intermediates forming on addition of chloroaminobutyrate to n-amino acid oxidase are unusual, the spectral patterns are not entirely novel. At high pH α ly-sine is a substrate for the enzyme and a green complex is developed which arises from some interaction of the cyclized reaction product with E-FAD (15). A similar long wave length absorption band is observed on interaction of the oxidized

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enzyme with \( \Delta^1 \)-piperidine-2-carboxylate, the oxidation product of the substrate piperolic acid (16). Also sarcosine generates long wave length spectral patterns with \( \alpha \)-amino acid oxidase.\(^3\)

The green spectral pattern is not restricted to substrates. Anthranilate (\( \alpha \)-aminobenzoylate), a good competitive inhibitor of amino acid substrates, produces a spectrum on interaction with \( E{-}FAD \) that is remarkably similar in shape and extinction coefficients (16) to that generated with chloroaminobutyrate. These complexes have all the spectral characteristics of charge transfer complexes between a nitrogenous donor and oxidized flavin.

A kinetic analysis of the formation of the green long wave length intermediates in the chloroaminobutyrate reaction reveals the occurrence of at least three processes, with rate constants of 2000 \( \text{min}^{-1} \), 40 \( \text{min}^{-1} \), and 1 \( \text{min}^{-1} \) (Structures I, II, and III of Scheme 3). Since the over-all turnover number of the catalytic process with chloroaminobutyrate is 6 to 9.5 \( \text{min}^{-1} \), neither of the first two processes can be rate-determining, and the third process is probably not important in the catalytic reaction. Furthermore, the fast process (\( k = 2000 \text{min}^{-1} \)) shows a substrate deuterium isotope effect of 5 while the over-all catalytic process (measured as keto acid formation) shows an isotope effect of less than 2.

The large isotope effect on the fast phase in the stopped flow experiments indicates that removal of the \( \alpha{-}H \) of the substrate has occurred by this point in the reaction. The isotope effect is not carried over into the second phase (\( k = 40 \text{min}^{-1} \) for either \( \alpha{-}H \)-or \( \alpha{-}H \)-labeled substrate) and the steady state concentration of the second intermediate is the same for the \( \alpha{-}H \)- and \( \alpha{-}H \)-labeled substrate.\(^4\) The rate-determining process in product formation with the isotope effect of 1.7 to 1.9 must be a step (or steps) subsequent to the formation of the second intermediate and is not spectroscopically detectable.

Since the isotope effect of 5 occurring in the fast phase indicates that the \( \alpha{-}C{-}H \) bond has been broken early in the reaction sequence, and, if the elimination of \( \text{H}^+ \) and \( \text{CF}^+ \) is in fact a concerted process, then the elimination will be complete with the first, fast spectroscopically detectable, phase. If this is true, then the first green complex consists of oxidized flavin and initial reaction product (either enamine or imine). If the elimination is not concerted, and there is a discrete \( \alpha \)-carbanion prior to elimination of the \( \beta \)-chloro group, then the first green complex must contain oxidized flavin and a substrate-derived carbanion and the second complex could consist of flavin and enamine.

It is highly unlikely that either of the green complexes is the adduct of the amino acid and oxidized flavin proposed by Hamilton (17) (Structure 1 of Fig. 6) since formation of this adduct would not show an isotope effect (the \( \alpha{-}H \) would not have been removed) nor have the spectrum of the fully oxidized flavin-enzyme. The spectral pattern also makes it unlikely that the green complex is a covalent adduct from reaction between oxidized flavin and substrate-derived carbanion (Structure 2 of Fig. 6) since model compounds show different spectra (18). If such a covalent adduct between substrate and flavin is found it is not detectable by spectroscopic analysis.

Although the conversion of \( \alpha{-}NH_2 \)-\( \beta \)-chlorobutyrate to keto-butyrate shows a deuteron isotope effect, it appears reasonably certain that the rate-limiting step is not the breaking of the \( \text{C}-\text{H} \) bond. It has been shown in a number of cases that the rate-determining step in reactions catalyzed by \( \alpha \)-amino acid oxidase is dissociation of products from enzyme-FAD (19). It is important to note that in the elimination reaction, the final complex, \( E{-}FAD{-}\text{imine or } E{-}FAD{-}\text{enamine is identical or very similar to the complex formed in the oxidative process. Therefore it is very likely that product dissociation is also rate-limiting in the elimination reaction. If that is the case then the observed isotope effect must be associated with some proton transfer step involved in or prior to product release. We tentatively propose that proton transfer between basic groups on the enzyme occurs prior to product release as shown in Scheme 4. A rate-determining proton transfer has also been proposed (20) for the reaction of L-leucine with L-amino acid oxidase. The observed isotope effect is then on the equilibrium between \( I \) and \( II \) on Scheme 4 rather than a rate-isotope effect.

The data available so far suggest that an early step between substrate and flavin coenzyme involves abstraction of the \( \alpha{-}H \) as a proton. The subsequent mechanism of electron transfer in substrate oxidation is still uncertain. A fundamental problem to be resolved is whether a covalent adduct between substrate and flavin at position C-4a or N-5 occurs, or whether the electron transfer to flavin occurs without covalent intermediates such as by hydride transfer from the amino nitrogen of the substrate (20) or through direct electron transfer from a molecular complex (21).

So far, no direct evidence for covalent intermediates has been obtained. However, several suggestive pieces of evidence can...
be considered. (a) Although no net electron transfer to the flavin coenzyme occurs in the elimination reactions catalyzed by the flavoprotein oxidases, the absolute requirement for oxidized flavin-enzyme complex could be construed as a requirement for flavin participation in covalent catalysis. On the other hand, the role of the coenzyme may be to provide the correct active site geometry for positioning of substrate and basic groups of the apoprotein. (b) Adducts between the flavin of d-amino acid oxidase and nitromethane (22) and between the flavin of lactate oxidase and 2-hydroxy-3-butyrate (23) have been reported recently. However, both of these adducts have been isolated after inactivation of the respective enzymes, and the role of such adducts as intermediates in catalysis has yet to be determined.

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