REACTIONS OF β-FLUOROALANINE AND β-BROMOALANINE WITH D-AMINO ACID OXIDASE

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SUMMARY. D,L-β-Bromoalanine hydrobromide has been prepared from D,L-β-chloroalanine hydrochloride, and shown to be a good substrate for pig kidney D-amino acid oxidase, undergoing the oxygen-independent elimination of HBr exclusively. D-Fluoroalanine, however, undergoes solely the normal oxidation reaction to fluoropyruvate.

INTRODUCTION. During our investigation on the elimination reaction catalyzed by D-amino acid oxidase (1,2) with β-chloroamino acids, we have been interested in preparing β-bromoamino acids, to be used as substrates for that reaction. To our knowledge, the detailed synthesis of such compounds have not appeared in the literature.[1] We report here a straightforward preparation of D,L-β-bromoalanine, and its use as a substrate for the elimination reaction. We have also tested D-β-fluoroalanine (3) as an enzyme substrate, and present these results in this communication.

MATERIALS AND METHODS. D,L-β-Chloroalanine was purchased from P.L. Biochemicals. [2-^{1}H]- and [2-^{2}H]D-β-fluoroalanine were gifts from Dr. Janos Kollonitsch (Merck, Sharp and Dohme Co.)

D-Amino acid oxidase was purified from pig kidneys (4) to a specific activity of 20 U/mg with D-alanine as substrate.

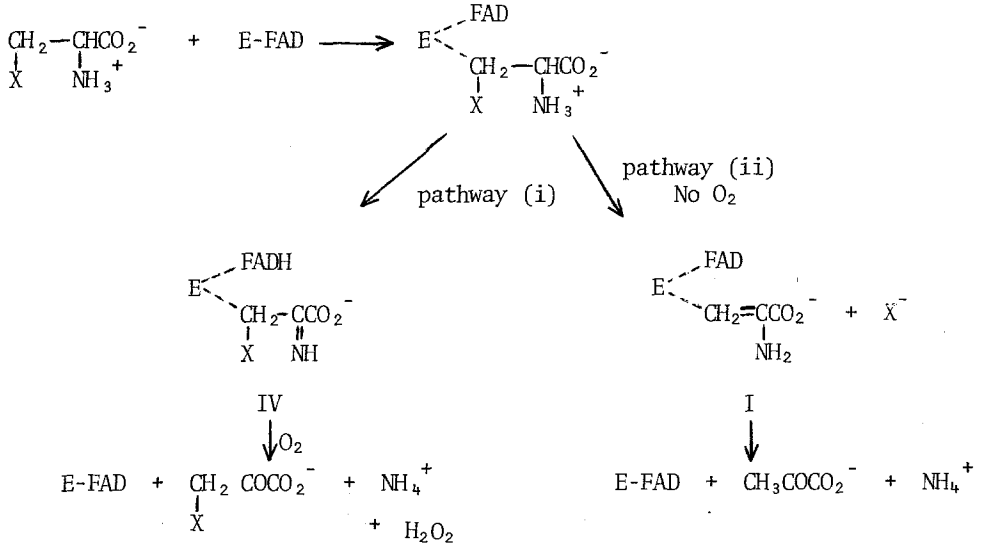
D,L-β-Bromoalanine was prepared from D,L-β-chloroalanine. Typically, 100 mg of D,L-β-chloroalanine·HCl in 2ml of 40%(w/w) anhydrous HBr in acetic acid contained

[1] L-Bromoalanine has been used by Morino and Okamoto (10); but neither the preparation not any physical datum was reported.

in a sealed tube, was heated in a steam bath for 14 hrs. Solvent was then removed by rotavaporation. The light brown solid left behind was crystallized from acetic acid/ethyl acetate. 50 mg of β-bromoalanine.HBr a white crystalline solid (32% yield), was obtained, m.p. 159-160°C. The melting point of β-chloroalanine·HBr is 133-135°C. The NMR spectrum of β-bromoalanine is distinct from that of the starting material: the two doublets due to the β-protons are now at ca. δ3.9 instead of δ4.1 for β-chloroalanine. When β-chloro-D-alanine was the starting material, somewhat surprisingly the product was again the racemic β-bromo-D,L-alanine (as assayed with both D- and L-amino acid oxidases), suggesting that bromination occurs by an elimination-addition sequence with an enamine intermediate under the forcing conditions used.[2]

α-Keto acid productions were assayed by monitoring NADH oxidation with lactic dehydrogenase. Oxygen consumption was monitored with a Clark-type oxygen electrode.

Scheme 1



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[2] Curiously, our preliminary results (Y. Cheung, and C.Walsh, unpublished) indicates that only one diastereomeric pair of β-bromo-α-aminobutyrate is formed from erythro-β-chloro-α-aminobutyrate, under similar reaction conditions.

RESULTS AND DISCUSSION. D-β-Chloroalanine has been shown to be a substrate for D-amino acid oxidase, partitioning between the normal oxidation (yielding chloropyruvate) and the oxygen-independent elimination reaction (yielding pyruvate)(1), with the minimum mechanism as shown in Scheme 1 (X = Cl).

The partition between the two pathways depends on the oxygen concentration Under atmospheric conditions, 65% of the D-β-chloroalanine molecules undergo the elimination pathway (1); 100% of them eliminate in the absence of oxygen.

In contrast, we now show that D-β-bromoalanine undergoes the elimination reaction exclusively (pathway (ii) in Scheme 1, X = Br). Within the sensitivity limit of our oxygen electrode, no O_2 consumption was observed, while coupled lactic dehydrogenase assay showed rapid α-keto acid (pyruvate) production. Under anaerobic conditions, turnover proceeds with no decrease in V_{max} consistent with catalytic loss of HBr.

On the other hand, a third β-halo amino acid, D-β-fluoroalanine was observed to undergo oxidation (pathway (i) in Scheme 1, X = F) without detectable elimination of F^- ion. The rate of O_2 consumption equals that of total α-keto acid production (measured in parallel assays), within experimental uncertainties, which we estimated to be ±5%. Moreover, no anaerobic turnover was detectable with D-β-fluoroalanine. Comparative data are presented in Table 1.

Table 1. β-Haloalanines as Substrates for D-Amino Acid Oxidase.

Substrate	V_{max} (μmol/min/mg)	K_m(mM)	%Elimination[a]
D-Alanine	20.0	1.3	none
D-β-Fluoroalanine	18.6	2.0	<<5
D,L-β-Chloroalanine	5.4 (5.5)[b]	0.2	65[b]
D,L-β-Bromoalanine	10.4	0.5	>99
D-β-Fluoro-[2-^{2}H]alanine	10.8	2.0	c

a. Under atmospheric oxygen. b. Reference (1) c. Not determined

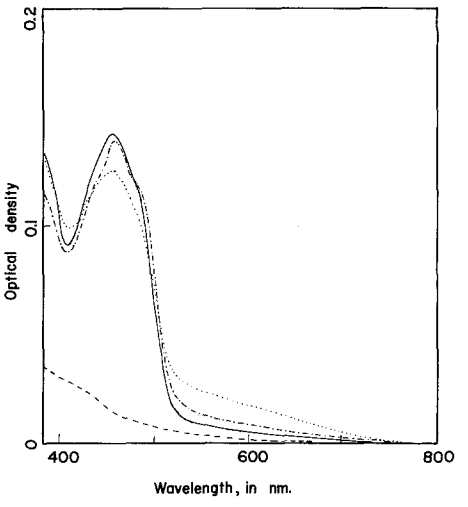


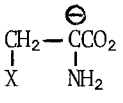
Figure I. Steady state spectra during D-amino acid oxidase catalysis with, as substrate: (A) - , none (i.e. oxidized enzyme-FAD); (B) -·-·, 6 mM D,L-β-bromoalanine·HBr; (C) ····, 6 mM D,L-β-chloroalanine·HCl; and (D) ---, 6 mM D-β-fluroalanine. Each solution contained 30 μmoles of NaPPi (pH 8.3), and 0.27 mg of D-amino acid oxidase. All substrate solutions were adjusted to neutrality before using. The spectra were taken immediately after addition of the substrates, under aerobic conditions on a Perkin-Elmer 200 spectrophotometer.

Further evidence that D-β-bromoalanine molecules always react by loss of HBr is obtained by examination of the steady state spectra of the FAD enzyme during catalysis, as shown in Figure 1. Curve A is the spectrum of Enzyme-bound FAD in the oxidized form, B is the spectrum in the presence of saturating D,L-β-bromoalanine. There is no reduction in the peak at 455 nm, showing that FAD is still in the oxidized form. An identical spectrum is obtained (during product formation) under anaerobic conditions, showing that $FADH_2$ does not accumulate as it would after stoichiometric reaction with a normal oxidizable substrate. Two useful characteristics are the vibronically resolved shoulder at 480 nm, and the long wave-length absorbance in the region 500-600 nm, indicating the presence of a charge transfer complex. This is almost identical to the

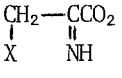
steady state spectrum seen with D,L-β-chloroalanine (C), except that in the latter, the vibronic resolution is less pronounced, and there is some reduction in A_{455}. On the other hand, in the presence of saturating D-β-fluoroalanine, the spectrum is that of reduced $FADH_2$, as O_2 in the solution is completely consumed. We suggest that the essentially identical steady state spectra with β-bromoalanine and β-chloroalanine represent the complex of oxidized enzyme with the common enzyme-bound enamine aminoacrylate (I in Scheme 1).

Accumulation of the enzyme-enamine complex is consistent with earlier observations that product release is rate limiting in the HCl elimination reactions (5,6,7), and that the Cl^- release step is very fast with β-chloro-α-aminobutyrate (5).

Examining the behavior of the three β-halo alanines of Table I, one sees a correlation between the leaving group ability and the percentage of elimination. Thus with the substituents bromide, chloride, and fluoride (relative leaving group abilities: 1, 0.02, and 0.0001 respectively (8)), the mode of reaction ranges from 100% elimination of HX to 0% elimination. This raises the question of which intermediate is partitioning between the oxidation and elimination pathways. The two most likely species are the carbanion (II) formed on abstraction of the α-proton, and an initial β-halo imino acid (III) product which could form by two electron oxidation of substrate.



II



III

The mechanism involving partitioning through III (5,7) (presumably by attack of $FADH_2$ on carbon 2 and subsequent loss of X^- from a "tetrahedral adduct") should not display a dependence of percentage elimination on leaving group ability, unless X^- release is slow compared to reoxidation of $FADH_2$ by O_2, and <u>all</u> interconverting steps are fully reversible. It is known that Cl^- release is actually

very fast, at least in the case of β-chloro-α-aminobutyrate (5). On the other hand, this leaving group dependency is what we would expect when II is the partitioned intermediate. (it is also consistent with two concerted processes, elimination and oxidation, in competition with each other.)

The relative total reaction rates of β-bromo- and β-chloroalanine warrant comment at this point. Bright and Porter (7) have suggested that the reason β-chloroalanine reacts with a lower V_{max} than alanine (see Table 1) is that 50% or more of the enzyme is trapped as the slowly reacting enamine intermediate (I in Scheme 1), since they argue that I releases the product enamine about 12 times slower (7) than IV (as shown in Scheme 1), the product complex formed from the normal oxidation reaction, releases iminopyruvate. They further suggested that with β-chloro-α-aminobutyrate, the reaction rate is even slower (V_{max} = 0.02 that of alanine) because all enzyme molecules are tied up in a slowly dissociating enamine form. However, if their hypothesis is correct, it will predict that also for β-bromoalanine, which undergoes elimination exclusively, essentially all enzyme molecules would be trapped as the slowly dissociating oxidized enzyme-enamine complex I.

Consequently, the V_{max} reaction rate should be much smaller than with β-chloroalanine. Yet we observe a V_{max} rate twice as fast for β-bromoalanine. Thus, that hypothesis (7) about V_{max} rates is probably incorrect.

One further test that both β-bromoalanine and β-chloroalanine give rise to the same enamine intermediate (I) when reacting in the elimination mode is to carry out incubations in ^3H_2O. We have previously shown that during conversion of the β-chloromethyl group of chloroalanine to the methyl group of pyruvate, the third hydrogen derives in part from solvent and in part from the original substrate α-hydrogen (2). Since we have also demonstrated with the 4 carbon analogue β-chloroaminobutyrate that tritium incorporation from solvent on ketonization of the enamine intermediate results only from prior release of the enamine to solvent (6), it is likely that for chloroalanine as well that tritium incorporation from ^3H_2O monitors that fraction of product which has been released from the D-

amino acid oxidase active site as the enamine. If the same enamine is formed from β-bromoalanine and its partitioning between protonation at the active site and protonation free in solution is identical then the specific radioactivity of the pyruvates from β-bromoalanine and β-chloroalanine in ^3H_2O should be equal. The data of Table II verify this prodiction when the product β-[^{3}H]-pyruvates were trapped as β-[^{3}H]-lactate, isolated, and specific radioactivities determined.

Table II. Specific Radioactivity of β-[^{3}H]-lactate from coupled Reaction of β-Bromoalanine (Aerobically) and β-Chloroalanine (Anaerobically) with D-Amino Acid Oxidase and Lactate Dehydrogenase in ^3H_2O.

Conditions	Compound	μmoles reacted	Specific Radioactivity cpm/μmole
aerobic	β-bromoalanine	0.66	13,000
anaerobic	β-chloroalanine	0.48	12,700

The incubation with β-bromoalanine combined in a volume of 0.99 ml of ^3H_2O (6.4×10^9 cpm): 3 μmoles NADH; 75 μmoles NaPPi, pH 8.3; 21 nmoles FAD, 68 μg lactate dehydrogenase, 1.32 μmole β-bromoalanine and 100 μg D-amino acid oxidase to initiate reaction. After quantitative conversion of the substrate to lactate, 5 μmoles of [^{14}C]pyruvate (1.4×10^4 cpm) was added to generate carrier [^{14}C]-lactate. The sample was placed on a 1 x 18 cm column of Dowex 1 Cl form and eluted with water until ^3H_2O counts had ceased to elute. The [^{14}C], [^{3}H]-lactate was then eluted with 5 mM HCl. Tritium specific activities were calculated after determining isolated product yield from the [^{3}H]/[^{14}C] ratio and correction for 30% ^{14}C crossover in the tritium channel.

The β-chloroalanine incubation was identical except that it was conducted under anaerobic conditions in a nitrogen atmosphere with glucose and glucose oxidase as an oxygen scavenging system. Conversion of added chloroalanine to lactate was quantitative and [^{14}C], [^{3}H]-lactate isolation followed the protocol of the β-bromoalanine experiment.

Since both β-bromo- and β-chloroalanine give rise to the same enamine intermediate (I) when reacting in the elimination mode, and since product release is slow for β-chloroalanine,it must also be slow for β-bromoalanine. Thus,

simplistically, V_{max} of the reaction should be proportional to the steady state concentration of the intermediate I, which is common in both cases for β-bromo- and β-chloroalanine. To account for the 2 fold faster V_{max} for the former, we have to postulate that bromide ion, being a much better leaving group, has resulted in a preequilibrium where twice as high a steady state concentration of the intermediate I is attained. Because of the high turnover number of bromoalanine, this relationship will have to be proved using rapid reaction stopped-flow kinetics.

We are currently unable to explain the deuterium kinetic isotope effect on V_{max} in the oxidation of D-β-fluoroalanine. D-alanine and D-β-fluoroalanine are about equally good substrates for D-amino acid oxidase (see Table 1). Yet only fluoroalanine displays a kinetic isotope effect (of 1.7). [2-^{1}H]- and [2-^{2}H]D-alanine show identical V_{max}'s. If indeed product release is the rate limiting step in the overall catalysis (5,7) for D-alanine than one might have expected it to be so in fluoroalanine oxidation as well, given the similar V_{max} value. This problem is under investigation.

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