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ENZYMATIC RELEASE OF NITRIC OXIDE FROM L-ALANOSINE, AN ANTINEOPLASTIC ANTIBIOTIC

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L-Alanosine is an antineoplastic drug which is the 3-iso-nitramino analog of L-aspartic acid. The drug is known to be metabolized to the corresponding 2-oxo acid. Unlike the parent amino acid, the 2-oxo acid is unstable under mild conditions. When the 2-oxo acid is generated *in vitro* by the aerobic action of L-amino acid oxidase on L-alanosine, the reaction mixture contains products capable of diazotizing sulfanilamide and of reducing ferricytochrome c to ferrocytochrome c. It is thus likely that, as expected from model reactions, the unstable 2-oxo acid derived from L-alanosine decomposes into nitric oxide and other reactive free-radical species. Enzymatically promoted production of highly cytotoxic nitric oxide may pertain to the biological activity of the antibiotic. The reaction should prove extrapolable to the design of other enzyme-activated cytotoxic agents.

INTRODUCTION

Compounds bearing ionizable nitroalkyl, nitramino, or iso-nitramino groups in place of carboxylate groups may serve as substrates and inhibitors of enzymes ordinarily acting on the corresponding carboxylic acids (1-4). For instance, mitochondrial succinate dehydrogenase (EC 1.3.99.1) is irreversibly inactivated by the toxic antibiotic 3-nitropropionic acid (bovinocidin) in a "suicide" reaction (1,5). Another flavin-dependent enzyme, renal D-amino acid oxidase (EC 1.4.3.3), is inactivated by the suicide substrate 1-chloro-1-nitroethane (D.J.T. Porter and T.A. Alston, in preparation). Pyridoxal 5'-phosphate-dependent enzymes are inactivated by the suicide substrates 5-nitro-L-norvaline and

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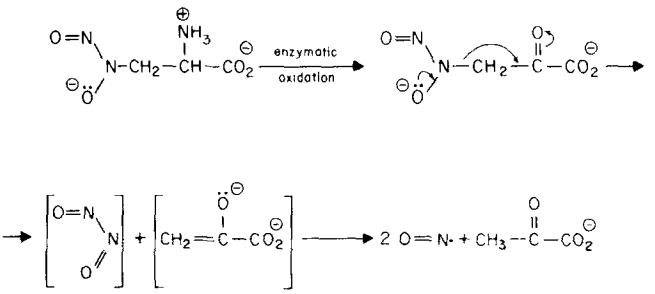

Figure 1. One mechanism for the enzymatic release of nitric oxide from L-alanosine. Decomposition of the enzymatically generated oxo acid proceeds nonenzymatically. Nitric oxide undergoes further reactions to yield many additional products. Other mechanisms are also plausible.

3-nitro-L-alanine (6). In other cases C-nitro and N-nitro compounds serve as competitive inhibitors or as substrates that are released into free solution as reactive products (7,8).

An isonitramino analog of L-aspartic acid is elaborated by Streptomyces alanosinicus. This antibiotic, L-alanosine (Fig. 1), exhibits antineoplastic activity, and there is evidence that this activity is to some degree secondary to metabolism of the analog by the L-aspartate-utilizing enzymes of purine biosynthesis (2,3). However, L-aspartate serves a multitude of metabolic functions, and the interactions of diverse enzymes with alanosine may thus be responsible for the therapeutic as well as adverse effects of the drug. When administered to the mouse, rat, dog, or monkey, a significant proportion of the drug is metabolized to the corresponding 2-hydroxy acid and is excreted as such (3). This finding indicates that the drug is converted to the 2-oxo acid in vivo. Oxidation of L-alanosine to this 2-oxo acid by pyridoxal 5'-phosphate-dependent transminases and by flavin-dependent oxidases has been demonstrated in vitro (3). The product, 3-(isonitramino)-pyruvate, is unstable. We report here that reactive free radicals probably including nitric oxide are among the decomposition products of the oxo acid obtained by the aerobic action of L-amino acid oxidase (EC 1.4.3.2) on L-alanosine. Nitric oxide and its auto-

oxidation products, such as nitrogen dioxide and nitrous acid, engage in deleterious reactions with cellular constituents. Since the evolution of nitric oxide from L-alanosine under mild conditions requires enzyme action, this example of "lethal synthesis" may well pertain to the selective toxicity of the drug.

MATERIALS AND METHODS

L-Alanosine (NSC 153,353) was a gift from Dr. D.A. Cooney, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute. L-Amino acid oxidase, a gift from Dr. D.J.T. Porter, was isolated (9) from lyophylized Crotalus adamanteus venom supplied by the Sigma Chemical Company. Equine heart cytochrome c and bovine liver catalase (EC 1.11.1.6) were Sigma products while bovine erythrocyte superoxide dismutase (EC 1.15.1.1) was supplied by Miles Laboratories Limited.

Visible spectra were recorded on the Cary 15 spectrophotometer. Oxygen consumption was measured by means of a Clark-type polarigraphic electrode from Yellow Springs Instruments. Inorganic nitrite was detected by means of a chromogenic diazo coupling reaction (10,11).

One unit of L-amino acid oxidase was taken to be that which converts 1.0 μmol of alanosine to products per min in a reaction mixture containing 5.0 mM L-alanosine, 0.24 mM oxygen, 100 mM KCl, 0.1 mM EDTA, and 10 mM 3-(N-morpholino)propanesulfonate at 25° and pH 7.2. L-Leucine (5.0 mM) is oxidized 1800 times as rapidly as L-alanosine by the enzyme under these conditions, while 5.0 mM L-aspartate is oxidized 0.6 times as rapidly as L-alanosine. One unit of oxidase corresponded to 220 mg of protein having an absorbance ratio of 10.6 at 278 and 462 nm.

RESULTS

In order to test the hypothesis that the oxo acid derived from L-alanosine decomposes into reactive free-radical products, we employed ferricytochrome c to detect one-electron-donating reductants in aerobic reaction mixtures of L-alanosine and L-amino acid oxidase. As reported by others (12), we have confirmed that the enzyme does not generate reductants of cytochrome c during oxidation of ordinary amino acids. However, as illustrated in Fig. 2, cytochrome c reduction does occur during the aerobic incubation of L-alanosine and the oxidase with initially oxidized cytochrome c. The actual heme-reductant is not the superoxide ion, $O_2^{\cdot-}$, because the reaction is not inhibited by superoxide dismutase

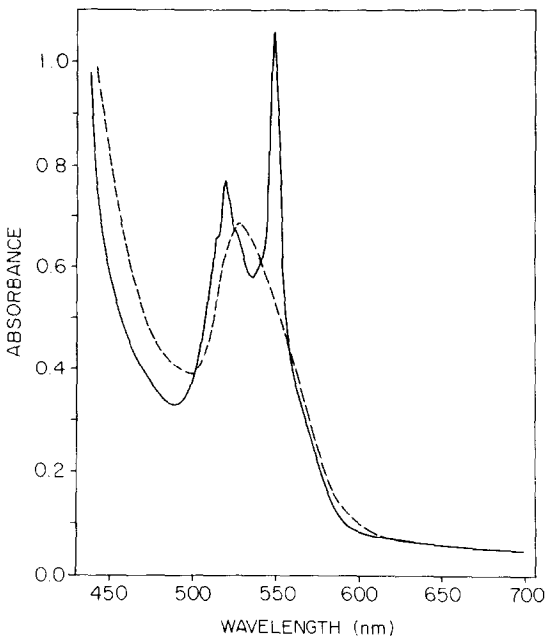

Figure 2. L-Amino acid oxidase-dependent reduction of ferricytochrome c by L-alanosine. L-Alanosine (40 mM) was incubated with L-amino acid oxidase (75 µg/ml) in a 1.0 cm open cuvette also containing 55 µM cytochrome c (initially ferric), 100 mM KCl, 0.1 mM EDTA, and 10 mM 3-(N-morpholino)propanesulfonate at 25° and pH 7.2. The electronic spectrum shown (solid line) was obtained after 4.0 h of reaction time and indicates reduction of 49% of the cytochrome c. The other spectrum (broken line) is that of a control experiment lacking the L-alanosine. In other control experiments, no cytochrome reduction occurred when the alanosine was replaced by 40 mM L-leucine or L-aspartate. Slight increase in the cytochrome absorbance at 550 nm (about 0.01 per cm) and 521 nm occurred during 4.0 h in the presence of 40 mM L-alanosine in the absence of L-amino acid oxidase. Less than 10% of the L-amino acid oxidase-dependent cytochrome reduction was inhibited by superoxide dismutase (1000 unit/ml as defined in ref. 13). Identical quantities of ferrocytochrome c appear in reaction mixtures exposed to room light as in those kept in the dark.

(12,13). The heme-reductant may prove to be nitric oxide (14,15), but the experiment described in Fig. 2 does not strictly quantitate nitric oxide production from alanosine since the reduction of ferricytochrome c by nitric oxide is a reversible reaction and because autoxidation products of nitric oxide are capable of oxidizing ferrocytochrome c.

Nitric oxide is known to give rise to inorganic nitrite in autoxidation and disproportionation reactions. We therefore assayed reaction mixtures for inorganic nitrite by means of a

chromogenic diazo coupling reaction that is highly specific for inorganic nitrite (10,11). The assay involves the diazotation of sulfanilamide by inorganic nitrite under acidic conditions followed by reaction of the diazonium product with N-(1-naphthyl)ethylenediamine to afford a pink diazo dye. L-Alanosine itself gives a negative test for nitrite when incubated in the chromogenic assay reaction mixture for the recommended 10 min (11), although the chromophore does slowly appear when the color is permitted to develop over several days. The alanosine is synthesized by the action of nitrite on 2-amino-3-(hydroxyamino)propanoic acid, and this reaction is slowly reversible under strongly acidic conditions. However, aliquots of reaction mixtures give rapidly positive tests for nitrite after L-alanosine is incubated aerobically with L-amino acid oxidase. For instance, when 500 nmol of L-alanosine is incubated for 20 h with L-amino acid oxidase (0.25 mg) in 0.1 ml of solution also containing catalase (.05 mg), 100 mM KCl, 0.1 mM EDTA, and 10 mM 3-(N-morpholino)propanesulfonate at 25^{o} and pH 7.2, then 30 nmol of inorganic nitrite is detected. No release of nitrite occurs under these conditions in the absence of the oxidase.

Discussion

We have found that, upon enzymatic oxidation, the antibiotic L-alanosine gives rise to species that are capable of reducing ferricytochrome c and of diazotizing sulfanilamide. Further experiments are required to establish the chemical details responsible for these observations. The hypothesis which prompted these experiments is outlined in Fig. 1. It is likely that the oxo acid derived from L-alanosine decomposes into nitric oxide under mild conditions. The suggested reaction may be viewed as analogous to evolution of carbon dioxide from oxalacetate derived from L-aspartate and also analogous to evolution of sulfur dioxide from 3-sulfinylpyruvate derived from cysteine sulfinic acid (16). The

reverse reaction, the synthesis of an isonitramino ketone from nitric oxide and a ketone, was reported by W. Traube in 1898 (17). Traube obtained the 2-isonitramino derivative of ethyl acetoacetate and also of other "active methylene" compounds by reaction with nitric oxide. Unlike 3-(isonitramino)pyruvate (Fig. 1), 2-(isonitramino)acetoacetic ethyl ester can be isolated as a stable disodium salt under anhydrous conditions.

Nitric oxide, nitrogen dioxide and other autoxidation products are highly cytotoxic. They convert hemoglobin, myoglobin, cytochromes and other metalloproteins to nonfunctional species (18,19). They react with amines and amides to afford N-nitroso species which then covalently modify other biomolecules (20). They oxidize sulfhydryl compounds (21) and furthermore damage membrane lipids, nucleic acids and other vital cellular constituents (22).

Vascular guanylate cyclase (EC 4.6.1.2) is covalently modified by nitric oxide, and several nitric oxide-generating compounds may thus serve as vasodilating agents (23). It is plausible that the experimental antihypertensive agent dopastin releases nitric oxide by a route analogous to that proposed in Fig. 1 for alanosine. Like alanosine, dopastin [N-(3-methyl-2-isonitraminobutyl)-2-butenamide] is a microbial product which may be viewed as a β-isonitramino amine (24).

The results suggest the possibility of pharmacologically exploiting the lethal synthesis of nitric oxide from congeners of L-alanosine.

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