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INHIBITION OF DOPAMINE β-HYDROXYLASE BY THIAZOLINE-2-CARBOXYLATE, A SUSPECTED PHYSIOLOGICAL PRODUCT OF D-AMINO ACID OXIDASE

Nariman Naber, Prasanna P. Venkatesan and Gordon A. Hamilton

Department of Chemistry
The Pennsylvania State University
University Park, PA 16802

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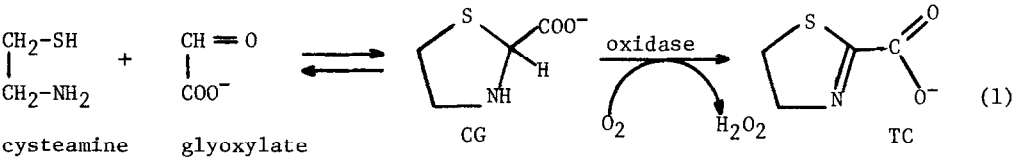
SUMMARY: Thiazoline-2-carboxylate was chemically synthesized and shown to be identical in all respects to the product formed in a D-amino acid oxidase catalyzed reaction involving cysteamine and glyoxylate. Both the chemically synthesized and enzymically prepared thiazoline-2-carboxylate are effective inhibitors of dopamine β-hydroxylase but they do not appreciably affect the activity of several other metalloenzymes that require copper, iron or zinc. The inhibition of dopamine β-hydroxylase is competitive with respect to the reactant ascorbic acid and uncompetitive with respect to tyramine. The possible physiological significance of this inhibition is briefly considered.

Recently we reported (1) that a solution containing cysteamine and glyoxylate contains some species that is an excellent substrate for D-amino acid oxidase, and furthermore we presented considerable circumstantial evidence that this reaction is probably occurring physiologically. The results strongly implied that the reactions occurring are those outlined in eq.1, namely,



(1)

that the adduct thiazolidine-2-carboxylate (CG), formed non-enzymically from cysteamine and glyoxylate, is the substrate and thiazoline-2-carboxylate (TC) the product of the enzymic reaction, but this was not conclusively established. As reported here, a direct comparison of chemically synthesized TC with the enzymic product now confirms this conclusion.

In considering what function TC might have in biological systems we were led to consider that one of its roles might be to complex metal ions, and thus control the activity of various metalloenzymes. One suspects that the metal ion complexing ability of TC should be similar to that of the anion of picolinic acid (pyridine-2-carboxylate), a compound known (2) to be a good complexing agent for several metal ions of biological interest, especially Cu(II), Fe(II)

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and Zn(II). Since picolinic acid derivatives are particularly good inhibitors of dopamine β-hydroxylase (3,4), we have especially investigated the inhibition of this copper containing enzyme by TC, but have also studied its effect on several other metalloenzymes as well. The results show that TC is a good inhibitor of dopamine β-hydroxylase but does not appreciably affect the rate of most of the other enzymes investigated.

EXPERIMENTAL PROCEDURE

MATERIALS: Unless otherwise noted, commercial materials were used as received. Thiazolidine-2-carboxylate (CG) was usually prepared *in situ* by leaving an aqueous solution containing 100 mM cysteamine and 105 mM glyoxylic acid sit for 1 hr at room temperature. For some experiments, crystalline CG, prepared by the method of Johnson and Smissman (5), was used; no differences in the reactivity of the CG's prepared by these methods was noted.

Thiazoline-2-carboxylate (TC) was chemically synthesized by hydrolysis of its ethyl ester which was prepared by the method of Erikson (6). Since Erikson's procedure has not appeared in the general literature, it will be given here. In the initial step ethyl-N-(2-mercaptoethyl) oxamate ($HSCH_2CH_2NHCOCOOCH_2CH_3$) was prepared as follows. Cysteamine hydrochloride (28.4 g, 0.25 mole), dissolved in 400 ml ethanol, was added with stirring over a period of 3 hrs to a solution of 36.5 g (0.25 mole) diethyl oxalate and 25.3 g (0.25 mole) triethylamine in 300 ml ethanol kept in an ice bath and under N_2. After sitting overnight at 0°C, the crystals of triethylamine hydrochloride were removed and the solution evaporated to dryness. To remove more of the triethylamine hydrochloride the residue was taken up in ether, stored overnight at 0°C, filtered, evaporated to dryness and the same procedure repeated. The oxamate (27 g, 62% yield) was obtained from the final pale yellow viscous residue by vacuum distillation (b.p. 120°C at 10 μ). Its NMR spectrum in CCl_4 is as follows: δ 7.80 (broad triplet, 1 H), 4.23 (q, 2 H), 3.50 (m, 2 H), 2.70 (m, 2 H), 1.57 (s, 1 H) and 1.37 (t, 3 H).

The oxamate (27 g) was dissolved in 250 ml anhydrous ether, cooled to 0°C, saturated with dry HCl gas, flushed with N_2 and left overnight at 0°C. The hygroscopic crystals (24 g, 80%) of ethyl thiazoline-2-carboxylate hydrochloride (m.p. 135°C with decomposition) were obtained by filtering in a dry box under inert conditions. This ester hydrochloride (13.5 g) was neutralized by dissolving in 200 ml ethanol and adding 15 ml triethylamine dropwise at 0°C. After flushing with N_2 and sitting for 30 min, the amine hydrochloride was removed by filtration, the solvent was evaporated and the ethyl thiazoline-2-carboxylate (9 g, 82%) obtained by vacuum distillation (b.p. 85°C at 20 μ). Its spectral characteristics are as follows: UV (in H_2O buffered at pH 7.0) λ_{max} 290 nm (ε = 1700 M^{-1}), IR (neat) 1745, 1725, 1605, 1280, 1250, 1065 cm^{-1} (all strong); NMR (in CCl_4) δ 1.39 (t, 3 H), 3.33 (t, 2 H), 4.26 (q, 2 H), 4.33 (t, 2 H); MS (electron impact) m/z 159, 115, 114, 87.

To 6.8 g of the ethyl ester in 300 ml carbonate free distilled water held at 55°C is added carbonate free NaOH until the pH remains stable at pH 8 (*ca.* 1.5 hr). After evaporation of the water the solid sodium thiazoline-2-carboxylate is washed with dry ether and dried (6.1 g, 90%). The solid is recrystallized by dissolving in hot methanol and adding ether to turbidity. Elemental analysis: found (theory in parentheses) C 31.62 (31.37), H 2.85 (2.63), N 9.16 (9.15), S 20.71 (20.93); UV (in 20 mM phosphate buffer, pH 7.0) λ_{max} 269.5 (ε = 1640 M^{-1}); IR (KBr) 1627, 1604, 1383, 1071, 1009, 800, 781 cm^{-1} (all strong); ^1H NMR (D_2O) triplets at δ 3.3 and 4.2 of equal intensity; ^{13}C NMR (D_2O-acetone D_6) δ 184.1, 179.5, 77.1, 46.4; MS (after treatment with CF_3COOH) m/z 131 and 87.

Solutions (*ca.* 20 mM) of thiazoline-2-carboxylate (TC) were also prepared enzymically as follows: O_2 was bubbled through a sintered glass disc into a

vigorously stirred solution containing 10 mM potassium phosphate, pH 7.4, 20 mM thiazolidine-2-carboxylate (CG), 0.15 mg/ml D-amino acid oxidase, 0.04 mg/ml catalase and 100 μM FAD, until the absorbance at 270 nm reaches a maximum (*ca.* 1 hr). The concentration of TC in these solutions was calculated from the absorption at 270 nm assuming the extinction coefficient found for synthetic TC.

ENZYMES AND ENZYME ASSAYS: Dopamine β-hydroxylase was purified from bovine adrenals by the procedure of Rush et al (7) with some modifications (8). All kinetic runs with this enzyme were performed at 37°C using an air atmosphere above solutions containing 200 mM acetate buffer, pH 5.0, 12 mM fumarate and 33 μg/ml catalase in addition to ascorbate and tyramine as given in the figure. The reactions were initiated by adding an aliquot of a dopamine β-hydroxylase solution and they were followed by measuring the initial rate of O_2 uptake using an O_2 electrode. Rates are given in katals (kat) per kg; 1 kat is equal to 1 mole per sec. Protein concentration was determined by the Lowry method (9).

Cytosolic phosphoenolpyruvate carboxykinase was partially purified from rat liver through the steps required to remove the ferroactivator protein by the method of MacDonald and Lardy (10). For inhibition studies the enzyme activity was assayed as described by Bentle and Lardy (11). Pig heart aconitase was partially purified (through step 3) by the procedure of Villafranca and Mildvan (12) and assayed as they describe. DNA polymerase was purified by R. Henrie from E. coli strain B according to the procedure of Jovin et al (13). Its activity was measured as described by Richardson et al (14). Hog kidney diamine oxidase was purified by T. Scalzo using a modification of the procedure of Klimova et al (15) and assayed as described by Bardsley et al (16). Hog kidney *myo*-inositol oxygenase was purified by the procedure of Reddy et al (17) and assayed by measuring O_2 uptake with an O_2 electrode. The following enzymes were obtained from Sigma Chemical Co. and assayed by the procedures given in the respective references: beef liver and bovine intestine alkaline phosphatase (18), human carbonic anhydrase B (19), porcine pancreas carboxypeptidase B (20), horse liver alcohol dehydrogenase (21), rabbit liver D-fructose-1,6-bisphosphatase (22), catalase, and D-amino acid oxidase (1). Mushroom tyrosinase was obtained from the United States Biochemical Corp. and was assayed by O_2 uptake using L-3,4-dihydroxyphenylalanine or 4-methylcatechol as substrate.

In the initial survey of possible effects of TC on the above enzymes, TC (*ca* 1 to 10 mM) was added directly to the assay mixtures in one set of experiments, and in another was incubated with the enzymes for 15 min to 1 hr at 25 or 37°C prior to initiation by adding substrate.

RESULTS

The chemical synthesis of thiazoline-2-carboxylate (TC) was accomplished as outlined in the Experimental Procedure section. In all respects tested this material was found to be identical to the product formed when a solution containing cysteamine and glyoxylate is oxidized by O_2 in the presence of D-amino acid oxidase. Thus, both forms of TC in aqueous solution at pH 7.0 absorb at 269 to 270 nm, and the absorbance seen with the enzymically prepared TC is that predicted from the extinction coefficient (1640 M^{-1}) of the synthetic material, and the amount of CG used in its preparation (although CG is racemic, D-amino acid oxidase ultimately converts all of it to TC (23,24) because CG racemizes rapidly under the reaction conditions). At pH 7 both the chemically synthesized and enzymically prepared TC's are relatively stable (for hrs at room temperature), but at pH 5 and below they react at identical rates to give the same products (these will be described more fully in a subsequent

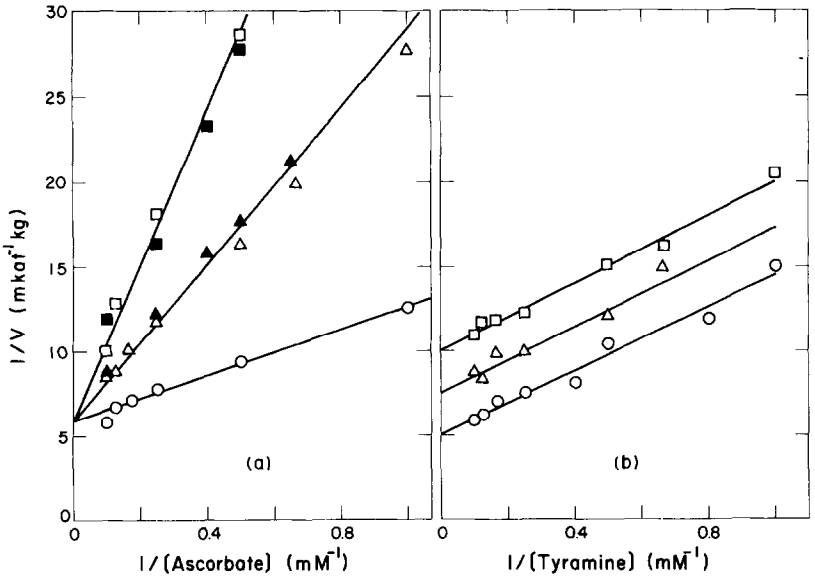

FIGURE 1. Inhibition of dopamine β-hydroxylase by TC, (a) tyramine concentration constant at 10 mM, and (b) ascorbate concentration constant at 10 mM. O, no TC present; Δ, 34 μM enzymically prepared TC; ▲, 34 μM chemically synthesized TC; □, 68 μM enzymically prepared TC; ■, 68 μM chemically synthesized TC. For other reaction conditions see the Experimental section.

publication). Furthermore, the NMR spectrum of synthetic TC is identical to the spectrum obtained from the enzymically prepared compound (23). Finally, dopamine β-hydroxylase is inhibited to the same extent by the synthetic and enzymically prepared TC's (Fig.1).

As the results in Fig.1 indicate, TC is a potent inhibitor of dopamine β-hydroxylase. Its inhibition is competitive with respect to the reactant ascorbic acid (Fig.1a) and uncompetitive with respect to tyramine (Fig.1b). Such results are typical for inhibitors that complex with the oxidized form of the enzymic copper (3). From the data of Fig.1a one can calculate that K_i for the TC inhibition that is competitive with ascorbate is 13 μM, and from the results in Fig.1b K_i for the uncompetitive inhibition is estimated to be approximately 70 μM. In contrast to the inhibition by TC, CG at 1 mM concentration has no detectable effect on the activity of dopamine β-hydroxylase

The inhibition of dopamine β-hydroxylase by TC is a relatively specific phenomenon because the activities of several other metalloenzymes are not appreciably affected. Thus, TC even at mM concentrations has no detectable effect on the activities of the following enzymes: the copper containing enzymes diamine oxidase and tyrosinase, the iron containing enzymes *myo*-inositol oxygenase and aconitase (no effect of TC on both the overall reaction and on its rate of activation by Fe(II) and cysteine was seen), the iron activated enzyme phosphoenolpyruvate carboxykinase, and the zinc containing enzymes

alkaline phosphatase, carbonic anydrase, carboxypeptidase B, DNA polymerase and alcohol dehydrogenase. The activity of the zinc containing enzyme fructose-1,6-bisphosphatase is increased by TC (25) as it is by other complexing agents (26). However, because mM or higher concentrations of TC are required to observe an effect (the rate is approximately doubled with 4 mM TC in the presence of 5 mM Mg^{++}), and because CG is as effective an activator as TC, such activation is probably not physiologically significant.

DISCUSSION

The results reported here, showing that chemically synthesized TC is identical to the product formed when a solution containing cysteamine and glyoxylate is acted upon by D-amino acid oxidase, prove conclusively that TC is the enzymic product. This, therefore indicates that thiazolidine-2-carboxylate (CG), is the true substrate for the reaction as had been earlier implied (1).

The remarkable specificity shown by TC in inhibiting dopamine β-hydroxylase and not a number of other metalloenzymes suggests that the inhibition of dopamine β-hydroxylase may be of physiological significance. From the known concentrations of cysteamine and glyoxylate in cells (1) one suspects that greater than micromolar concentrations of TC could be produced, and at such concentrations the activity of dopamine β-hydroxylase would be affected. In order to inhibit this enzyme however, the TC would have to be able to enter either the blood stream or chromaffin granules where most of the dopamine β-hydroxylase is located (27,28). Whether it does this or not remains to be determined.

Several naturally occurring dopamine β-hydroxylase inhibitors have been partially characterized (28), and some of them have properties suggesting that they may be either TC itself or closely related to it. For example, Hogue-Angeletti and Lysiak (29) reported that a dialysable inhibitor present in human plasma is destroyed during incubation at mildly acidic pH's (as indicated earlier, TC is hydrolyzed under such conditions). Also, Chubb et al. (30) isolated a small molecule inhibitor from bovine heart that had an absorption maximum at 263 nm (TC absorbs at 269 to 270 nm). Because it appears to contain carbohydrate and organic phosphate, it is unlikely that this material is TC itself, but it might be a substituted thiazoline-2-carboxylate. Our previous results(1) indicated that a glyoxylate adduct of any cysteine derivative that contains the cysteine thiol and amino groups free is a potential substrate for D-amino acid oxidase, and the expected products of such reactions would be substituted thiazoline-2-carboxylates. Consequently, one should be sensitive to the possibility that several naturally occurring dopamine β-hydroxylase inhibitors might contain the thiazoline-2-carboxylate structure, formed as the result of a D-amino acid oxidase catalyzed reaction.

By correlating the physiological effects of various types of compounds with their ability to inhibit the D-amino acid oxidase reaction, we have recently suggested (31) that TC or one of its metabolites may play an important role in the control of metabolism, and its inhibition of the dopamine β-hydroxylase reaction is certainly consistent with that. However, the lack of inhibition of a large number of other metalloenzymes as found here indicates that, if TC itself performs such a function, it must usually operate by some mechanism other than by merely complexing metal ions. Other possible mechanisms consistent with the correlations would be that TC covalently modifies various enzymes as previously proposed (1), or that some metabolite of TC is the important effector. Oxalate is one expected TC metabolite that does affect the activity of several enzymes that control the flux through important metabolic pathways (32), but one suspects that other metabolites may also be involved. Such possibilities are being explored.

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