Essential arginine residues occur in or near the catalytic site of L-amino acid oxidase

M. F. Christman and Janet M. Cardenas[1,2]

The Department of Chemistry, University of North Carolina, Chapel Hill (North Carolina 27514, USA), 1 September 1981

Summary. Butanedione in borate buffer irreversibly inactivates L-amino acid oxidase. L-Phenylalanine and L-methionine, which are good substrates for the enzyme, protect against inactivation but glycine, which is a very poor substrate, and D-phenylalanine, which is neither substrate nor inhibitor, do not provide significant protection. These results are consistent with the modification by butanedione of one or more arginine residues located in or near the catalytic site of L-amino acid oxidase.

Snake venom L-amino acid oxidase (EC 1.4.3.2), a glycoprotein containing 2 subunits of 70,000 mol. wt each and 2 FAD moieties per molecule[3], catalyzes the oxidative deamination of amino acids, particularly those having large hydrophobic side chains. O_2 is utilized and the immediate products of the reaction are hydrogen peroxide and the α-imino acid[4]. The latter compound then hydrolyzes nonenzymatically to form the α-keto acid and ammonia. Little is known about the nature of the amino acid residues that occur in the catalytic site of L-amino acid oxidase.

Experimental Procedures. Butanedione was generously provided by J. H. Harrison of this university. All other reagents including L-amino acid oxidase were purchased from Sigma Chemical Company (St. Louis, MO).

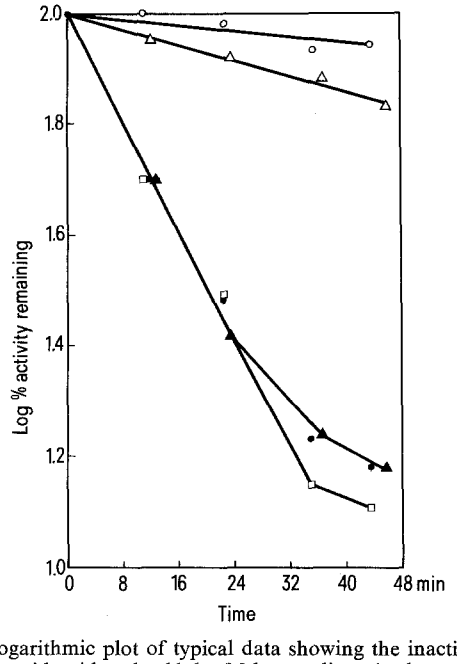
Enzymatic assays were based on the fact that the substrate L-phenylalanine is essentially transparent at 280 nm while the molar extinction coefficient of the product phenylpyruvate is 408 M^{-1} at this wavelength[5]. Enzymatic assays were performed in 0.5 ml volumes in 1.0 ml cuvettes having a path length of 1.0 cm. Standard assays contained 2 mM L-phenylalanine in 50 mM Tris-HCl, pH 7.0, to which 12.5 µg of bovine liver catalase was added in order to lessen decarboxylation of the product α-keto acid by another reaction product (hydrogen peroxide). Oxygen was bubbled through each cuvette using microtubing for 5 min at a flow rate of approximately 40 ml/min before adding L-amino acid oxidase. Enzymatic activity was monitored continuously by measuring the increase in absorbance at 280 nm in a Beckman Acta III spectrophotometer with the temperature regulated at 25 °C. The measured reaction velocity varied linearly with enzyme concentration over the range tested (20–100 µg of L-amino acid oxidase) and produced a specific activity in the range expected based on commercial information accompanying the enzyme.

L-amino acid oxidase was treated with butanedione under conditions similar to those used by Foster and Harrison for malate dehydrogenase[6]. Equal volumes of L-amino acid oxidase (0.4 mg/ml) in 50 mM potassium borate, pH 8.0 and various concentrations of butanedione in water were mixed in 5 ml conical test tubes and incubated at 25 °C. At various times, aliquots were withdrawn and assayed immediately for residual enzymatic activity. Appropriate controls lacking butanedione were assayed simultaneously and were found to retain essentially full activity throughout the course of the experiment.

Results. Incubation of solutions of L-amino acid oxidase with butanedione under the conditions described above resulted in a pseudo first order inactivation of the enzyme at rates that depended on the concentration of butanedione. The observed half times for inactivation were: 47.0 min at 2.3 mM butanedione; 23.0 min at 9.3 mM; 16.7 min at 7.0 mM; and 4.9 min at 11.6 mM butanedione. Inactivation of L-amino acid oxidase was irreversible in borate buffer, results that are typical of the formation of a guanidino-butanedione-borate complex (see Foster and Harrison[6]). Neither dialysis in borate buffer nor the addition of phenylalanine after the enzyme had been partially inactivated produced reactivation of the enzyme. The effect of butanedione on L-amino acid oxidase therefore appears to differ from reversible pH-dependent inactivation or the inactivation upon freezing that was observed for this enzyme by Curti et al.[7] and later confirmed by Coles et al.[8]. L-phenylalanine and L-methionine, known to be good substrates for L-amino acid oxidase[9,10], protected against inactivation by 11.6 mM butanedione (see fig. 1). Neither glycine, a poor substrate, nor D-phenylalanine, which is not a substrate, offered significant protection.

Discussion. The lack of protection by glycine and D-phenylalanine rule out protection of the enzyme via reaction of the butanedione itself with the added free amino acids. The chain-specific and stereospecific protection by amino acids against inactivation of L-amino acid oxidase is consistent with the affinity of the enzyme for these amino acids. While the reactivity of butanedione with free amino acids could well be dependent on the nature of the amino acid side chain, such reactivity would not be expected to be stereospecific.

Attempts to inactivate L-amino acid oxidase with other specific reagents have so far been unsuccessful; neither trinitrobenzenesulfonic acid, a reagent known to react primarily with amino groups, nor 5,5′-dithiobis-(2-nitrobenzoic acid), which reacts with sulfhydryl groups, had any effect on enzymatic activity. The fact that butanedione inacti-



A semilogarithmic plot of typical data showing the inactivation of L-amino acid oxidase by 11.6 mM butanedione in the presence of the following amino acids: (□), no amino acids added; (●), 5 mM D-phenylalanine; (▲), 5.0 mM glycine; (△), 5.0 mM L-methionine; and (○), 5.0 mM L-phenylalanine. Experimental procedures are described in the text.

vates the enzyme while neither trinitrobenzenesulfonic acid nor 5,5′-dithiobis-(2-nitrobenzoic acid) have any effect points to the likely presence of one or more essential guanidinium groups in or near the catalytic site of L-amino acid oxidase.

D- and L-amino acid oxidase both are known to be capable of utilizing a variety of amino acids as substrates, but their stereospecific requirements appear to be nearly absolute. The only known good substrate that they have in common is L-3,4-dehydroproline[11]. Massey and Curti[12] proposed that the 2 enzymes operate via similar reaction mechanisms, based on kinetic studies. We note that Nishino et al.[13] have recently reported the inactivation of hog kidney D-amino acid oxidase by butanedione. Thus, in spite of stereospecific differences in the substrate specificities of D- and L-amino acid oxidases, it appears likely that they utilize similar reaction mechanisms and may indeed have chemically similar catalytic centers.

1 Acknowledgments. We thank Drs D. Porter and S. Johnson for advice and assistance; Ms D. Hurt for electrophoretic analyses of protein samples; NIH for grant No. AM-25247, and NSF for grant No. SP176-83182.
2 Reprint requests should be addressed to: J.M.C., National Eye Institute, Bldg 31, Rm 6A49, National Institutes of Health, Bethesda (MD 20205, USA).
3 A. DeKok and A.B. Rawitch, Biochemistry *8*, 1405 (1969).
4 E.W. Hafner and D. Wellner, Proc. natl Acad. Sci. USA *68*, 987 (1971).
5 H.A. Sober, ed., in: Handbook of Biochemistry, p. B-76. Chemical Rubber Co., Cleveland, Ohio 1970.
6 M. Foster and J.H. Harrison, Biochem. biophys. Res. Commun. *58*, 263 (1974).
7 B. Curti, V. Massey and M. Zmudka, J. biol. Chem. *243*, 2306 (1968).
8 C.J. Coles, D.E. Edmondson and T.P. Singer, J. biol. Chem. *252*, 8035 (1977).
9 D. Wellner, Meth. Enzym. *17B*, 597 (1971).
10 L.A. Lichtenberg and D. Wellner, Analyt. Biochem. *26*, 313 (1968).
11 H. Weissbach, A.F. Robertson, B. Witkop and S. Udenfriend, Analyt. Biochem. *1*, 286 (1960).
12 V. Massey and B. Curti, J. biol. Chem. *242*, 1259 (1967).
13 T. Nishino, C.H. Williams, Jr, and V. Massey, 11th int. Congr. Biochem. Proc., p. 295. 1979.

3′-O-Methyl-(+)-catechin glucuronide and 3′-O-methyl-(+)-catechin sulphate: new urinary metabolites of (+)-catechin in the rat and the marmoset

A.M. Hackett, I.C. Shaw and L.A. Griffiths[1,2]

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (England), 7 September 1981

Summary. The major urinary metabolites of (+)-catechin (cyanidanol-3) in the rat were (+)-catechin glucuronide, 3′-O-methyl-(+)-catechin glucuronide and 3′-O-methyl-(+)-catechin sulphate. The latter conjugate was the major metabolite in marmoset urine.

The naturally occurring flavanol, (+)-catechin, which is known to occur in a number of green plants and plant products of significance in the human diet[3], has recently been reported to possess liver-protective properties[4] and to be beneficial in the therapy of acute viral hepatitis in man[5]. Previous metabolic investigations have shown that oral administration of (+)-catechin (Cyanidanol-3) to rats results in the excretion of 3-hydroxyphenyl-propionic acid, 3-hydroxyhippuric acid[6] and δ-(hydroxyphenyl)-γ-valerolactones[7] in urine. Metabolites extractable from unhydrolyzed urine with diethyl ether (including these compounds) accounted for only ⅙ of the 50–63% of the dose appearing in urine. Our recent finding[8] that 3′-O-methyl-(+)-catechin glucuronide is the major metabolite of (+)-catechin in rat bile led us to examine rat and marmoset urine for the presence of such conjugates.

Materials and methods. [U^{14}C](+)-Catechin (radiochemical purity 95%, sp. act. 698 μCi/mmole) was donated by Zyma S.A., Nyon, Switzerland.

Male albino Wistar rats (S.P.F., 250 g) and male marmosets (300 g) were maintained as previously described[6,9]. [U^{14}C]-(+)-Catechin was administered (40 mg/kg, 2 μCi) to rats by stomach intubation in water and to marmosets orally in Cytacon Syrup (Glaxo Labs., Greenford, Middlesex). Animals were housed in all glass metabolism cages allowing separate collection of urine, faeces (over solid CO_2) and expired CO_2[10].

Measurement of radioactivity, paper chromatography[8,10], enzymic hydrolysis of metabolites[11], HPLC and bile duct cannulation surgery[8] were carried out by techniques previously described. Diazotised 4-nitroaniline[12] was used to locate phenolic compounds on chromatograms. Individual metabolites were quantified following separation on thin layers of cellulose (Type 20, Sigma Chemical Co., Poole, Dorset; butan-2-ol:acetic acid:water 5:1:2) using a Berthold Series 272 radiochromatogram scanner linked to a PET computer. (+)-Catechin and 3′-O-methyl-(+)-catechin were quantified by similar means following separation on silica gel 'G' (300-μm layers) developed in toluene:ethyl acetate:formic acid (5:4:1 v/v/v).

Results and discussion. The urinary excretion of ^{14}C in the rat and marmoset following the oral administration of [^{14}C]-(+)-catechin is shown in table 1. Urinary excretion of ^{14}C (66% of dose) in the rat was close to that observed in earlier studies[7]. In the present study radioscanning of TLC separations of metabolites showed that 3 conjugates accounted for 84% of the ^{14}C of rat urine (table 2). These conjugates were identified by the use of specific enzyme treatments and by co-chromatography of the aglycones liberated (table 2). The evidence obtained indicated that these conjugates were a glucuronide of (+)-catechin and a glucuronide and a sulphate of 3′-O-methyl-(+)-catechin. The latter aglycone was chromatographically identical to the aglycone of the major biliary metabolite of (+)-catechin in the rat which we previously characterized by mass spectrometry[8]. The intact glucuronide conjugate of 3′-O-methyl-(+)-catechin isolated from urine did not however show identical chromatographic properties to that obtained from