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STUDIES ON THE STABILITY OF L-AMINO-ACID OXIDASE OF SNAKE VENOM

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SUMMARY

Snake venom L-amino-acid oxidase from *Crotalus adamanteus* is very unstable at pH 9.0 in the absence of substrate amino acid. Some amino acids such as L-leucine offer complete protection of the enzyme from inactivation while others such as L-arginine afford only partial protection. However, aliphatic and aromatic monocarboxylic acids protect the enzyme even in the absence of substrate. It is also shown that the type of buffer modifies the protective action of the acid on the activity of the enzyme.

INTRODUCTION

A previous publication from this laboratory has indicated that the activity of snake venom L-amino-acid oxidase is very unstable at alkaline pH in glycine–NaOH buffer[1]. This instability of the enzyme varied with the nature of the substrate used. In the present communication, we present results which indicate that acetic acid as well as a large number of aliphatic and aromatic monocarboxylic acids protect the enzyme from inactivation at the high pH.

MATERIALS AND METHODS

Crude L-amino-acid oxidase from *Crotalus adamanteus* venom and L-amino acids were obtained from Nutritional Biochemical Inc., and twice crystallized L-amino-acid oxidase from *C. adamanteus* venom from Sigma Biochemical Co. [1,2-$^{14}C_2$]-Acetic acid (specific activity, 25.6 mC per mmole) was purchased from New England Nuclear. The rest of the chemicals were purchased from various local sources.

Enzymatic activity was determined manometrically by the use of Warburg vessels with double side arms. The main compartment of the Warburg flask contained

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0.1 M glycine–NaOH buffer, pH 9.0, and the compounds to be tested, while one side arm contained 0.5 ml of the enzyme suspension and the other 0.5 ml of substrate solution. All the reagents were prepared in glycine–NaOH buffer, pH 9.0, except the enzyme which was suspended in water. A 2 cm × 2 cm piece of filter paper saturated with 0.2 ml of 20% KOH was placed in the center well to absorb CO_2. Measurement of O_2 consumption started by introducing the substrate, and the amount of O_2 consumed in the first 30 min was recorded. The enzymatic activity is expressed as μl O_2/h per mg protein. Protein was determined by the method of LOWRY *et al.*[2]. For assay of the radioactivity, 0.2 ml of the sample was introduced into 10 ml of scintillation solution and the radioactivity was measured with a Nuclear-Chicago model 8401 scintillation counter with an efficiency of approximately 70%. The scintillation solution contained 8 g of 2,5-diphenyloxazole, 200 mg of 1,4-bis-(5-phenyloxazolyl-2)-benzene, 600 mg of ethanol, and 1400 ml of toluene.

RESULTS AND DISCUSSION

Effect of acetate on the stability of L-*amino-acid oxidase*

Fig. 1 illustrates the effect of acetate on the stability of snake venom L-amino-acid oxidase at pH 9.0. When L-leucine was used as substrate, the enzymatic activity did not change over a 45-min period, indicating that the enzyme was not destroyed. However, with L-arginine as substrate, the activity started to decrease after 20 min,

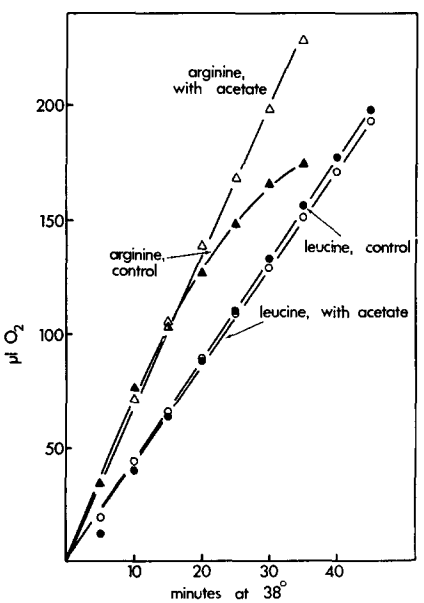

Fig. 1. Stability of the L-amino-acid oxidase in the presence of substrate at pH 9.0. The Warburg flask contained the following: 0.5 ml of 0.1 M amino acid, 1.5 ml of 0.1 M glycine–NaOH buffer at pH 9.0, and 0.5 ml of 2.0 M sodium acetate in the main compartment. The side arm contained 0.5 ml of an aqueous suspension of enzyme (0.5 mg crude, *C. adamanteus*). In the control experiments sodium acetate was omitted. The flask was equilibrated at 38° for 10 min prior to the addition of the enzyme to the main compartment. The rest of the procedures are as outlined in MATERIALS AND METHODS.

suggesting that the stability of L-amino-acid oxidase is dependent on the nature of the amino acid used as substrate. On the other hand, when acetate was added to L-arginine, a linear relationship with time occurred over the experimental period. It is evident from Fig. 1 that acetate protected the enzyme from inactivation rather than stimulating the enzyme, since the initial rates of activity in the presence and in the absence of sodium acetate were the same. Furthermore, the presence of acetate did not increase the rate of oxidation of L-leucine by the enzyme. This effect is also not due to a possible shift of pH during the reaction, since the pH of sodium acetate solution is 9.0. The decrease of the enzymatic activity observed after 20 min with L-arginine as substrate was not due to end-product inhibition as is evident from the result shown in Fig. 2. When the enzyme was preincubated at pH 9.0 for different

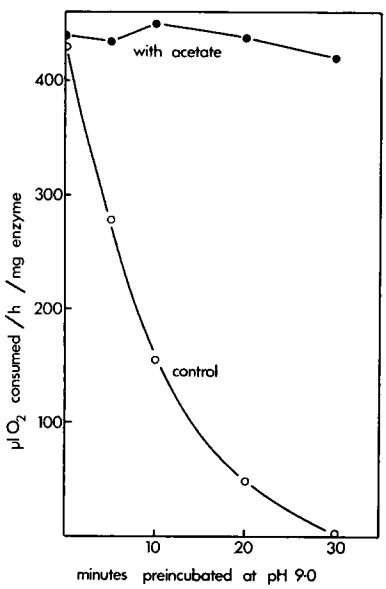

Fig. 2. Stability of the L-amino-acid oxidase in the absence of substrate at pH 9.0. The Warburg vessel contained the following: 0.5 ml of 2.0 M sodium acetate and 1.5 ml of 0.1 M glycine–NaOH buffer, pH 9.0, in the main compartment, and 0.5 ml of 0.05 M L-leucine and 0.5 ml of the enzyme suspension in each of two side arms. After 10 min equilibration at 38° the enzyme suspension was tipped into the main compartment. The enzyme was preincubated for varying time periods in the pH 9.0 buffer prior to the introduction of L-leucine. As a control, buffer was introduced in the place of sodium acetate. The rest of the procedures are the same as in Fig. 1.

intervals in the absence of substrate and the activity measured with L-leucine the rate of the enzymatic activity decreased as the period of preincubation increased. However, preincubation with acetate in the absence of substrate completely prevented the inactivation of the enzyme.

Relation of concentration of acetate and the degree of protection

Fig. 3 shows the effect of concentration of acetate on the degree of protection of the enzyme. It should be noted from Fig. 3 that rather high concentrations of acetate appear to be required for protection.

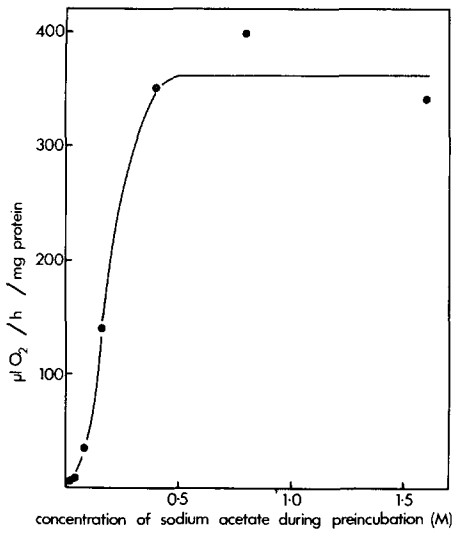

Fig. 3. Effect of concentration of acetate during preincubation. All the conditions are the same as in Fig. 2.

Effect of buffers on the protective action of acetate

Glycine–NaOH, Tris–HCl and veronal buffers did not prevent the inactivation of snake venom L-amino-acid oxidase at pH 9.0, although more activity remained when NH_4OH–NH_4Cl buffer was used (Table I). Acetate was most effective only in the glycine–NaOH buffer (see also Fig. 2). Since it is impossible to test the effect of the various buffers used in this study at a pH of 9.0, one can not rule out the possibility that the buffers themselves caused an inhibition of the enzyme. It should be noted, however, that snake venom L-amino-acid oxidase in these buffers at pH 7.2 showed no inhibitory effect. The data in Table I suggest a complex interaction between the enzyme, acetate and buffer ions.

TABLE I

EFFECT OF VARIOUS BUFFERS ON THE PROTECTIVE ACTION OF ACETATE

The main compartment of a Warburg flask contained 1.5 ml of a 0.1 M (pH 9.0) solution of each of the buffers and 0.5 ml of sodium acetate at a final concentration of 0.33 M. After 10 min equilibration at 38°, 0.5 ml of a suspension of L-amino-acid oxidase (0.5 mg) was introduced from a side arm into the main compartment and the enzyme preincubated at pH 9.0 for 30 min. After this enzyme preincubation period, 0.5 ml of 0.05 M L-leucine (pH 9.0) was added from the other side arm to start the reaction. The assay was carried out as outlined in MATERIALS AND METHODS. The control flasks contained no sodium acetate.

Buffer	*Enzymatic activity (μl O_2/h per mg enzyme)*	
	Control	*With acetate*
Glycine–NaOH	37.4	553.5
Tris–HCl	28.8	255.6
Veronal	32.2	303.8
NH_4OH–NH_4Cl	114.4	353.8

TABLE II

EFFECT OF SOME INORGANIC ANIONS ON THE PROTECTIVE ACTION OF ACETATE ON INACTIVATION OF L-AMINO-ACID OXIDASE AT pH 9.0

The main compartment of a Warburg flask contained the following: 0.2 ml of 2.0 M sodium acetate (final concentration during preincubation 0.16 M), 1.0 ml of 0.4 M inorganic salts (1.8 ml of 0.116 M sodium borate), and 0.8 ml of the buffer. Each of the anions was the sodium salt. The preincubation period and substrate concentration were as outline in Table I.

Anions added with acetate ion	*Final concentration of anions during preincubation (M)*	*Remaining enzyme activity (μl O_2/h per mg protein)*	*Per cent protection*
None		109	100
Chloride	0.16	107	98
Nitrate	0.16	133	121
Azide	0.16	136	124
Bicarbonate	0.16	19	17
Sulphate	0.16	14	12
Borate	0.08	0	0
Arsenate	0.16	33	30
Phosphate	0.16	12	11

Antagonistic effect of some inorganic anions on the action of acetate

Table II shows the effect of various inorganic anions on the protective action of acetate on snake venom L-amino-acid oxidase. Monovalent anions such as chloride, nitrate or azide ions did not prevent the effect of acetate, although polyvalent anions such as bicarbonate, sulfate, borate, arsenate and phosphate were effective. Borate ion seems to be most active in this respect.

Specificity of the protection of the enzyme inactivation

The specificity of protection of snake venom L-amino-acid oxidase from inactivation at pH 9.0 is illustrated in Table III. Although all the aliphatic and aromatic monocarboxylic acids used had some protective activity, the length of the carbon chain was important. Octanoic acid had no effect, although surprisingly, formaldehyde protected better than other aldehyde. DL-Lactate maintained the activity of the enzyme to a greater degree than the D isomer. When an additional carboxyl group was introduced into the molecule such as glutamic acid, aspartic acid, maleic acid and oxalic acid, or a carboxyl group is replaced with hydroxyl (ethyl alcohol, *n*-propyl alcohol, *n*-butyl alcohol, glycerol and glucose), the protective activity disappears.

The foregoing results were obtained with a crude snake venom L-amino-acid oxidase from *C. adamanteus*. However, the use of twice crystallized enzyme gave results which agreed completely with the data described above. Further thermodynamic and crucial kinetic studies carried out with crystalline enzyme will be published elsewhere.

Since the molecular weight of crystalline L-amino-acid oxidase from *C. adamanteus* is approx. 130 000 (ref. 3), the concentration of acetate required for complete protection of the enzyme is beyond the amount needed for stoichiometric combina-

TABLE III

SPECIFICITY FOR THE PROTECTION OF SNAKE VENOM L-AMINO-ACID OXIDASE FROM INACTIVATION AT pH 9.0

Each of the compounds at a concentration of 0.2 M was dissolved in 0.1 M glycine–NaOH buffer pH 9.0 and 2.0 ml was added to the main compartment of a double side arm Warburg flask. All other experimental conditions are the same as in Table I.

	Compound added to the buffer at pH 9.0 during preincubation	*Remaining enzyme activity (μl O_2/h per mg protein)*
1	None	0
2	Formic acid	92
3	Acetic acid	163
4	Propionic acid	447
5	Butyric acid	207
6	Valeric acid	368
7	Caproic acid	422
8	Heptanoic acid	160
9	Octanoic acid	3
10	Oleic acid	0
11	*p*-Aminobenzoic acid	405
12	Benzoic acid	429
13	Phenylacetic acid	383
14	Phenylpropionic acid	294
15	Glyoxalic acid	109
16	Formaldehyde	164
17	Acetaldehyde	46
18	DL-Lactic acid	236
19	D-Lactic acid	93
20	Pyruvic acid	0
21	DL-Glyceric acid	6
22	α-Ketobutyric acid	172
23	DL-β-Hydroxybutyric acid	154
24	DL-γ-Hydroxybutyric acid	357
25	Glutamic acid	0
26	Aspartic acid	0
27	Maleic acid	0
28	Oxalic acid	0
29	Ethyl alcohol	0
30	*n*-Propyl alcohol	0
31	*n*-Butyl alcohol	0
32	Glycerol	0
33	Glucose	0
34	Ascorbic acid	0
35	EDTA	0

tion. Furthermore, the specificity of the buffer (Table I) suggests a very complex interaction between the enzyme, acetate and buffer ions. When [^{14}C]acetate and the enzyme were incubated at pH 9.0 and the incubation mixture passed through Bio-gel P-10, the radioactivity peak did not coincide with the peak of enzyme activity, suggesting the absence of bond formation between these two components. The exact mechanism of the action of acetate on the enzyme L-amino-acid oxidase at pH 9.0 is not clear at present, and further studies are in progress.

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