A Factor which Counteracts the Stabilizing Activity of Acetate Ion on Ophioxidase at Alkaline pH

We have reported that L-amino acid oxidase (L-amino acid: O_2 oxidoreductase, EC 1.4.3.2) from *Crotalus adamanteus* venom exhibits a variety of pH curves depending on the amino acids used as the substrate[1]. This enzyme is highly unstable at high alkaline pH, though acetate ion, as well as a large number of aliphatic and aromatic monocarboxylic acids, protects it from inactivation[2]. In this communication, evidence is presented that beef liver contains a factor which partially counteracts the effect of acetate ion.

Materials and methods. Twice crystallized L-amino acid oxidase from *Crotallus adamanteus* venom and the proteins used in compiling the data in Table II were purchased from Sigma Chemical Co.

Preparation of a factor. Approximately 10 g of frozen beef liver was thawed, homogenized in 3 volumes of water, and passed through a double layer of cheese cloth. 20 ml of the homogenate was centrifuged at 39,000 g for 20 min and 15.5 ml of the supernatant was obtained. The supernatant was treated with an equal volume of $Ca_3(PO_4)_2$ gel (17.5 mg solid/ml) and washed successively with 30 ml of water and 15 ml of 0.02 *M* phosphate buffer at pH 7.2. The gel was eluted with 4 ml of 0.1 *M* pH 7.2 phosphate buffer at 39,000 *g* for 10 min. 8 ml of the obtained eluate was treated with an equal volume of saturated ammonium sulfate, and the precipitate was collected after 10 min. The precipitate was dissolved in 8 ml of water, and 4 ml of hydroxyapatite (98.5 mg solid/ml) was added to the suspension. This was washed once with 30 ml of water and finally eluted with 4 ml of 0.1 *M* pH 7.2 phosphate buffer by centrifugation at 39,000 *g* for 10 min. The purification schedule and recovery of the factor is shown in Table I.

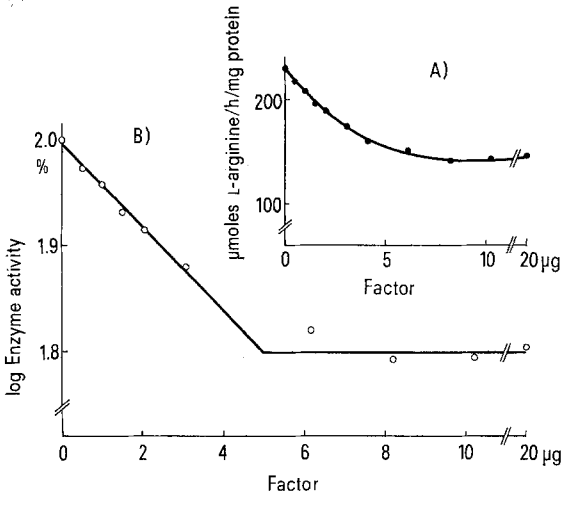

Fig. 1. Effect of concentration of the factor on the protective action of acetate of L-amino acid oxidase. During preincubation of L-amino acid oxidase in pH 9.0 for 30 min, 0.4 *M* acetate was present. Detailed conditions are described under Methods.

Enzyme assay. Into the main compartment of a double side arm Warburg flask 0.5 ml of 2.0 *M* sodium acetate (water solution has a pH 9.0), 1.0 ml of 0.1 *M* glycine-NaOH buffer, pH 9.0, and 0.5 ml of the factor suspended in the above buffer were placed. In the case of control, the factor was replaced with buffer. The 2 side arms contained 0.5 ml of 0.1 *M* L-arginine and 0.5 ml of crystalline enzyme solution containing 0.09 mg protein, respectively. All the reagents were prepared in the buffer except the L-amino acid oxidase, 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 carbon dioxide. The vessels were equilibrated at 37 °C for 10 min. The enzyme was then tipped into the main compartment of the flask and preincubated at pH 9.0 for 30 min before the addition of substrate from the second side arm. The oxygen consumption in the first 12 min was recorded and the enzyme activity expressed as µmoles of substrate oxidized/h/mg of enzyme protein. Protein concentration was determined by the method of LOWRY et al.[3].

Results and discussion. As shown previously[1,2], when L-amino acid oxidase was preincubated for 30 min at pH 9.0 in the absence of substrate, the enzyme activity was abolished. However, the presence of 0.4 *M* sodium acetate during preincubation completely protected the enzyme from inactivation. As shown in Figure 1, the activity of L-amino acid oxidase is completely protected by sodium acetate during preincubation in the absence of the factor. Increasing concentrations of the factor decreases the protective activity of the acetate ion (Figure 1A). However, after counteracting the protective activity of acetate about 40% (60% of the enzyme activity remains), the increasing concentration of the factor does not further influence the degree of inhibition. It is unlikely that the phosphate ion, introduced when eluting the factor from hydroxyapatite, counteracts the acetate since the final

[1] W. K. PAIK and S. KIM, Biochim. biophys. Acta *96*, 66 (1965).
[2] W. K. PAIK and S. KIM, Biochim. biophys. Acta *139*, 49 (1967).
[3] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).

Table I. Purification of the factor from beef liver[a]

Purification step	Volume (ml)	Protein (mg/ml)	Factor activity (units)[b]	Total units	Yield (%)	Purification
Whole homogenate	20.0	58.9	41.0	48,298	100.0	1.0
Supernatant at 39,000 *g* for 20 min	15.5	38.8	73.5	44,203	91.5	1.8
Eluate from calcium phosphate gel	8.0	8.6	138.9	9,556	19.8	3.4
Precipitate from ammonium sulfate	8.0	4.4	312.5	11,000	22.8	7.6
Eluate from hydroxyapatite	4.9	2.1	500.0	5,145	10.7	12.2

[a] 20 days frozen beef liver was used. [b] Unit activity is defined as mg of the factor to decrease the effect of acetate ion from 100% to 80%. For detail see the text.

Table II. Specificity of the factor to counteract the effect of acetate on inactivation of L-amino acid oxidase at pH 9.0

Compound	Amount (mg)	Remaining enzyme activity (μmole/h/mg enzyme protein)	%
None		226.8	100.0
Factor (isolated from beef liver)	0.01	150.7	66.4
Acetone powder extract of *Bact. cadaveris*	0.10	153.5	67.7
Extract of acetone powder of pigeon liver	0.10	155.3	67.8
Lysine-rich histone	0.10	232.8	102.6
Globulin	0.10	209.9	92.5
Protamine	0.10	222.1	97.9
Arginine-rich histone	0.10	216.8	95.6
Lysozyme	0.10	218.0	96.1
Trypsin inhibitor	0.10	215.2	94.9
Polylysine	0.10	219.1	96.7
Ribonuclease	0.10	221.4	97.6
Bovine serum albumin	0.10	222.2	98.0

The conditions are described in the text.

concentration of phosphate was less than 10^{-4} *M*. In a previous study, phosphate in this concentration had no effect[2]. When the values in Figure 1A were plotted as logarithm of percent activity vs. the amount of the factor (Figure 1B), a straight line was obtained at regions where the concentration of the factor had an effect on the degree of counteraction. Therefore, as in the case of Table I, unit activity of the factor was defined as mg of the factor necessary to decrease the logarithmic value from 2 to 1.9. This corresponds to a decrease of enzyme activity from 100% to 80%.

A reciprocal plot of acetate concentration and the rate of L-amino acid oxidase is shown in Figure 2. It is evident from this figure that the factor and acetate act competitively. However, since the counteraction does not increase indefinitely with increase of the factor concentration (Figure 1), this inhibition is of a partially competitive nature[4].

Several lines of evidence suggest that the factor is a protein nature: a) it is inactivated by heating at 60 °C for 5 min; b) the factor was purified by conventional methods of enzyme purification. Various proteins were tested for their capacity to counteract the protective effect of acetate on L-amino acid oxidase. As shown in Table II, histone, globulin, protamine, lysozyme, trypsin inhibitor, polylysine, albumin and ribonuclease have no effect. There seems to be no relation between basicity of protein and the inhibitory activity, since albumin is acidic and the rest of the above proteins are basic. On the other hand, acetone powder extracts of *Bact. cadaveris* and of pigeon liver demonstrate counteracting ability. Therefore, it appears that the factor is quite common in nature.

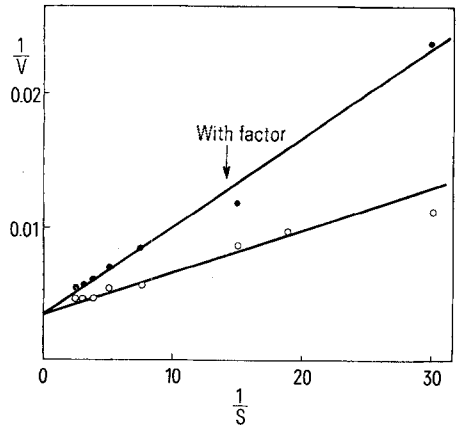

Fig. 2. Reciprocal plot of acetate concentration and the rat of L-amino acid oxidase. 10 μg of the factor were used, and V_{max} value in the figure was calculated as 285.7 μmole L-arginine oxidized/h/mg enzyme protein.

When the factor was preincubated with acetate-^{14}C and this mixture was passed through Bio-Gel P-10, it was found that all of the acetate-^{14}C was recovered in the same position as if it alone were present during the preincubation, as was the protein peak which contained no radioactivity. The factor itself was excluded by this gel, indicating molecular weight greater than 10,000. These results suggest that the factor neither destroys nor combines with the acetate-^{14}C. It is highly probable that the factor might regulate the effect of acetate on the L-amino acid oxidase in some allosteric fashion. Further investigation into this problem will be continued.

Presently, the biological significance of these findings remain clouded, however, the apparent common occurrence of this factor in nature might indicate a form of L-amino acid oxidase regulatory mechanism based on the proportion of factor: acetate ion in the liver of higher animals.

Résumé. La L-aminoacide oxydase de venin de serpent est très instable en milieu alcalin et les ions d'acetate soutiennent cette inactivation. Le foie de bœuf contient un facteur qui inhibe partiellement l'effet des ions d'acetate. Cette inhibition est en partie de type compétitif.

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26 August 1974.

[4] M. Dixon and E. C. Webb, *Enzymes* (Longmans, Green and Co. Lts., London 1962), p. 320.