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Characterization of sodium borohydride-reduced histaminase–histamine intermediate

Pig kidney histaminase (diamine oxidase; diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6) has been crystallized and characterized as a copper-pyridoxal phosphate-dependent enzyme[1,2]. Pyridoxal phosphate is stably bound to the enzyme and cannot be liberated by common methods. Thus, the possible role of pyridoxal phosphate in the oxidase reaction has not been established. It is, however, possible that the enzyme–substrate complex may be formed through the aldimine bonding between the carbonyl group of pyridoxal phosphate and the amino group of substrate amine, as occurs in many pyridoxal phosphate-dependent enzymes. To elucidate this point, histaminase was treated with $NaBH_4$ in the presence of labeled histamine.

Crystalline histaminase was isolated from fresh pig kidney according to the method previously reported[1]. Enzyme activity was determined by measuring the formation of pyrroline, an oxidative product of putrescine. The assay was carried out colorimetrically as described by YAMADA *et al.*[3]. A unit was defined as the amount of enzyme which catalyzed the formation of 1 μmole of pyrroline per min under standard assay conditions. The protein concentration was determined spectrophotometrically by measuring the absorbance at 280 mμ. The A value of 1.63 for 1 mg/ml and for 1-cm light path, which was used throughout, was obtained by absorbance and by dry weight determinations.

Pyridoxylhistamine was synthesized from pyridoxal hydrochloride and [^{14}C]-histamine by the slightly modified[4] method of HEYL *et al.*[5]. The synthesized sample was identified by measurement of its melting point, elemental analyses and NMR spectrum analysis.

0.5 ml of a reaction mixture containing 50 μmoles of sodium phosphate (pH 8.0) and 1.0 mg of crystalline enzyme (specific activity, 1.07) was equilibrated at 30° in air in a 10-ml glass tube. The enzymatic reaction was initiated by the addition of 0.05 ml of a solution of 0.01 M [$^{14}C_2$]histamine (576 000 counts/min). After a 2-min incubation, 5 mg of solid $NaBH_4$ were added, and the system was incubated at 30° for an additional 30 min before 0.5 ml of 10% trichloroacetic acid solution was added. The precipitated protein was collected by centrifugation in the same glass tube. The supernatant was discarded, and the precipitated protein was washed with 2 ml of 5% trichloroacetic acid solution by mixing it with a flash mixer and was then centrifuged. This washing procedure was repeated 5 times. The washed precipitate was dissolved by the addition of 0.5 ml of 1 M NH_4OH solution. The entire contents of the glass tube were transferred quantitatively to a stainless steel planchet (2.5 cm in diameter) and dried at 50°. Radioactivity measurements were carried out with a gas-flow counter. Results are shown in Table I. When the effects of inhibitors were to be examined, each inhibitor was added to the initial reaction mixture, and after a 15-min preincubation, the labeled substrate was added. The subsequent procedure is the same as previously described.

Reductive incorporation of labeled histamine into the enzyme protein was limited to reaction systems having the catalytically active enzyme. No combination was

TABLE I

REDUCTIVE STABILIZATION OF INTERMEDIATES IN THE INTERACTION OF HISTAMINASE AND [^{14}C]-HISTAMINE

Reactions were carried out as described in the text. The concentrations of semicarbazide and cuprizone were $2 \cdot 10^{-4}$ M and $3.3 \cdot 10^{-5}$ M, respectively. Heat inactivation of the enzyme was made at 80° for 20 min.

Reaction system	*Protein-bound ^{14}C (counts/min)*
Histaminase (0.5 mg)	
+ [^{14}C]histamine + $NaBH_4$	443
+ [^{14}C]histamine	204
Histaminase (1.0 mg)	
+ [^{14}C]histamine + $NaBH_4$	1040
+ [^{14}C]histamine	324
+ semicarbazide + [^{14}C]histamine + $NaBH_4$	284
+ semicarbazide + [^{14}C]histamine	187
+ cuprizone + [^{14}C]histamine + $NaBH_4$	466
+ cuprizone + [^{14}C]histamine	250
Histaminase (1.0 mg) (heat inactivated)	
+ [^{14}C]histamine + $NaBH_4$	200

effected with enzyme protein with heat and inhibitors such as semicarbazide and cuprizone. The net counts trapped by the histaminase correspond to about 0.115 mole of histamine per 1 mole of enzyme (mol. wt., 185 000, ref. 1). Inactivation of the enzyme was observed using the $NaBH_4$ treatment under the same conditions mentioned above. The rate of inactivation was similar to that of the incorporated labeled substrate per 1 mole of enzyme.

5 mg of labeled histaminase were obtained as previously described, except that a specific radioactivity of the substrate 10 times higher was used. The washed ^{14}C-labeled protein was hydrolyzed anaerobically in a sealed glass tube for 12 h with 6 M HCl at 105°. The contents of the tube were dried in a vacuum and dissolved in 0.5 ml of water. A 0.1-ml aliquot was spotted on a 15 cm × 55 cm Toyoroshi No. 51A paper strip. The paper strip was wetted with 0.2 M sodium acetate buffer (pH 5.0) from the two ends and submitted to high-voltage electrophoresis. The electrophoresis was carried out for 30 min at a constant potential of 2 kV, corresponding to a potential

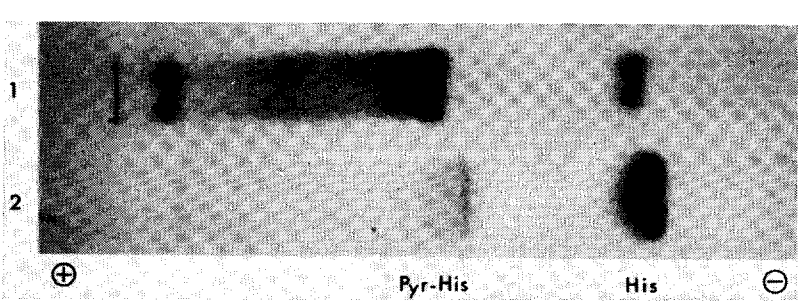

Fig. 1. Autoradiogram of the ionogram of labeled protein hydrolysate. 1, hydrolyzed [^{14}C]protein; 2, authentic samples. His, histamine; Pyr-His, pyridoxylhistamine.

gradient of about 50 V/cm. Simultaneously, synthesized ^{14}C-labeled pyridoxylhistamine and [^{14}C]histamine were applied to electrophoresis for comparison. After electrophoresis was complete, the paper strip was dried, and the position of labels was detected by autoradiography. Exposure time to the X-ray film was 30 days. The hydrolysate of the labeled protein was shown as three distinctly separated spots which migrated to the cathode side of the paper. The one having the greatest mobility corresponds to histamine and another to pyridoxylhistamine (Fig. 1).

The interaction of crystalline pig kidney histaminase with histamine initially involves an enzyme–substrate intermediate. The results presented indicate that this intermediate was trapped as a stable ^{14}C-labeled protein derivative by reduction with $NaBH_4$ and that the hydrolysate of this labeled protein derivative contained pyridoxylhistamine. A possible interpretation of these results is that the initial combination of enzyme and substrate was due to the formation of aldimine bonding (azomethine formation) between the aldehyde group of pyridoxal phosphate and an amino group of the substrate. $NaBH_4$ reduced the aldimine bonding (I) to form the stable secondary amine bonding (II) as follows.

$$\underset{\text{(I)}}{\text{R–CH=N–R'}} \xrightarrow{H_2} \underset{\text{(II)}}{\text{R–CH}_2\text{–NH–R'}}$$

A similar observation has been reported by BUFFONI[6], using a crystalline preparation of pig plasma amine oxidase.

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1 H. YAMADA, H. KUMAGAI, H. KAWASAKI, H. MATSUI AND K. OGATA, *Biochem. Biophys. Res. Commun.*, 29 (1967) 723.
2 B. MONDOVI, M. T. COSTA, A. FINAZZI-AGRÓ AND G. ROTILIO, *Arch. Biochem. Biophys.*, 119 (1967) 373.
3 H. YAMADA, A. TANAKA AND K. OGATA, *Mem. Res. Inst. Food Sci. Kyoto Univ.*, 26 (1965) 1.
4 H. KUMAGAI, H. YAMADA AND H. FUKAMI, *Agr. Biol. Chem. Tokyo*, in preparation.
5 D. HEYL, E. LUZ, S. A. HARRIS AND K. FOLKERS, *J. Am. Chem. Soc.*, 70 (1948) 3669.
6 F. BUFFONI, *Proc. 2nd Intern. Symp. Chem. Biol. Aspects Pyridoxal Catalysis, Moscow 1966*, John Wiley, New York, 1968, p. 363.

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