γ-Hydroxyarginine, a New Guanidino Compound from a Sea-cucumber

III. Actions of Arginase and Arginine Decarboxylase

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(Received for publication, February 10, 1961)

The isolation and identification of γ-hydroxy-L-arginine from a sea-cucumber, Polychoer a rufescens (1), and the determination of its stereoconfiguration (2) were reported previously by the present author. In these studies it was found that the amino acid was susceptible to arginase and L-amino acid oxidase and thereby converted to γ-hydroxy-L-ornithine and γ-guanidino-β-hydroxybutyric acid, respectively. Recently, the occurrence of γ-hydroxyarginine in a sea-anemone, Anthopleura japonica, was found by Makisumi in this laboratory, who isolated simultaneously from this animal γ-guanidino-β-hydroxybutyric acid and γ-hydroxyagmatine corresponding to the oxidation and decarboxylation products of γ-hydroxyarginine, respectively (3). To identify γ-hydroxyagmatine, a new guanidine compound, Makisumi synthesized it enzymically by using arginine decarboxylase from Escherichia coli 7020 and No. 1 (4).

In all these enzymatic reactions, it was observed that the rates of conversion of γ-hydroxyarginine to each of the reaction products were considerably low in comparison with those of arginine. It is obvious that the steric hindrance caused by the presence of a hydroxy group at γ-position of this compound is responsible for the decrease in the rates. Accordingly, it is interesting to measure quantitatively the effect of the presence of γ-hydroxy group on the velocity of enzymatic reaction.

In the present experiment, the rates of reaction of γ-hydroxyarginine catalyzed by arginase and arginine decarboxylase were determined and compared with those of arginine catalyzed by the same enzymes, and the Michaelis constants for γ-hydroxyarginine in these enzymatic reactions were also calculated.

MATERIALS

Substrates—L-Arginine monohydrochloride is a commercial product. γ-Hydroxy-L-arginine monohydrochloride is a preparation isolated from the sea-cucumber. The stereoconfiguration of its γ-hydroxy group is erythro-form; m. p. 190-191°C (decomp.), [α]D +5.4° (c=2, in 5N hydrochloric acid).

Arginase—A partially purified beef liver arginase preparation obtained as a solution in the Step D according to Greenberg (5) was used without dilution in the incubation with γ-hydroxyarginine, while in the case of arginine the arginase solution was diluted with water ten times.

Urease—For determination of urea a crude urease preparation purchased from Ishizu Pharmaceutical Co. Ltd. was used without further purification.

Arginine Decarboxylase—Though it is well known that Escherichia coli 7020 is specific for the decarboxylation of arginine alone (6), it was tested for the decarboxylation of γ-hydroxyarginine as Hagihara (7) and Suzuki et al. (8) found recently that the organism also decarboxylated 1-canavanine which is a structural analogue of arginine. After preparing an acetone powder of the organism according to the method of Gale (9), it was suspended in 0.2M phosphate-citrate buffer pH 5.2 (10mg./ml.) to obtain an arginine decarboxylase solution.

EXPERIMETNALS AND RESULTS

Arginase

pH Optimum of Arginase for the Hydrolysis of γ-Hydroxyarginine—An assay mixture containing each 0.5ml. of 0.1M γ-hydroxyarginine, 0.01 M manganous sulfate, 0.4 M Michaelis’ veronal buffer (pH range from 7 to 9.6)
and the arginase solution was incubated at 30°C for 10 minutes. For the suppression of arginase action, the mixture was added with 0.5 ml. of 1.2 N sulfuric acid and heated in a boiling water bath for 5 minutes, then cooled to room temperature. For determination of urea to be produced, an urease method was applied as follows. To the resulting mixture 0.5 ml. of 1.6 N phosphate buffer was added (the pH of the mixture should be 6.8, otherwise, it should be corrected by adding an adequate amount of 2 N sodium hydroxide or 2 N sulfuric acid). After further addition of 0.5 ml. of 0.05 M EDTA and the same volume of an urease solution (10 mg. of the urease/ml. of water) to the mixture, it was incubated again at 30°C for 60 minutes. Then ammonia in an aliquot of the incubation mixture was analyzed by using the Conway microdiffusion method. The pH optimum of arginase for the hydrolysis of γ-hydroxyarginine was approximately 9.2 under these experimental conditions (Fig. 1). Consequently, as there was no appreciable difference between the pH optima of arginase action on arginine and on γ-hydroxyarginine, subsequent experiments were carried out at this pH.

Determination of the Hydrolysis Rates—The rates of hydrolysis by arginase of arginine and γ-hydroxyarginine having several substrate concentrations at pH 9.2 were determined in a similar manner to the case of the determination of pH optimum (Fig. 2). The apparent velocity constants for the hydrolysis of the two substrates and the ratio of the two constants at the same substrate concentration were calculated. The results are summarized in Table I. The rate of hydrolysis of γ-hydroxyarginine by arginase was approximately 11 per cent that of arginine with the substrate concentration from 0.005 to 0.05 M.

**Table I**

<table>
<thead>
<tr>
<th>Substrate Concentration (10⁻² M)</th>
<th>( k_a )</th>
<th>( k_{HA} )</th>
<th>( k_{HA}/k_a )</th>
<th>Average Ratio</th>
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1) Velocity constant for arginine
2) Velocity constant for γ-hydroxyarginine

The Michaelis Constant—By using the velocity constants obtained above, the Michaelis
constants for these substrates were calculated according to the Lineweaver-Burk's equation (Fig. 3). The constant for γ-hydroxyarginine was $1.6 \times 10^{-2} M$, while for arginine was $5 \times 10^{-3} M$.

The plots of initial substrate concentration [S] divided by initial velocity $V_i$ versus [S] for the hydrolysis of the substrates by arginase at 30°C and pH 9.2.

**Arginase Decarboxylase**

**Determination of the Decarboxylation Rates** — The decarboxylations of arginine and γ-hydroxyarginine were carried out in the Warburg manometers filled with air at 30°C. A mixture consisting of 1.0 ml. of the freshly prepared suspension of acetone powder of *E. coli* and of 0.5 ml. of 0.2 M phosphate-citrate buffer (pH 5.2) was placed in the main compartment, and 1.0 ml. of 0.0025 to 0.0125 M substrate solution was placed in the side arm.

The evolution of carbon dioxide in 5 minutes was measured after equilibration of the temperature (Fig. 4). The apparent velocity constants for decarboxylation of the two substrates and the ratio between the two constants at the same substrate concentration were calculated. The results are summarized in Table II. The rate of decarboxylation of γ-hydroxyarginine by arginine decarboxylase from *E. coli* 7020 was approximately 16.5 per cent that of arginine with the substrate concentration from 0.0015 to 0.003 M.

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate Concentration (0.001 M)</th>
<th>$k_A$</th>
<th>$k_{HA}$</th>
<th>$k_{HA}/k_A$ %</th>
<th>Average Ratio %</th>
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1) Velocity constant for arginine
2) Velocity constant for γ-hydroxyarginine

The plots of initial substrate concentration [S] divided by initial velocity $V_i$ versus [S] for the decarboxylation of the substrates by arginine decarboxylase at 30°C and pH 5.2.

**The Michaelis Constant** — The constants for the two substrates were calculated in a similar manner to the case of hydrolysis by arginase...
and the following values were obtained; $7.6 \times 10^{-3} M$ for γ-hydroxyarginine and $4.1 \times 10^{-4} M$ for arginine (Fig. 5). The value for arginine was in good accordance with that reported by Gale (10).

**SUMMARY**

The reaction rates of hydrolysis and decarboxylation of γ-hydroxyarginine catalyzed by arginase and arginine decarboxylase were determined and compared with those of arginine. The apparent reaction velocity of hydrolysis of γ-hydroxyarginine was about 11 per cent of that of arginine, and that of decarboxylation was about 16.5 per cent. The Michaelis constants of arginase and arginine decarboxylase for γ-hydroxyarginine were also calculated and the following data were obtained; $1.6 \times 10^{-2} M$ in the arginase reaction and $7.6 \times 10^{-3} M$ in the decarboxylase reaction.

The author wishes to express his cordial thanks to Prof. S. Shibuya for his guidance and encouragement. His thanks are also due to the Ministry of Education for financial support of this research.

**REFERENCES**