STUDIES ON THE ENZYMIC DECOMPOSITION OF UROCANIC ACID III. 4(5)-IMIDAZOLONE-5(4)-PROPIONIC ACID HYDROLASE[1]

D. Rajagopal Rao and D. M. Greenberg

Department of Biochemistry, School of Medicine
University of California
San Francisco 22, California

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The enzymic conversion of urocanic acid to formimino-L-glutamic acid has been shown previously to involve two enzymic steps with the formation of 4(5)-imidazolone-5(4)-propionic acid as an intermediate (Feinberg and Greenberg, 1959; Revel and Magasanik, 1958). Evidence for the structure of the latter compound has been published recently by Brown and Kies (1959), in agreement with observations made independently by us with highly purified preparations of beef liver urocanase (Rao and Greenberg, 1960). We report here preliminary results on the properties of imidazolonepropionic acid hydrolase, the enzyme which catalyses the formation of formimino-L-glutamic acid from 4(5)-imidazolone-5(4)-propionic acid.

Imidazolonepropionic acid hydrolase has been partially purified from rat liver homogenates[2] (Table I). The purified enzyme was free of imidazolonepropionic acid oxidase (hydantoinpropionic acid forming enzyme). The enzyme was quite stable to dialysis and the enzymic activity was unaffected by 8-hydroxyquinoline-5-sulfonic acid, α,α'-bipyridyl, ethylenediamine tetraacetic

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[2] The same procedure can be used with slight modifications to purify the enzyme from guinea pig liver homogenates. Evidence for the presence of the enzyme in liver homogenates of other species of animal (Beef, Hog and Sheep livers) and also in crude extracts of bacteria (Pseudomonas fluorescens ATCC. 11299) has been obtained earlier by an independent coupled assay procedure involving formiminoglutamic acid - tetrahydrofolic acid transfer enzyme (Rao and Greenberg, 1960).

acid, azide or cyanide at concentrations of 10^{-3} M. p-Chloromercuribenzoate and p-chloromercuriphenyl sulfonate were 100% inhibitory at a concentration of 10^{-4} M.

TABLE I

PURIFICATION OF 4(5)-IMIDAZOLONE-5(4)-PROPIONIC ACID HYDROLASE FROM RAT LIVER

Fraction	Total Volume (ml)	units/ml	Total units	Protein conc. (mg/ml)	Specific activity (units/mg)	Recovery (%)
Homogenate*	70	300	21,000	21.2	14	100
40-50% saturation ammonium sulfate precipitate**	20	500	10,000	11.1	45	45
Calcium phosphate gel and alumina C gel treatments***	20	434	8,680	4.3	100	41
0-50% saturation ammonium sulfate precipitate	5	800	4,132	7.75	103	20

* 20 grams of fresh rat liver were homogenized with 4 vols of 0.1 M potassium phosphate buffer, pH 7.2, for 1 min. in a Waring blendor and centrifuged at 6000 x g for 15 min. at 0-5°. The supernatant fluid was again centrifuged at 20,000 x g for 30 min. at 0-5° in a Spinco preparative ultracentrifuge. The opalescent supernatant fluid (vol = 70 ml) was used for fractionation studies. The specific activity of the supernatant at this stage can vary from 3-15.

** Partial purification of the enzyme at this stage can also be achieved by removal of inactive protein by heat treatment at 52-55° for 5 min. at pH 7.2 in 10% w/v ammonium sulfate.

*** The gel adsorption procedure was carried out under conditions described elsewhere (Rao and Greenberg, 1960).

Imidazolonepropionic acid hydrolase activity was measured by the rate of disappearance of 264 mμ absorption maxima of 4(5)-imidazolone-5(4)-propionic acid at pH 7.2 (Rao and Greenberg, 1960). Imidazolonepropionic acid was generated enzymically from urocanic acid and purified beef liver urocanase. In a typical experiment the reaction mixture in Quartz spectrophotometric cuvettes (in a Cary recording spectrophotometer Model 14) contained 300 μmoles

of potassium phosphate buffer, pH 7.2, 0.2 μmoles of urocanic acid and a large excess of purified beef liver urocanase in a final volume of 3.1 ml. The rate of conversion of urocanic acid to imidazolonepropionic acid was followed by disappearance of ultraviolet absorption at 264 mμ (instead of at 277 mμ) and when the rate had fallen to less than an optical density change of 0.005 per min. (nonenzymic degradation of imidazolonepropionic acid to formylisoglutamine imidazolonepropionic acid hydrolase was added. The blank contained all components except urocanic acid. Forty units of enzyme caused an optical density change of 0.050 per min. under the above conditions. Good proportionality (over a six fold range from 10-60 units of enzyme activity) between the rate of disappearance of the 264 mμ absorption maximum and enzyme concentration was obtained in several experiments after correction for nonenzymic degradation.

In order to establish the identity of the product of the hydrolase reactio a large scale incubation of urocanic acid (3 millimoles of urocanic acid-2-C^{14} containing 5.75×10^5 c.p.m. of radioactivity), with 22,000 units of purified beef liver urocanase and 6,000 units of purified imidazolonepropionic acid hydrolase was carried out at 37° for 5 hours under partial anaerobic conditions. Formimino-L-glutamic acid[3] (3.33×10^5 c.p.m. -58% of total radioactivity) was isolated from the reaction mixture after deproteinization on a Dowex 50-H^+ (X-8, 200-400 mesh), 8 x 1 inch column by the method of Silverman, Gardiner and Bakerman (1952), except that 2N HCl was used for elution. Approximately 1.52×10^5 c.p.m. of radioactivity (26.5% of total radioactivity) was not held on the column and was identified as formylisoglutamine by methods described earlier (Rao and Greenberg, 1960). A considerable amount of radioactivity (0.9×10^5 c.p.m., about 15.6% of total radioactivity) was held on the column as a pigment[4]. Formimino-L-glutamic acid was purified (to remove traces of

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[3] Because of the labile nature of the intermediate 4(5)-imidazolone-5(4)-propionic acid and the probable inhibitory effects of the nonenzymic degradation products on the imidazolonepropionic acid hydrolase reaction the over-all conversion of urocanic acid to formiminoglutamic acid was not stoichiometric.

[4] 4(5)-Imidazolone-5(4)-propionic acid can probably undergo nonenzymic oxidative dimerization reactions to form colored pigments (Rao and Greenberg, 1960).

glutamic acid) on a Dowex-1-acetate (X-8, 200-400 mesh) 6 x 1 inch column (Tabor and Rabinowitz, 1957) and identified unequivocally[5].

The properties of imidazolonepropionic acid hydrolase thus closely resemble those of "220 fold purified fraction of imidazoleacetic acid oxidase". (The conversion of imidazoleacetic acid to formiminoaspartic acid presumably involves 4(5)-imidazolone-5(4)-acetic acid as an intermediate). Evidence as to whether two enzymes are involved in the latter sequence is lacking (Kny and Witkop, 1959). This is also the case in the sequence of reactions involved in the formation of formiminoglycine from 4(5)-aminoimidazole (Freter, Rabinowitz and Witkop, 1957).

The discovery and separation of imidazolonepropionic acid hydrolase completes the enzymic picture involved in the metabolism of histidine according to the urocanic acid pathway, as the remaining sequence of reactions have been described (Tabor and Wyngarden, 1959).

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[5] The isolated and synthetic samples of formimino-L-glutamic acid (Tabor and Rabinowitz, 1957) had the same R_f values of 0.48 and 0.54 in t-butanol: water: formic acid (70:15:15) and phenol-citrate-phosphate solvents (Broquist, 1956). Equimolar amounts of the isolated and synthetic formimino-glutamic acid had the same chromogenicity with the ferricyanide-nitroprusside reagent and also gave the same yield of 5,10-methenyltetrahydrofolic acid in the enzymic assay of Tabor and Wyngarden (1959). In addition the infrared spectrum (KBr pellet) of the enzymic product was superimposable with that of the synthetic material in every respect and was also in good agreement with the data reported by Miller and Waelsch (1954).

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