PURIFICATION AND PROPERTIES OF IMIDAZOLONE PROPIONIC ACID HYDROLASE

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Studies pertaining to histidine metabolism via the urocanic acid pathway have yielded evidence for the existence of 4(5)-imidazolone-5(4)-propionic acid as an intermediate in the conversion of urocanic acid to formimino-glutamic acid. The intermediate is the direct product of urocanase action, and a second enzyme, imidazolone propionic acid hydrolase, is required to convert it to formimino-glutamic acid (Revel and Magasanik, 1958; Feinberg and Greenberg, 1959). Brown and Kies (1959) have stabilized, purified and studied some of the characteristics of this intermediate, imidazolone propionic acid. Rao and Greenberg (1960) have reported a seven fold purification of imidazolone propionic acid hydrolase, the enzyme which converts imidazolone propionic acid to formimino-glutamic acid. However, under the aerobic conditions of their assay the spontaneous degradation of imidazolone propionic acid to formylisoglutamine competes with the enzymatic conversion to formiminoglutamic acid. Thus, even when employing partially anaerobic conditions in an effort to get a high yield of product, Rao and Greenberg were unable to attain a stoichiometric conversion of imidazolone propionic acid to formimino-glutamic acid. Moreover they did not effectively isolate and stabilize the substrate, hence

were unable to rigorously control and characterize the nature of the enzymatic reaction.

An independent investigation of this enzymatic reaction has been underway in this laboratory. This paper reports sixty fold purification of imidazolone propionic acid hydrolase from rat liver homogenates with an improved assay procedure (cf. Table I). The reaction was shown to proceed 99% to formimino-glutamic acid (formimino-glutamic acid assayed by the method[1] of Tabor and Wyngarden (1958).

The substrate, imidazolone propionic acid, was enzymatically synthesized, stabilized, and purified according to the method of Brown and Kies (1959). Because of the instability of imidazolone propionic acid at neutral pH under aerobic conditions, incubation of imidazolone propionic acid hydrolase with its substrate was always conducted anaerobically in a nitrogen atmosphere. Imidazolone propionic acid was found to be quite stable under nitrogen atmosphere in the absence of enzyme. Brown and Kies (1959) have shown that the only metabolic products of imidazolone propionic acid are formimino-glutamic acid, formylisoglutamine, and hydantoin propionic acid. Since catabolism to the latter two compounds requires molecular oxygen (Brown and Kies 1959), the only product of imidazolone propionic acid degradation in the anaerobic system is formimino-glutamic acid.

Assay of imidazolone propionic acid hydrolase was carried out as follows: 3.2 ml of 0.1 M phosphate buffer (pH 7.4) and 0.1 ml enzyme were placed in a Thunberg tube with 0.35 μmoles of pure imidazolone propionic acid in the side arm. The Thunberg tube was repeatedly evacuated and flushed with nitrogen. Four minu

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after the enzyme and substrate were mixed, the Thunberg tube was opened and the absorption of the incubation mixture was comparted with that of an enzyme-buffer blank at 260 mμ, the ultraviolet absorption peak of imidazolone propionic acid at neutral pH (Brown and Kies, 1959). The rate of decrease in absorption at 260 mμ was shown to be proportional to enzyme concentration. In the presence of boiled enzyme, no decomposition of substrate occurred.

Since under our assay conditions, decrease in imidazolone propionic acid concentration can be due only to enzymatic conversion to formimino-glutamic acid, it was possible to study the reaction rate at limiting concentrations of substrate. By this procedure, the Km value for imidazolone propionic acid hydrolase was calculated to be 0.7×10^{-4} M.

The enzyme was inhibited 100% by 10^{-4} M concentrations of p-chloromercuribenzoate. Enzymatic activity was unaffected by sodium versenate, potassium cyanide, hydrogen peroxide, hydroxylamine, and sermicarbazide at 10^{-3} M concentrations. The two latter compounds are known to inhibit urocanase at 10^{-3} M concentration (Feinberg and Greenberg (1959)).

Maximal activity of imidazolone propionic acid hydrolase occurred at pH 7.4 with a considerably narrower pH optimum than that observed by Feinberg and Greenberg (1959) for urocanase.

The reversibility of the reaction catalyzed by imidazolone propionic acid hydrolase was studied aerobically by incubating synthetic formimino-glutamic acid with an excess of purified imidazolone propionic acid hydrolase in quartz cuvettes in a Beckman Model DU spectrophotometer at 25° and anaerobically by identical incubations in evacuated Thunberg tubes at 25°. The absorption at 260 mμ, the absorption maximum of imidazolone propionic acid, and at 227 mμ were followed for 30 minutes. The measurement at 227 mμ was made to

Table I

PURIFICATION OF IMIDAZOLONE PROPIONIC ACID HYDROLASE FROM RAT LIVER

Rat Liver Fraction	Specific Activity Units/mg Protein (1)	Total Protein mg	Total Acti Units
Soluble supernatant fraction	1.24	6450	8000
50-60% sat. $(NH_4)_2SO_4$ ppt.	4.90	583	2860
Supernatant of pH 5 acid precipitation	6.74	414	2790
Calcium phosphate gel eluate, Step 1 (2)	20.0	118	2360
Calcium phosphate gel eluate, Step 2 (3)	77.0	39	3000

1. One unit equals 2×10^{-2} μmoles of imidazolone propionic acid degraded per minute. The concentration of imidazolone propionic acid was calculated from its molar extinction coefficient determined by the method of Silverman et al (1958). The imidazolone propionic acid preparation used for this study had an extinction coefficient of 4000 at neutral pH and 6000 in acid pH.

2. Supernatant fraction of pH precipitation mixed with calcium phosphate gel in ratio of 1.4 mg gel/mg protein. Protein eluted twice with 0.05 M phosphate buffer (pH 7.4).

3. Eluate of Step 1 diluted with an equal volume of distilled H_2O, mixed with calcium phosphate gel in a ratio of 2.3 mg of gel/mg protein, and eluted twice with 0.05 M phosphate buffer (pH 7.4).

detect the presence of formylisoglutamine if any should be formed spontaneously from imidazolone propionic acid. Incubations were performed at pH values of 6.6, 7.4, and 7.9, and with formiminoglutamic acid samples of 0.22 and 18.4 μmoles respectively. No increase in absorption at either wave length was observed under any of the conditions described, and thus there was no evidence that the reaction was reversible under these conditions.

The purified enzyme was assayed for urocanase activity according to the method of Mehler and Tabor (1953) and found to be inactive. Since imidazolone propionic acid hydrolase is associated

with urocanase in the soluble supernatant fraction of rat liver, this result reaffirms the fact that two separate enzymes are required to carry out the reaction from urocanic acid through imidazolone propionic acid to formimino-glutamic acid.

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