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Reversible reaction via a carbanion intermediate in the elimination of ammonia from L-histidine catalysed by histidine ammonia-lyase

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ABSTRACT

L-[5′-$^{2}H_2$]Histidine was used as a substrate to investigate the enzymatic reaction mechanism with histidine ammonia-lyase from *Pseudomonas fluorescens*. The study was performed to determine the exchange rate of deuterium at C-5′ of the imidazole ring with solvent hydrogen relative to the net urocanic acid production. The extent of hydrogen exchange at C-5′ of histidine or urocanic acid was measured by gas chromatography–mass spectrometry–selected ion monitoring, monitoring the molecular ion intensities of the respective gas chromatographic derivatives, at *m/z* 380 and 379 for histidine and at *m/z* 267 and 266 for urocanic acid. The observed hydrogen exchange at C-5′ suggested a reversible mechanism via a carbanion intermediate in the reaction with histidine ammonia-lyase.

INTRODUCTION

Histidine ammonia-lyase (EC 4.3.1.3) catalyses β-elimination of ammonia from L-histidine to produce urocanic acid [1–15]. The elimination involves initial abstraction of 3-H and cleavage of the C-2–N bond to form the *trans* double bond. Our recent studies [16–18] demonstrated the involvement of a stepwise mechanism via a carbanion intermediate in the elimination reaction, based on the finding of the enzyme-catalysed hydrogen exchange at C-5′ of the imidazole ring in the reaction of L-[3,3,5′-$^{2}H_2$,3′-^{15}N]histidine with histidine ammonia-lyase.

This study was designed to determine the rate of hydrogen exchange at C-5′ relative to the net urocanic acid production in the enzymatic reaction of L-[5′-^{2}H]-histidine. The results demonstrated a reversible mechanism via a carbanion intermediate in the elimination of ammonia from L-histidine catalysed by histidine ammonia-lyase.

EXPERIMENTAL

Materials

L-Histidine free base (Wako, Osaka, Japan), urocanic acid (Aldrich, Milwaukee, WI, U.S.A.), trifluoroacetic anhydride (Nakarai, Kyoto, Japan) and diethyl

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pyrocarbonate (Aldrich) were obtained commercially. Histidine ammonia-lyase (*Pseudomonas fluorescens*) was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals and reagents were of analytical-reagent grade and were used without further purification.

Synthesis of L-[5′-^{2}H]histidine

A solution of L-histidine free base (2.0 g, 12.9 mmol) in ^2H_2O (100 ml) was acidified with ^{2}HCl (99 atom-%) to p^{2}H 5.0 and heated at 180°C for 15 h in a nitrogen-filled, sealed tube. After lyophilization, the residue was dissolved in ^2H_2O (100 ml) and heated again. After evaporating the solvent at <60°C under reduced pressure, the product (DL-[2,2′,5′-$^2H_3$]histidine) was dissolved in H_2O (100 ml) and then heated at 120°C for 2 h or at 80°C for 12 h. The solution was neutralized to pH 7.2 with 1 *M* LiOH and evaporated to dryness. The residue was washed with ethanol and recrystallized from aqueous ethanol to give DL-[2,5′-$^2H_2$]histidine (1.95 g, 98%). A solution of DL-[2,5′-$^2H_2$]histidine (1.57 g, 10.0 mmol) in acetic acid–acetic anhydride (10:1, v/v) (22 ml) was heated at 80°C for 15 min under a stream of nitrogen. After removal of the solvent at less than 50°C under reduced pressure, the residue was taken up twice in H_2O (*ca.* 20 ml) and the solution was evaporated to dryness each time to give N-acetyl-DL-[5′-^{2}H]-histidine quantitatively. The almost pure material was dissolved in H_2O (50 ml) and the solution was brought to pH 7.2 with 0.5 *M* LiOH. Hog renal acylase (Tokyo Kasei, Tokyo, Japan) in three portions (50 mg, 50 000 U each) was added at 24-h intervals, being incubated at 37°C. After three days, the reaction was stopped by acidification with acetic acid (pH 5.0) and the protein was filtered with the aid of charcoal. The solution was neutralized to pH 7.2 with LiOH solution. The reaction mixture was purified by ion-exchange column chromatography (IRC-50 resin) to give L-[5′-^{2}H]histidine (1.66 g, 85%) as a free base. The overall scheme is shown in Fig. 1.

Optical purity (>99.9%) was determined by high-performance liquid chromatography (HPLC) with a chiral stationary phase (Chiralpak WH column; Daicel, Tokyo, Japan).

^{1}H NMR (^2H_2O): δ 3.15 (2H, d, 3-H), 3.97 (1H, t, 2-H) and 7.75 (1H, s, 2′-H). No proton signal at C-5′ (δ 7.05) was detected.

Gas chromatography–mass spectrometry–selected ion monitoring (GC–MS–SIM)

Capillary GC–MS–SIM analysis was done on a Shimadzu QP 2000 GC–MS system equipped with a data processing system. GC was performed on a Durabond (DB-5) fused-silica capillary column (40 m × 0.32 mm I.D.) with the stationary phase coated at a 0.1-μm film thickness (J&W Scientific, Rancho Cordova, CA, U.S.A.). Helium was used as the carrier gas at a column head pressure of 78.4 kPa. A split–splitless injection system operated in the splitless mode was used with a septum purge flow-rate of 10 ml/min and a split flow-rate of 60 ml/min. The purge activation time was 2 min. The initial column temperature was set at

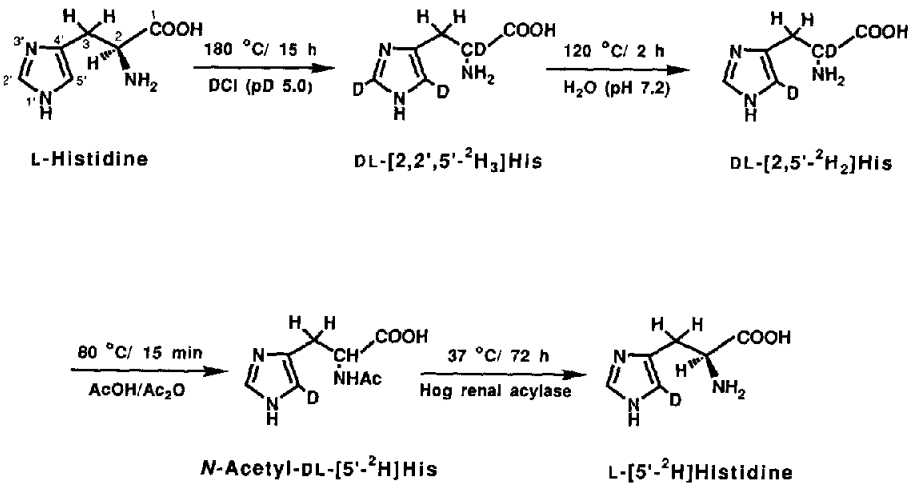

Fig. 1. Synthesis of L-[5'-^{2}H]histidine.

100°C and, 2 min after the sample injection, was increased at 25°C/min to 200°C and then at 10°C/min to 250°C. The electron energy was set at 70 eV and the ion source temperature was 280°C. The multiple-ion detector was focused on the molecular ions at *m/z* 379 and 380 for $^{\alpha}$N-(trifluoroacetyl)-^{im}N-(ethoxycarbonyl)-histidine *n*-butyl ester (His-TEB) [19,20] and at *m/z* 266 and 267 for ^{im}N-(ethoxycarbonyl)urocanic acid *n*-butyl ester (UA-EB).

Sample preparation for GC–MS–SIM

The enzymatic reaction mixture (0.1 ml) was deproteinated and extracted with 2 ml of ethanol on a vortex mixer. The ethanol phase was transferred into a 2-ml V-vial and evaporated to dryness at 50°C under a stream of nitrogen. To the residue containing histidine and urocanic acid were added 200 μl of 3 *M* hydrogen chloride in *n*-butanol. The reaction mixture was sealed under a nitrogen atmosphere and heated at 100°C for 15 min. After removal of the solvent at 70°C under a stream of nitrogen, 200 μl of dichloromethane were added and the solution was evaporated to dryness again. The residue was dissolved in 2 ml of dichloromethane and then applied to a Sep-Pak silica cartridge (Waters Assoc., Milford, MA, U.S.A.). The cartridge was washed with 5 ml of dichloromethane and then eluted with 5 ml of methanol. After evaporation of the eluate in a 10-ml conical centrifuge tube under a stream of nitrogen, 1.5 ml of methanol were added to the residue and the sample was transferred into a 2-ml V-vial, and then the solvent was evaporated. The residue was reconstitued in 250 μl of dichloromethane–trifluoroacetic anhydride (TFAA) (4:1, v/v) and then heated at 150°C for 5 min. After the reaction, the excess of reagent and solvent were evaporated at room temperature under a gentle stream of nitrogen. The residue was dissolved in 200

μl of 1.5% diethyl pyrocarbonate (DEPC) in dichloromethane, heated at 150°C for 20 min and then evaporated to dryness. The residue was reconstituted with 100 μl of dichloromethane for the GC–MS–SIM analysis. The derivative was stable at −20°C for at least two months.

Enymatic reaction

Histidine ammonia-lyase (200 U, *Pseudomonas fluorescens*) was incubated in 13.2 ml of 0.2 *M* Tris buffer at 25°C for 30 min at either pH 7.0 or 9.0 in the presence of 3.8 m*M* $MgCl_2$ and 3.8 m*M* glutathione. To the incubation mixture was added L-[5′-^{2}H]histidine (5.0 mg, 2.45 m*M*) and incubated at 25°C for 24 h. The reaction mixture (0.1 ml) was collected every 2 h. After excess of absolute ethanol (*ca.* 2 ml) had been added to terminate the reaction, the solvent was evaporated at 50°C under a stream of nitrogen.

RESULTS AND DISCUSSION

This study was carried out to determine the rate of hydrogen exchange at C-5′ of the imidazole ring with solvent hydrogen relative to the net urocanic acid production in the reaction of L-[5′-^{2}H]histidine catalysed by histidine ammonia-lyase. For the synthesis of L-[5′-^{2}H]histidine, the deuterium–hydrogen exchange reaction was employed for the selective deuteriation at C-5′ of the imidazole ring of L-histidine. As shown in Fig. 1, unlabelled L-histidine was first treated with ^2HCl–^2H_2O (p^{2}H 5.0) at 180°C for 15 h to give DL-[2,2′,5′-$^2H_3$]histidine. Selective back-exchange of deuterium at the C-2′ position of DL-[2,2′,5′-$^2H_3$]histidine with H_2O (pH 7.2) at 120°C for 2 h gave DL-[2,5′-$^2H_2$]histidine. Treatment of this material with acetic acid–acetic anhydride (10:1, v/v) at 80°C for 15 min provided N-acetylation with concomitant racemization at C-2 to N-acetyl-DL-[5′-^{2}H]histidine. The resolution of DL-histidine was carried out by the stereospecific hydrolysis of its N-acetyl derivative with hog renal acylase at 37°C for 72 h. The optical purity of the labelled L-histidine (more than 99.9%) was determined by HPLC with a chiral stationary phase.

Fig. 2 shows the electron-impact mass spectra of GC derivatives of unlabelled and stable isotopically labelled histidine (L-[5′-^{2}H]histidine). The isotopic purity was calculated as 97.4%, based on the ion intensities in the region of the molecular ion of each compound.

L-[5′-^{2}H]Histidine (2.45 m*M*) was incubated at 25°C with histidine ammonia-lyase (*Pseudomonas fluorescens*) at either pH 7.0 or 9.0 in the presence of $MgCl_2$ and glutathione. The time course of the reaction was followed every 2 h for 24 h. Histidine and urocanic acid were derivatized to His-TEB and UA-EB, respectively. The extent of hydrogen exchange at C-5′ of histidine or urocanic acid was measured by GC–MS–SIM [19,20], monitoring the molecular ion intensities of the respective GC derivatives at *m/z* 380 and 379 for histidine and at *m/z* 267 and 266 for urocanic acid.

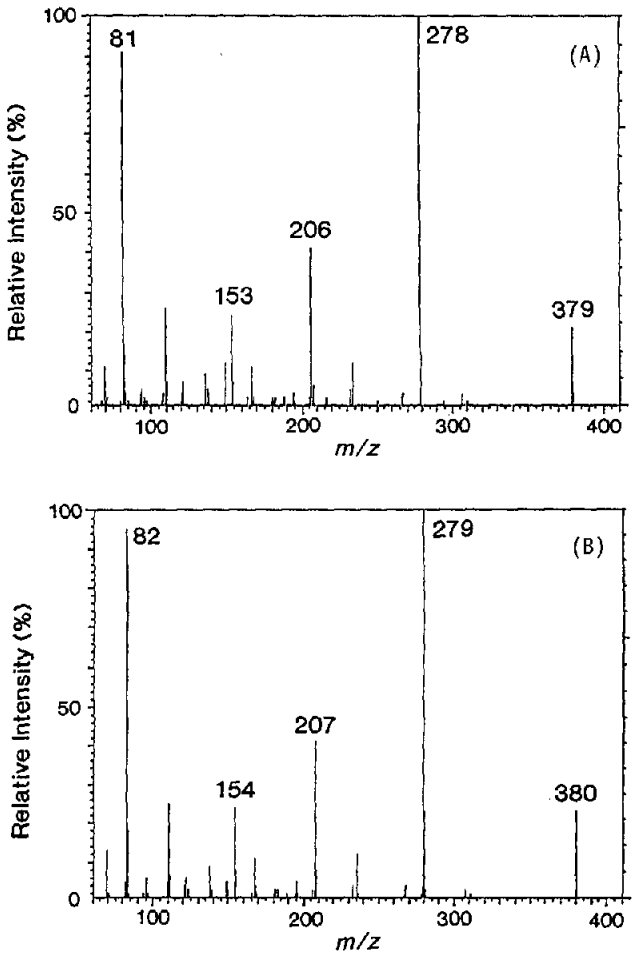

Fig. 2. Electron-impact mass spectra of TEB derivatives of (A) unlabelled L-histidine and (B) L-[5'-$^2H_2$]-histidine.

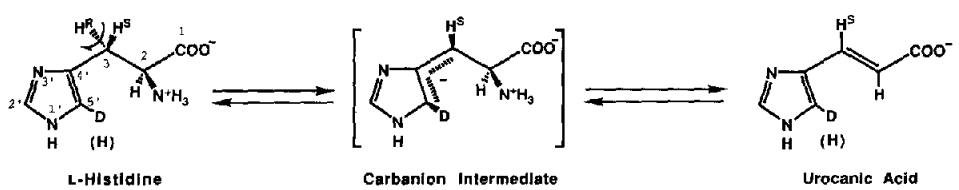

Fig. 3. Reversible mechanism via a carbanion intermediate for the formation of L-histidine and urocanic acid.

The production rates of histidine and urocanic acid exchanged with hydrogen increased with the reaction time, being 5.0 μM/h (pH 7.0) and 1.4 μM/h (pH 9.0) for histidine and 27.7 μM/h (pH 7.0) and 25.6 μM/h (pH 9.0) for urocanic acid. The observed hydrogen exchange at C-5′ suggested a reversible mechanism via a carbanion intermediate in the reaction with histidine ammonia-lyase (Fig. 3).

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