Evidence for a Carbanion Intermediate in the Elimination of Ammonia from L-Histidine Catalyzed by Histidine Ammonia-Lyase

Takashi Furuta,* Hidenori Takahashi, and Yasuji Kasuya

Contribution from the Department of Medicinal Chemistry and Clinical Pharmacy, Tokyo College of Pharmacy, Hachioji, Tokyo 192-03, Japan. *Received June 19, 1989*

Abstract: Stable isotopically labeled histidines, i.e., L-[3,3,5'-$^2H_3$,3'-^{15}N]histidine (His-*3,3,5'-d*$_3$) and DL-[2,5'-$^2H_2$]histidine (His-*2,5'-d*$_2$), were used as substrates to investigate the enzymatic reaction mechanism with histidine ammonia-lyase (EC 4.3.1.3) from *Pseudomonas fluorescens* in order to determine whether the elimination is concerted or involves a carbanion intermediate. The labeled L-histidine (His-*3,3,5'-d*$_3$) (100 mM) was incubated with histidine ammonia-lyase (400 units) for 24 h at 25.0 °C (pH 9.0, 0.2 M Tris buffer) in the presence of 0.01 M $MgCl_2$ and 0.1 M glutathione. The 400-MHz ^1H NMR spectrum of the labeled urocanic acid product (UA-*3,5'-d*$_2$) revealed the presence of a proton signal at δ 7.75 corresponding to the C-5' position of the imidazole ring, indicating the incorporation of hydrogen at C-5'. The extent of deuterium loss was calculated to be 44%, which was practically the same as that obtained from the mass spectral data (45.3%). When His-*2,5'-d*$_2$ was incubated, the same extent of deuterium loss at C-5' was observed, being 42% based on the ^1H NMR data. The evidence presented in this study supports a stepwise mechanism via a carbanion intermediate for the elimination of ammonia catalyzed by the enzyme.

Histidine ammonia-lyase (EC 4.3.1.3) catalyzes β-elimination of ammonia from L-histidine to produce urocanic acid.[1-6] The formation of the trans double bond in the urocanic acid molecule results from the initial abstraction of 3-hydrogen followed by the cleavage of the C-N bond of L-histidine. In spite of extensive studies on the action of the enzyme,[7-12] the precise mechanism involved in the elimination of ammonia still remains obscure. A concerted elimination mechanism proposed for this enzymatic reaction was supported by several investigators on the basis of the observation that the incorporation rate of solvent tritium into the histidine molecule (at *pro*-3*R*) was essentially identical with[5] or somewhat faster than[13] that of histidine regeneration from urocanic acid.

With the use of 4'-nitro-L-histidine as a substrate, Klee et al.[14] observed that the electron-withdrawing substituent at the C-4' position of the imidazole ring enhanced the acidity of 3-hydrogen and the stability of the conjugated 3-carbanion. The increase in the acidity of 3-hydrogen then leads to the rate-limiting step progressing from concerted C-H/C-N bond breaking to C-N bond cleavage alone.[14,15] This model reaction seems to indicate carbanion formation in the deamination process. There is, however, no direct evidence that supports the involvement of a carbanion intermediate for the natural L-histidine.

The present study was carried out to elucidate the enzymatic reaction mechanism accounting for the elimination of ammonia from L-histidine. Two kinds of stable isotopically labeled histidines were uniquely used as substrates for the enzymatic reaction with histidine ammonia-lyase from *Pseudomonas fluorescens* in order to determine whether the reaction is concerted or a carbanion intermediate is involved. The evidence provided in this study strongly suggests a stepwise mechanism via a carbanion intermediate in the enzymatic elimination of ammonia.

Results and Discussion

L-[3,3,5'-$^2H_3$,3'-^{15}N]Histidine (His-*3,3,5'-d*$_3$)[17] was synthesized by our method starting from [3,3,5,5-$^2H_4$]-2,5-diamino-4-oxopentanoic acid.[18] The labeled L-histidine (His-*3,3,5'-d*$_3$) (100 mM) was incubated with histidine ammonia-lyase (400 units)[19] for 24 h at 25.0 °C (pH 9.0, 0.2 M Tris buffer) in the presence of 0.01 M $MgCl_2$ and 0.1 M glutathione in the H_2O-buffer system. The reaction was stopped with excess EtOH at room temperature. The urocanic acid product (UA-*3,5'-d*$_2$) was purified by anion-exchange column chromatography (Dowex 1-X8). The 400-MHz ^1H NMR spectrum of the labeled urocanic acid (UA-*3,5'-d*$_2$) revealed the presence of a proton signal at 7.75 corresponding to the C-5' position of the imidazole ring (Figure 1), indicating the incorporation of hydrogen into C-5'. On the basis of the H-2' or H-2 integration, the loss of deuterium at C-5' was calculated to be 44%. The extent of deuterium loss was practically the same as that obtained from the mass spectral data (45.3%)[20] (Figure 2). Without the use of the enzyme, the deuterium atom at C-5' of His-*3,3,5'-d*$_3$ was found to be retained completely. When DL-[2,5'-$^2H_2$]histidine (His-*2,5'-d*$_2$)[23] was substituted for the

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(14) Klee, C. B.; Kirk, K. L.; Cohen, L. A. *Biochem. Biophys. Res. Commun.* **1979**, *87*, 343.
(15) Isotope exchange of the 3-hydrogens of 4'-nitro-L-histidine easily occurs even under aqueous basic conditions (1 N NaOD).[14,16] The absence of the primary isotope effect has also been reported for the elimination of ammonia from the C-3 deuteriated 4'-nitrohistidine analogue.
(16) Klee, C. B.; Kirk, K. L.; McPhie, P.; Cohen, L. A. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **1974**, *33*, 1318.
(17) The deuterium-labeled position of His-*3,3,5'-d*$_3$ was confirmed by the 400-MHz ^1H NMR spectral analysis: (D_2O) δ 3.97 (1 H, s, 2-H) and 7.77 (1 H, d, 2'-H). Other proton signals at C-3 (δ 3.14) and C-5' (δ 7.05) completely disappeared. The isotopic purity (96.5 atom %) as the [$^2H_3$,^{15}N]-form was estimated by selected ion monitoring, on the basis of the ion intensities in the molecular ion region of the GC derivative [$^{\alpha}_{N}$-(trifluoroacetyl)-$^{im}_{N}$-(ethoxycarbonyl)histidine *n*-butyl ester].[21,22]
(18) Furuta, T.; Kasuya, Y.; Takahashi, H.; Baba, S. *J. Chem. Res., Synop.* **1987**, 86.
(19) One unit deaminates 1.0 nmol of L-histidine to urocanic acid per minute at pH 9.0 at 25 °C.
(20) The extent of hydrogen exchange of labeled urocanic acid formed by the enzymatic reaction was determined by GC-MS selected ion monitoring, measuring the ion intensities in the molecular ion region (*m/z* 269 and 268) of the GC derivative [$^{im}_{N}$-(ethoxycarbonyl)urocanic acid *n*-butyl ester].
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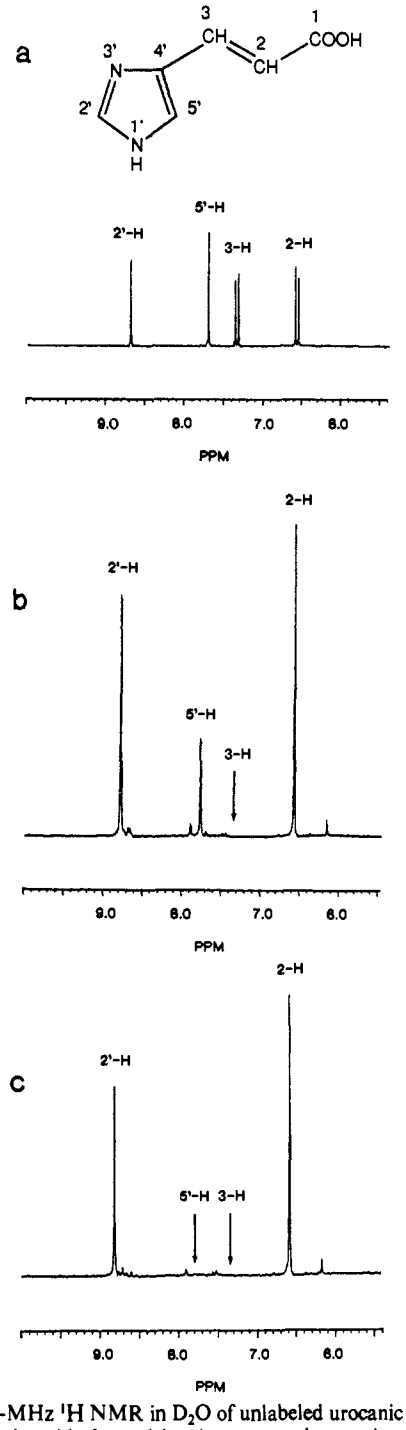


Figure 1. 400-MHz ^1H NMR in D_2O of unlabeled urocanic acid (a) and labeled urocanic acids formed by the enzymatic reaction of L-[3,3,5'-$^2H_3$,3'-^{15}N]histidine with histidine ammonia-lyase in the H_2O–buffer (b) and D_2O–buffer (c) systems.

substrate His-*3,3,5'-d*$_3$, the same extent of deuterium loss at C-5' was observed, being 42% based on the ^1H NMR data. There was no deuterium loss at C-2.

On the other hand, incubation of either His-*3,3,5'-d*$_3$ or His-*2,5'-d*$_2$ in D_2O–buffer resulted in no loss of deuterium at C-5' (Figure 1). This indicates that solvent-derived hydrogen was incorporated into C-5'. In addition, there was no deuterium incorporation into the C-3 and C-2' positions. That is, the enzymatic reaction products were [3,5'-$^2H_2$]- and [2,5'-$^2H_2$]urocanic acids from His-*3,3,5'-d*$_3$ and His-*2,5'-d*$_2$, respectively. Under the D_2O–buffer conditions employed, the incubation of unlabeled urocanic acid resulted in no incorporation of deuterium at the C-5' position. In the light of these results, the observed loss of deuterium at C-5' (ca. 45%) during the enzymatic reaction of His-*3,3,5'-d*$_3$ and His-*2,5'-d*$_2$ should be interpreted as the result of the enzyme-catalyzed hydrogen exchange.

(23) Only the L-isomer is a substrate for the enzymatic reaction with histidine ammonia-lyase.

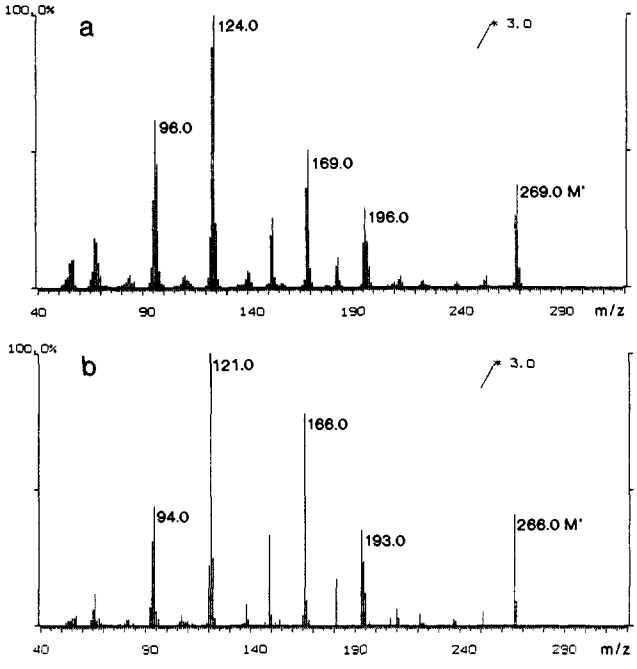


Figure 2. Electron-impact mass spectra of unlabeled urocanic acid (b) and labeled urocanic acids (a) formed by the enzymatic reaction of L-[3,3,5'-$^2H_3$,3'-^{15}N]histidine with histidine ammonia-lyase in the H_2O–buffer system.

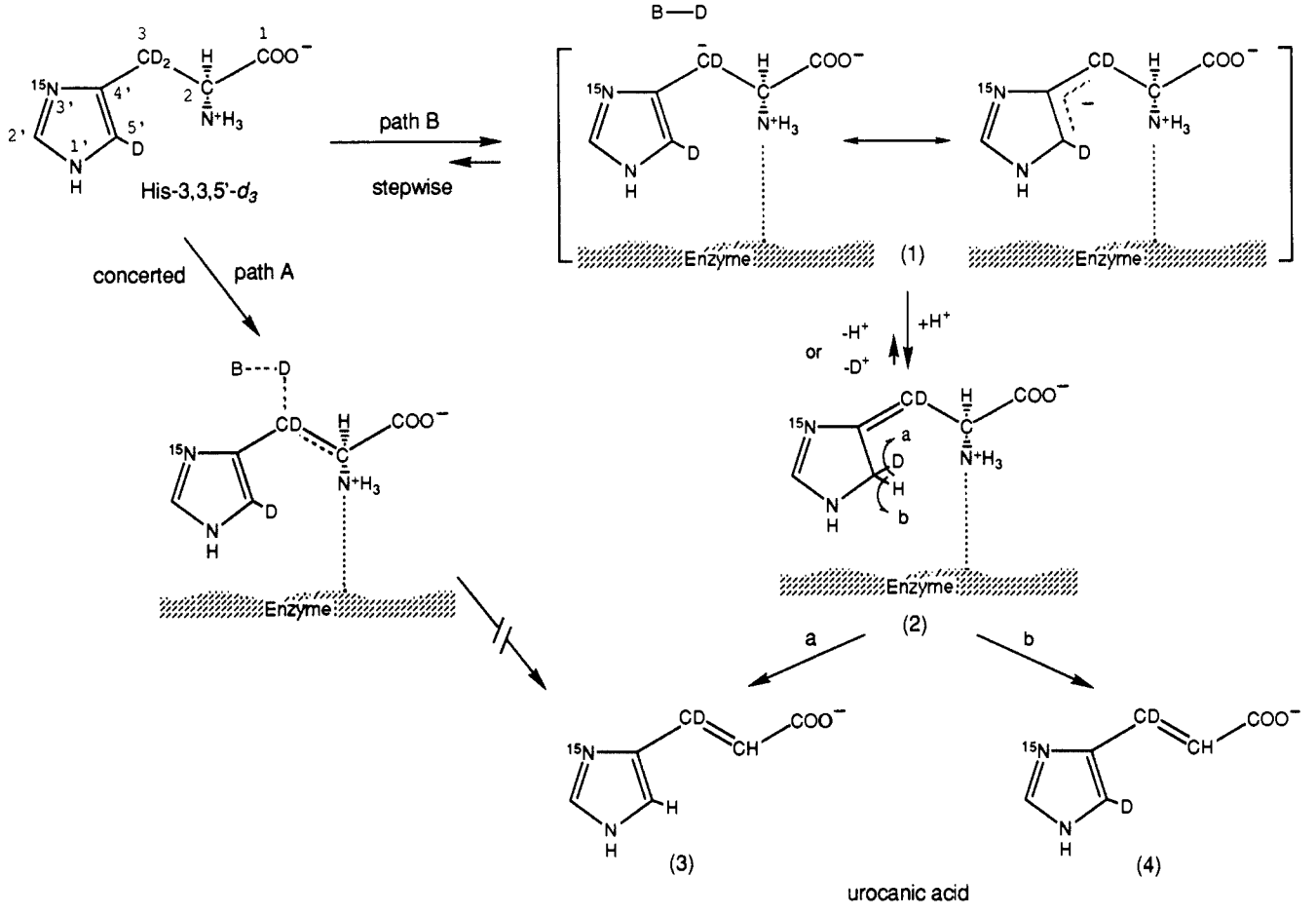
It is interesting to note that the incubation of unlabeled L-histidine with histidine ammonia-lyase under the D_2O–buffer conditions (pD 9.0) led to only ca. 20% deuterium incorporation at C-5' of urocanic acid,[24] as determined by ^1H NMR. The difference in the observed ratios between deuterium incorporation at C-5' (ca. 20%) and deuterium loss or hydrogen incorporation of ca. 45% when His-*3,3,5'-d*$_3$ is incubated under the H_2O–buffer conditions must be attributable to the solvent isotope effect for the incorporation.

The fact that the deuterium–hydrogen exchange occurred at C-5' of the deuterium-labeled substrates excludes the possibility of a concerted mechanism (see path A in Scheme I). Instead, the stepwise mechanism via a carbanion intermediate shown in Scheme I (path B) should provide a rational explanation for the enzymatic elimination of ammonia from L-histidine. The first step involves the abstraction of *pro*-3*R* hydrogen to form a carbanion delocalized by conjugation. This is followed by the electrophilic incorporation of hydrogen from solvent (H_2O) at C-5' of the carbanion intermediate. The subsequent loss of either D^+ or H^+ leads to the elimination of ammonia in the form of either NH_2D or NH_3.

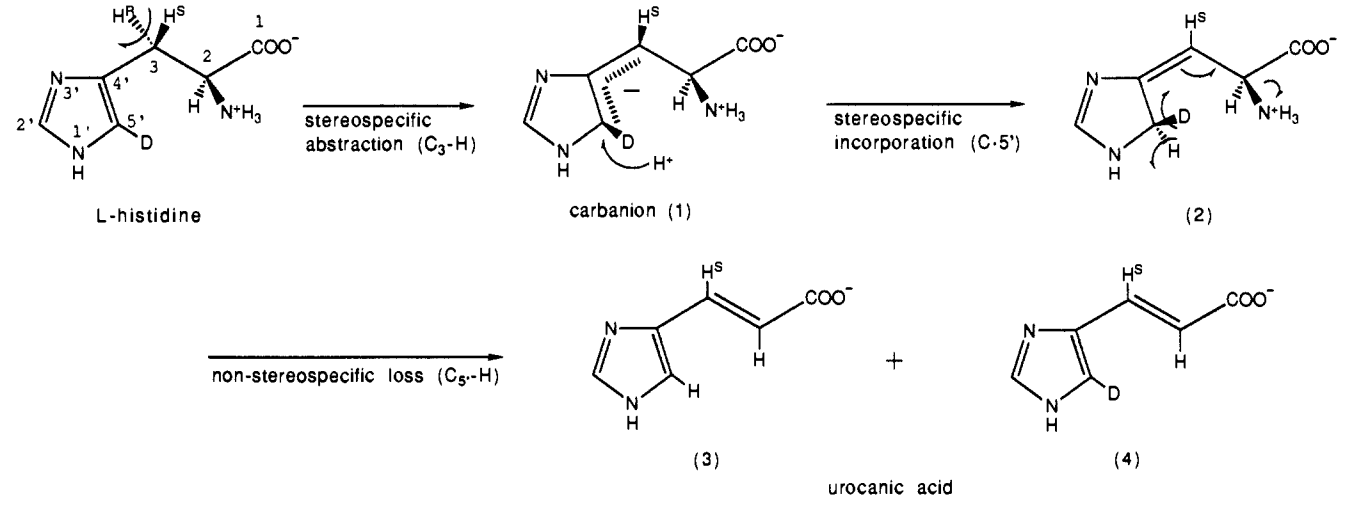
In the elimination process, the enzyme-catalyzed abstraction of C-3 hydrogen has been demonstrated to proceed stereospecifically.[1,3,4,6] The stereospecific abstraction of C-3 hydrogen (*pro*-3*R*) could be followed by delocalization of the 3-carbanion to C-5' on the same face as the allyl carbanion asymmetrically formed. This would lead to stereospecific hydrogen incorporation at C-5'. The stereospecific hydrogen incorporation from solvent and subsequent nonstereospecific loss of a proton at C-5' would result in the formation of urocanic acid retaining 50% deuterium atom (Scheme II). This provides a rational explanation for our

(24) Peterkofsky[5] reported that the reversible reaction in T_2O with enzyme resulted in tritium incorporation only at the C-3 position of the histidine molecule. Furthermore, the urocanic acid recovered from the tritiation experiment contained only a slight amount of radioactivity. Our finding of enzyme-catalyzed hydrogen or deuterium exchange at C-5' of the imidazole ring indicates that solvent tritium incorporation must have occurred at both C-3 (*pro*-3*R*) and C-5' for the histidine molecule and also at C-5' for the urocanic acid.

Scheme I



Scheme II



present results of ca. 45% deuterium exchange at C-5'. The subsequent abstraction of a C-5' proton leading to ammonia elimination may proceed nonenzymatically or spontaneously with concomitant aromatization to the imidazole. The loss of the C-5' proton would then occur nonstereospecifically.

The 3-substituents of the substances generally allow the 3-carbanion formed during the C–H bond-breaking process to be delocalized. The effect would be enhanced by an electron-withdrawing substituent on the imidazole ring, as in the enzymatic reaction of 4'-nitro-L-histidine demonstrated by Klee et al.[14] The delocalization may also be enhanced by coordination of the 3-imidazole group to a divalent metal cation (e.g., Mg^{2+}).[1] Using L-[5'-^{2}H]histidine as a substrate, further studies are now in progress in our laboratory to investigate pH and metal ion influences on the degree of kinetic deuterium isotope effect and on the extent of deuterium loss at the C-5' position. Kinetic isotope effects on the C_3–H bond breaking during the deamination process for the L-[3,3-$^2H_2$]histidine substrate under these various conditions will also give further insight into the formation of the unstable intermediate.

The mechanism of the histidine ammonia-lyase reaction has been investigated by Peterkofsky[5] and Klee et al.[13,14] Peterkofsky[5] demonstrated that the incorporation rates of both solvent tritium and ^{14}C-labeled urocanic acid into regenerated histidine substrates were almost equivalent. The exchange rate of tritium into histidine from T_2O in the enzymatic reaction represents that of C–H bond cleavage at C-3 (*pro*-3*R*). The regeneration rate of radioactive histidine from [^{14}C]urocanic acid indicates that of C–N bond

formation (=cleavage) by its reverse reaction. If the reaction is actually concerted, these exchange rates should be exactly identical. The identical value for both rates then implicates the simultaneous (concerted) occurrence of C–H/C–N bond breaking. Peterkofsky postulated that the enzyme accomplishes the β-elimination by a concerted mechanism. However, it should be noted that all studies that attempt to determine the sequence of bond cleavage by use of *isotope exchange* are generally faced with the difficulty of determining whether the exchange rate is indicative of covalent bond cleavage or dissociation of the group from the enzyme.[25-27] A comparison of these exchange rates of radioactivity may not be precise enough for evaluating such a microscopic reaction mechanism. Even if C_3–H bond cleavage proceeds slightly faster than C_2–N bond cleavage,[13] separation of the steps would not have been sufficient to invoke a carbanion mechanism, as postulated by Bright et al.[28] for the deamination of 3-methylaspartic acid. Experimentally *almost equivalent* rates do not necessarily mean a concerted mechanism. In addition, the solvent tritium isotope effect on the deamination reaction always has to be taken into account in assessing the net rate of C–H bond cleavage as long as T_2O or D_2O is used as a solvent.

In recent years, mechanistic studies of the enzymatic reactions catalyzed by the ammonia-lyases such as aspartase,[29,30] methylaspartase[31-33] and phenylalanine ammonia-lyase[34] have attracted much interest. The elimination mechanism of ammonia is still a subject of controversy. The present study using stable isotopes has provided evidence for carbanion involvement in the enzymatic reaction of L-histidine with histidine ammonia-lyase. The novel finding of hydrogen exchange at C-5′ of the imidazole ring sheds light on the formation of an unstable intermediate involved in the enzymatic reaction.

Experimental Section

General Procedures. ^{1}H NMR spectroscopy was performed on a Bruker AM-400 400-MHz spectrometer. Chemical shifts are reported relative to the isotopic impurity peak for a given solvent (D_2O, 4.75 ppm). Mass spectra were recorded on a Hitachi M-80 mass spectrometer (EI; 70 eV). Capillary GC–MS analysis was done on a Shimadzu QP2000 gas chromatographic–mass spectrometric system equipped with a data-processing system. GC–MS employed a DB-5 fused-silica capillary column (30 m × 0.32 mm i.d., film thickness 0.1 μm; J & W Scientific Inc.).

L-[3,3,5′-$^2H_3$,3′-^{15}N]Histidine. To a solution of [3,3,5,5-$^2H_4$]-2,5-diamino-4-oxopentanoic acid[18] (5.0 g, 22.4 mmol) in D_2O (15 mL, 99.75 atom %) was added dried NaSC^{15}N (3.25 g, 40.0 mmol)[18] in four portions at 30-min intervals, with heating at 95 °C. After the final addition of NaSC^{15}N, the reaction mixture was heated at 85 °C for 1 h. The solution was adjusted to pD 5.0 with saturated sodium acetate in D_2O and was then left at 0 °C for 12–24 h. The precipitate was collected and washed with a small volume of water to give DL-[3,3,5′-$^2H_3$,3′-^{15}N]-2-mercaptohistidine as a colorless solid (1.93 g, ca. 45% yield). The product was used without further purification in the preparation of labeled histidine. A mixture of labeled DL-mercaptohistidine (1.75 g, 9.2 mmol) and iron(III) sulfate (23.8 g) in H_2O (120 mL) was heated at 100 °C for 1 h. The reaction mixture was poured into boiling water (200 mL), followed by treatment with $Ba(OH)_2 \cdot 8H_2O$ (42 g, 140 mmol). The resulting precipitate was collected and suspended in boiling water (150 mL) and filtered off, and the process was repeated. The combined filtrate (pH 8) was heated to boiling and neutralized to pH 7.2 with dilute H_2SO_4. After removal of barium sulfate, the solution was concentrated to a volume of 50 mL and then filtered. The solution was evaporated to dryness under reduced pressure, and the residue was recrystallized from aqueous EtOH to give DL-[3,3,5′-$^2H_3$,^{15}N]histidine in ca. 90% yield quadrilateral plates: mp 285 °C dec; ^{1}H NMR (D_2O) δ 3.97 (1 H, s, 2-H) and 7.77 (1 H, d, 2′-H).

A solution of racemic labeled histidine (769 mg, 4.8 mmol) in 11 mL of acetic acid/acetic anhydride (10:1 v/v) was heated at 80 °C for 10–15 min under a stream of nitrogen gas. After removal of the solvent at 50 °C under reduced pressure, the residue was taken up twice in H_2O (5–10 mL) and the solution was evaporated to dryness each time to give the *N*-acetylhistidine in ca. 98% yield. The almost pure material was dissolved in H_2O (30 mL) and the solution was brought to pH 7.2 with 0.5 M LiOH. Hog renal acylase I in three portions (50 mg, 50000 units each) was added at 24-h intervals to the solution incubated at 37 °C. After 3 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-[3,3,5′-$^2H_3$,3′-^{15}N]histidine (366 mg, 95% yield) as a free base: ^{1}H NMR (D_2O) δ 3.97 (1 H, s, 2-H) and 7.77 (1 H, d, 2′-H). Other proton signals at C-3 (δ 3.14) and C-5′ (δ 7.05) completely disappeared. The isotopic purity (96.5 atom %) of the [$^2H_3$,^{15}N]-form was estimated by selected ion monitoring, on the basis of the ion intensities in the molecular ion region of the GC derivative [$^{\alpha}_{N}$-(trifluoroacetyl)-$^{im}_{N}$-(ethoxycarbonyl)histidine *n*-butyl ester].[21,22] The optical purity (more than 99.9%) was determined by HPLC with a chiral stationary phase (Chiralpak WH column, Daicel Chemicals).

DL-[2,5′-$^2H_2$]Histidine. A solution of L-histidine (3.1 g, 20 mmol) in D_2O (50 mL) was acidified with DCl (99 atom %) to pD 5.0 and heated at 180 °C for 13 h in a nitrogen-filled sealed tube. After lyophilization, the residue was redissolved in D_2O (50 mL) and heated again. The solvent was evaporated to dryness at 60 °C under reduced pressure to give DL-[2,2′,5′-$^2H_3$]histidine. Selective back-exchange of deuterium at the C-2′ position with H_2O (100 mL, pH 7.2) at 120 °C for 2 h gave DL-[2,5′-$^2H_2$]histidine: ^{1}H NMR (D_2O) δ 3.15 (2 H, s, 3-H) and 7.75 (1 H, s, 2′-H). No proton signals at C-2 (δ 3.97) and C-5′ (δ 7.05) were detected.

Enzymatic Reaction. Stable isotopically labeled histidine (100 mM), i.e., L-[3,3,5′-$^2H_3$,3′-^{15}N]histidine and DL-[2,5′-$^2H_2$]histidine, was incubated at 25 °C for 24 h with histidine ammonia-lyase (400 units) in 3.0 mL of 0.2 M Tris buffer (pH 9.0) in the presence of 0.01 M $MgCl_2$ and 0.1 M glutathione. The reaction was stopped with excess EtOH (ca. 15 mL) and the solvent was evaporated under reduced pressure at 50 °C. The anion-exchange resin (Dowex 1-X8, 100–200 mesh) was washed several times with 3 M hydrochloric acid, converted into its hydroxide form with 3 M sodium hydroxide, then transformed to its acetate form with 8 M acetic acid, and finally washed with water until the effluent was neutral. The reaction mixture (pH 8.5) was adsorbed on the column (200 mm × 15 mm i.d.), which was then washed with 25 mL of water. The flow rate was 15 mL/h. Elution was performed with 0.1 M acetic acid.

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