Histidine Ammonia-lyase

THE USE OF 4-FLUOROHISTIDINE IN IDENTIFICATION OF THE RATE-DETERMINING STEP

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SUMMARY

The α,β eliminations of NH₃ from L-histidine and 4-fluoro-L-histidine by histidine ammonia-lyase appear to occur by similar mechanisms, although a large difference in Vₘₐₓ for the two reactions was observed. Both reactions were shown to be reversible with an equilibrium constant of 4 to 5. The pre-steady state kinetics of the deamination of 4-fluoro-L-histidine indicates that the rate-determining step precedes the dissociation of ammonia from the enzyme. The isotope effect of 1.4 to 2.0 observed with 4-fluoro-DL-[α-2H]histidine or DL-[β-2H]histidine indicates that the C–H bond breakage is at least partially rate-determining for the deamination of both substrates.

Histidine ammonia-lyase effects an α,β elimination of ammonia from L-histidine to generate trans-urocanic acid (1–6). It has been postulated that the α-amino group of the substrate adds covalently to a "dehydroalanyl" residue in the active site of the enzyme (4, 7); removal of the β proton (4, 6) and rupture of the α carbon to nitrogen bond result in the formation of urocanic acid and the amino enzyme postulated by Peterkofsky (6).

Histidine + enzyme ⇌ NH₂-enzyme + H⁺ + urocanic acid (1)

NH₂-Enzyme + HOH ⇌ enzyme + NH₃ (2)

(Equations 1 and 2). Reaction 2, initially found to be experimentally irreversible (6), was later shown to be reversible under appropriate conditions (8) although at a very slow rate. The existence of an amino-enzyme intermediate was deduced from the observations that solvent tritium and [1⁴C]urocanic acid were incorporated into reisolated histidine, while [1⁴N]₂ was not incorporated (6). Additional support for the existence of this intermediate is based on the noncompetitive kinetic behavior of urocanate relative to histidine, as observed by Givot et al. (4). The existence of the exchange reactions was taken as evidence that the dissociation of the ammonia moiety from the enzyme is the slowest step in net product formation. Because the exchange reaction was similar in rate and, under some circumstances, considerably slower than the over-all reaction, Rose suggested that a common step, prior to the release of ammonia, was rate-limiting in both exchanges (9). Furthermore, the stimulatory effect of metal ions as well as the inhibitory effect of EDTA on the elimination reaction (1, 9) suggests that metal ions operate at the rate-determining step. It has also been proposed that metals operate to "facilitate the deprotonation of the substrate" (14).

The evidence presented in this paper confirms the reversibility of the over-all reaction and indicates that C–H bond cleavage is at least partially responsible for limiting the rate of deamination of histidine and of 4-fluorohistidine.

MATERIALS AND METHODS

Materials—L-Histidine, urocanic acid, and L-[U,¹⁴C]histidine (312 mCi/mmol) were products of Schwarz/Mann. Silica Gel GF and G plates were obtained from Analtech. H₂O (99.7%) was a product of Aldrich Chemical Co. H₂O (100 mCi/g) was purchased from New England Nuclear.

4-Fluoro-DL-histidine and 4-fluoro-L-histidine were synthesized as described previously (16, 17). To obtain 4-fluoro-DL-[¹⁴C]histidine, diethyl-α-acetamido[4-fluoro-5-imidazoyl methyl]malonate (17), 315 mg (1 mmol), in 0.3 ml of H₂O (30 mCi), and 0.3 ml of concentrated hydrochloric acid were heated on a steam bath for 18 hours. The resulting 4-fluoro-DL-[α-¹⁴C]histidine was isolated and purified by the published procedure (17). The yield was 151 mg (87%), the specific activity, 50 × 10⁵ cpm/amol. DL-[²H₂]-Histidine and 4-fluoro-DL-[β-²H]histidine were synthesized from the corresponding deuterated imidazole-5-methanols; the latter compounds were obtained by reduction of ethyl imidazole-5-carboxylate and of ethyl 4-fluorimidazole-5-carboxylate with LiAlH₄. Mass spectral analysis demonstrated the presence of 2 deuterium atoms per molecule. The amino acid side chain was elaborated, and the products were purified by the published methods (17). Concentrations of the deuterated and nondeuterated DL-amino acids were measured with an amino acid analyzer, and the concentrations of the L enantiomers were measured enzymatically with histidine ammonia-lyase (19) under the standard assay conditions at 0.03 and 0.06 mm NH₃-histidine and 0.2 mg of enzyme/ml or 0.04 and 0.07 mm 4-fluoro-DL-histidine and 0.9 mg of enzyme/ml.

[¹⁴C]Urocanic acid was obtained by treatment of L-[U,¹⁴C]-histidine (specific activity 0.06 mCi/mmol) with histidine ammonia-lyase. At the end of the reaction, the reaction mixture was heated at 100° for 2 min, and the pre-
pupification. The enzyme was freed of residual histidine on a column (1 × 15 cm) of Eicke Gel 60 (30 to 70 mesh), equilibrated with 0.1 M Tris-HCl buffer (pH 9.0) containing 0.1 mM MnCl₂ or 0.1 mM CdCl₂ and flash evaporated to dryness. With the solvent described above, all of the radioactive material co-chromatographed on thin layer plates with an authentic sample of urocanic acid. Its specific activity was 83 × 10³ cpm/pmol. 4-Fluorouraconate and 4-fluorohistidine, respectively, at 286 nm and its color value was 80% that of histidine. The concentrations of urocanic acid and fluorourocanic acid were measured spectrophotometrically as well as by radioactivity. Long term reversibility experiments were done in the presence of tolune, and the incubation mixtures were filtered through sterile Millipore filters prior to assay. At the end of these experiments a 50-μl aliquot of the incubation mixture was placed on plates of supplemented nutrient broth (28) with 1.5% agar; no bacterial growth was detected after 24 hours.

Reversibility of Histidine Ammonia-lyase Reaction—Reversibility of the histidine ammonia-lyase reaction was measured by conversion of [14C]urocanic acid and 4-fluoro[3H]urocanic acid to [14C]histidine and 4-fluoro[3H]histidine, respectively, and the presence of enzyme and NH₄HCO₃. Incubation mixtures without enzyme or NH₄HCO₃ served as controls. Aliquots were taken at various times; the reaction was stopped by heating the samples at 100° for 2 min, and 5 μl of a 0.02 M solution of histidine or 4-fluorohistidine was added then as carrier. These aliquots were subjected to thin layer chromatography, and the radioactivity present in the area corresponding to histidine or 4-fluorohistidine was measured.

The amount of histidine or 4-fluorohistidine formed was calculated from the known specific activities of the substrates and corrected for 75 to 85% recovery in order to calculate equilibrium constants. Formation of histidine and fluorohistidine was also measured directly with a Beckman Spinco model 120C amino acid analyzer (27). Protein that precipitated after the heating step was removed by centrifugation, and the supernatant fluid was lyophilized three times from water to remove NH₄HCO₃. The residue was suspended in 0.01 N HCl for amino acid analysis. 4-Fluorohistidine was eluted from the long column, using only the pH 3.5 buffer; it eluted at 106 to 107 min, and its color value was 80% that of histidine. The concentrations of fluorohistidine during any incubation were determined as described above. The labeled histidine and 4-fluorohistidine products were isolated by thin layer chromatography.

The specific activity of the urocanate or 4-fluorouraconate during any incubation was taken as the average of the specific activity at the beginning and the end of the interval. The embedded radioactivity of the enzyme was also measured and found to be between 80 and 90% of the starting material.

Exchange Reactions—Incorporation of [U-14C]urocanate into histidine and of 4-fluoro[3H]urocanate into 4-fluorohistidine was measured in the presence of 20 mM L-histidine or 4-fluoro-L-histidine, respectively, as stated in the legend to Fig. 5. The specific activity of product formation and exchange reaction was determined as described below. The labeled histidine and 4-fluorohistidine products were isolated by thin layer chromatography.

The specific activity of the urocanate or 4-fluorouraconate during any incubation was taken as the average of the specific activity at the beginning and the end of the interval. The embedded radioactivity of the enzyme was also measured and found to be between 80 and 90% of the starting material.

The incorporation of tritium from water into histidine or 4-fluorohistidine at 25° was measured in a complete histidine ammonia-lyase incubation mixture in the absence of added urocanate or 4-fluorouraconate. At various times, aliquots of 50 μl were diluted with 0.5 ml of 50% acid and lyophilized to constant specific activity from 50% acid (usually successive lyophilizations). After the last lyophilization, the residue was diluted in the appropriate volume of 0.05 M potassium phosphate buffer (pH 7.8) for spectrophotometric determination of urocanate and 4-fluorouraconate concentrations by their respective absorptions at 277 nm and 280 nm. When these samples were subjected to thin layer chromatography, all of the radioactivity co-chromatographed with histidine or 4-fluorohistidine, respectively.

**BIBLIOGRAPHY**

4-Fluoro-L-histidine behaves as a strong competitive inhibitor of histidine with a Ki of 1.27 nm, as compared with a Km for L-histidine of 2.7 nm (Fig. 1). It can be seen from Fig. 2 that 4-fluoro L-histidine is a very poor substrate. Since the imidazole ring of 4-fluorohistidine is a weaker base than that of histidine,
I/HISTIDINE (M-')

FIG. 1. Inhibition of deamination of n-histidine by 4-fluoro-n-histidine. The enzyme assays were carried out at 25° in 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-NaOH buffer (pH 8.0) containing 0.1 mM MnCl₂ and 0.67 μg/ml of reduced enzyme. V is expressed as micromoles of product formed per min per mg of protein. O, no addition; ●, in the presence of 1.6 mM 4-fluoro-n-histidine; and Δ, in the presence of 3.2 mM 4-fluoro-n-histidine. The increase in absorbance due to the deamination of 4-fluorohistidine was negligible (0.5 to 1%) compared to the increased absorbance due to the formation of urocanate under these conditions. Kᵢ = 1.24 and 1.29 mM 4-fluoro-n-histidine.

KH

FIG. 2. Variation of Kᵢ and Vₘₐₓ with pH. Assays were carried out at 25° in 0.05 M sodium carbonate-bicarbonate buffer, 0.1 mM CD⁻² (●), or in 0.05 M sodium carbonate-bicarbonate buffer, 10 mM EDTA (O), and 0.1 M N-2-hydroxyethylpiperazine,N'-2-ethanesulfonic acid (HEPES)-NaOH buffer, 0.1 mM CdCl₂ (△), or in 0.1 M HEPES buffer, 10 mM EDTA (Δ). The indicated pH was measured at the end of each reaction. When L-histidine was used, the enzyme concentration was 0.15 to 0.4 μg/ml, and the reaction was followed at 277 nm. When 4-fluoro-L-histidine was used, the conditions were the same as above, but the enzyme concentrations were 4 to 13 μg/ml, and the reaction was followed at 286 nm. The Vₘₐₓ values are expressed as units per mg of protein, and the Kᵢ as millimolarity.

FIG. 3. Rapid kinetics of deamination of 4-fluoro-L-histidine and L-histidine by histidine ammonia-lyase measured by stopped flow technique. Top, deamination of 4-fluoro-L-histidine in 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH buffer (pH 7.3), 0.1 mM CdCl₂, in the presence of 0.65 mg/ml (3.3 nmol) of enzyme (Experiment 1, Table I). The details of the experiments are described in the text. Bottom, deamination of L-histidine in 0.05 M HEPES-NaOH buffer (pH 7.3) and 1 mM EDTA, in the presence of 0.38 mg/ml (1.8 nmol) of enzyme (Experiment 4, Table I). Right ordinate, the absorbance change was converted to nanomoles of 4-fluorourocanic or urocanic acid formed per nmol of enzyme tetramer. The zero values correspond to the absorbance of the enzyme at the given wavelength.

by 3.5 pK units, the Michaelis constants for both substrates were measured as a function of pH, as shown in Fig. 2 (right). The Kᵢ-pH curves for the two substrates are very similar in shape. Both Kᵢ values are similarly affected by the presence of EDTA, which results in a significant increase in Kᵢ for both substrates without any change in the pH dependence. The Vₘₐₓ values are shown on the left of Fig. 2; the Vₘₐₓ with 4-fluoro-L-histidine is 30 to 100 times smaller than that with L-histidine. The Vₘₐₓ values for the two substrates show a similar pH dependence and are similarly affected by EDTA.

Rapid Kinetic Experiments—The existence of both a hydrogen and a urocanate exchange reaction with histidine had previously been taken as evidence that the slow step in the formation of urocanate is the dissociation of NH₃ from the enzyme (6,9). Since this step is common to both substrates, the difference in Vₘₐₓ shown above indicates that dissociation of ammonia from the enzyme cannot be a common rate-limiting step; alternatively, the slow step in both cases may precede the loss of NH₃, or the slow step may be different for the two substrates. The former hypothesis was tested by spectrophotometric measurement of presteady state kinetics, as shown in Fig. 3. At the top of the figure, a rapid kinetic experiment with 4-fluorohistidine is reported. No initial burst of activity is detected; if the rate-limiting step which controls the steady state rate followed the formation of 4-fluorourocanate, a burst of activity (29) should result in an increased absorbance of 0.08 to 0.3 at zero time. At all times the observed rate is linear with time and not significantly different from the steady state rate, as summarized in Table I. Further-

³ H. J. C. Yeh, K. L. Kirk, L. A. Cohen, and J. S. Cohen, J. Chem. Soc., in press.
Evidence for absence of initial burst of activity in deamination of 4-fluoro-L-histidine and L-histidine

| Experiment No. | Conditions of experiment | Product formed | Δε-Δε | Initial burst |
|---------------|--------------------------|----------------|--------|-------------|
| 1             | Fluorohistidine, pH 7.3, Cd²⁺ | 0.08            | 0.007-0.011 | 0.08 - 0.13 |
| 2             | Fluorohistidine, pH 8.5, Cd²⁺ | 0.17            | 0.14    | <0.3       |
| 3             | Histidine, pH 7.3, Cd²⁺ | 4.90            | 4.90    | 2.0       |
| 4             | Histidine, pH 7.3, EDTA | 0.50            | 0.45    | 0.5 - 1.5 |
| 5             | Histidine, pH 8.5, EDTA | 0.73            | 0.57    | 2.0       |

a The incubation was carried out at 27°C in the presence of the substrates indicated in the table at a concentration of 1 mM and in 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-NaOH buffer at the pH indicated above. When present EDTA was 1 mM and Cd²⁺ 0.1 mM. Experiments 1 and 4 are the same as described in Fig. 3.

The enzyme concentrations for initial rate measurements were 0.70 to 0.93 mg/ml (3.3 to 4.3 nmol) (Experiments 1 and 2) and 0.38 mg/ml (1.8 nmol) (Experiments 3 to 5). The reaction was followed for 1 to 30 s. The steady state rates were measured under identical conditions at concentrations of enzyme of 1 to 2 μg/ml. The micromoles of product formed were derived from the increase in absorbance at 286 nm for fluorouracanic and at 277 nm for urocanic acid as indicated under "Materials and Methods." Aa, absorbance extrapolated to zero time at 286 nm (Experiments 1 and 2) or at 277 nm (Experiments 3 to 5). A,, measured absorbance due to the enzyme at 286 nm (Experiments 1 and 2) or at 277 nm (Experiments 3 to 5).

The nanomoles of product formed during the putative initial burst was calculated for the difference of these values: Aa - Aa. The concentration of enzyme is expressed in nanomoles of 215,000 molecular weight species.

More, extrapolation to zero time (Table I) clearly indicates that no initial burst has occurred; the increase in absorption at 286 nm at zero time corresponds to less than 0.3 nmol of fluorouracanic formed per nmol of enzyme tetramer at 2 pH values and in the presence of metal ions. In the case of fluorohistidine, therefore, the rate-limiting step under these conditions precedes the dissociation of ammonia from the enzyme.

The results of the experiments performed with L-histidine are not as conclusive. A representative experiment is shown at the bottom of Fig. 3. At the earliest time measurable after mixing (25 ms at this electronic band pass setting), the absorbance change is occurring at the steady state rate (Table I), indicating the absence of any process with a rate constant greater than 100 s⁻¹. However, extrapolation to zero time consistently shows an initial absorbance which could correspond to the very fast release of as much as 2 nmol of urocanic acid per nmol of enzyme tetramer. The size of this initial absorbance is not very reproducible; at lower pH and in the presence of EDTA, both of which decrease the steady state rate, the zero time absorbance can be decreased to as little as 0.5 nmol of urocanic acid per nmol of enzyme tetramer. In the minimum time period between consecutive experiments (15 to 20 s) the absorbance of the solution will reach a high value (Fig. 3); therefore, we believe that the initial absorbance is an artifact, resulting from incomplete removal of product from the observation chamber, prior to the mixing of enzyme and substrate solutions for the next experiment. This objection is not true for 4-fluorohistidine experiments, because of the slow reaction rate.

Isotope Effects—The absence of a demonstrable initial burst of activity in the presteady state kinetic experiments described above indicates that the rate determining step in the deamination of 4-fluorohistidine could correspond to the breaking of the C—N bond, the C—H bond, or to a concerted mechanism. In the latter pathways, substitution of the hydrogen in the β position by a deuterium should decrease the rate of the overall reaction. Lineweaver-Burk plots of the velocity of the reaction as a function of concentration of 4-fluoro-L-histidine and of 4-fluoro-DL-[p-2D]histidine are shown in Fig. 4 (bottom). The Michaelis constants derived from these experiments are summarized in Table II. Isotopic replacement of the β-hydrogens does not affect the Kₘ values, but does decrease Vₘₚₖ both in the presence of metal ions and of EDTA. Isotope effects of 1.7 and 2.0 were calculated. As shown in Fig. 4 (top), isotope effects of 1.4 and 1.5 are observed with DL-histidine and its β deuterated analog. These findings suggest that the mechanism of the reaction is similar with both substrates and that breaking of the covalent C—H bond determines at least partially the reaction rate.

Since an exchange of ²H₂O with histidine was observed previously for the deamination of histidine (6), the rate of deamination of the deuterated substrate in ²H₂O was compared with that of DL-histidine in H₂O as a function of pH and pD, in order to eliminate the possibility that some exchange of deuterium in the β position will decrease the isotope effect under the conditions described above. No significant change in the isotope effect was observed under these conditions (data not shown).

Exchange Reactions—Peterkofsky has shown that solvent tritium and [¹⁴C]urocanic acid can be incorporated into reisolated...
Table II

Michaelis constants with DL-histidine, DL-\(\beta\)-H\(_2\)histidine, 4-fluoro-DL-histidine, and 4-fluoro-DL-\(\beta\)-H\(_2\)histidine

| Experiment No. | Substrate | \(K_m\) \(\times 10^{-3}\) | \(V_{max}\) \(\times 10^{-3}\) | \(V_p/\max\) |
|---------------|-----------|-----------------|-----------------|-------------|
| 1 | DL-Histidine | 10.0 ± 1.0 | 3.04 ± 0.13 | 1.5 |
|     | DL-\(\beta\)-H\(_2\)Histidine | 11.2 ± 2.0 | 2.07 ± 0.20 | |
| 2 | DL-Histidine | 9.7 ± 0.2 | 11.60 ± 0.06 | 1.4 |
|     | DL-\(\beta\)-H\(_2\)Histidine | 2.4 ± 0.2 | 8.30 ± 0.20 | 2.0 |
| 3 | 4-Fluoro-DL-histidine | 6.4 ± 1.0 | 0.0008 ± 0.0004 | |
|     | 4-Fluoro-DL-\(\beta\)-H\(_2\)histidine | 6.9 ± 1.0 | 0.0034 ± 0.0003 | |
| 4 | 4-Fluoro-DL-histidine | 1.25 ± 0.15 | 0.002 ± 0.003 | 1.7 |
|     | 4-Fluoro-DL-\(\beta\)-H\(_2\)histidine | 1.3 ± 0.19 | 0.003 ± 0.002 | |

\(\text{\textsuperscript{a}}\) The experimental conditions were as described in Fig. 4. The Michaelis constants were obtained by the curve-fitting method described under "Materials and Methods." 

\(\text{\textsuperscript{b}}\) The concentrations correspond to that of the DL mixture. 

\(\text{\textsuperscript{c}}\) Experiments 1 and 3 were done in the presence of 1 mM EDTA and Experiments 2 and 4 in the presence of 0.1 mM CdCl\(_2\). 

The rate of the tritium exchange reaction is shown in Fig. 6 for both L-histidine and 4-fluoro-L-histidine. As in the urocanic acid exchange, the rate of tritium exchange is much smaller with 4-fluoro-L-histidine than with histidine. For both exchanges, the reduced rates with the fluoro analog reflect the considerably lower value of \(V_{max}\). It can also be noted that the rate of tritium exchange is not greatly affected by urocanate concentration. At low pH, the rate of tritium exchange is linear, even at early times. When \(\text{\textsuperscript{3}}\)H exchange and \(\text{\textsuperscript{14}}\)Curocanate exchange into histidine were measured simultaneously, the rate of \(\text{\textsuperscript{3}}\)H exchange exceeds slightly that of \(\text{\textsuperscript{14}}\)Curocanate exchange (Table III).

Reversibility of Reaction—The existence of these exchange reactions and the lack of exchange of ammonia into histidine had led to the conclusion that the reaction occurs in two steps (6), a reversible formation of an amino enzyme intermediate and a slow irreversible dissociation of the amino enzyme, accounting for the apparent irreversibility of the over-all reaction (6). However, it was subsequently shown that after prolonged incubation, the reaction is reversible with an equilibrium constant of 3 to 5 (8). To clarify this situation, the inhibition of urocanate formation by NH\(_4\)HCO\(_3\) in the presence of EDTA\(\text{\textsuperscript{a}}\) was studied. The results could all be expressed as linear double reciprocal plots, compatible with NH\(_3\) being a competitive inhibitor with respect to histidine. However, the nature of the inhibition could not be proved unequivocally, due to the high apparent \(K_m\) of NH\(_4\)HCO\(_3\) (0.2 M). We were able to confirm the reversibility of the reaction with urocanic acid as well as with 4-fluorouracil acid within short incubation times by use of high concentrations of enzyme.

The time course of such reversibility experiments is summarized in Table IV. The equilibrium constants derived from these experiments are similar for both substrates and are comparable to those published earlier for urocanic acid (8).

The dependence of the rate of the reverse reaction on the concentration of histidine was found to be 0.015 mol/min/mg of enzyme. Under the same conditions, no exchange was observed with 4-fluorouracil. This experiment provides further support for the existence of a common amino enzyme intermediate.

\(\text{\textsuperscript{a}}\) In view of the ability of NH\(_4\)HCO\(_3\) to bind the metal activators (37) and thereby inhibit the enzyme noncompetitively, the reaction was carried out in the presence of EDTA.
In the presence of the metal-binding agent. Since both reverse reaction of histidine is not significantly affected by IGDTA, but the formation of 4-fluorohistidine is reduced to undetectable levels reactions were not performed in the presence of saturating those of the forward reaction. Furthermore, the rate of formation of urocanic acid and of 4-fluoro-urocanic acid was also measured. In contrast to the forward reaction, where the K, of NH₃, these values of (0.002 and 0.007 units/mg, respectively) are much smaller than (3 μM) values for the back reaction with these substrates used to calculate the micromoles per ml at 25 min was the average of the specific activities at zero time and at 25 min.

TABLE III
Comparison of tritium and urocanate exchange reactions

| Incubation time | Urocanic acid concentration | Urocanic acid incorporation | Tritium incorporation |
|-----------------|-----------------------------|-----------------------------|----------------------|
| Zero time       | 4.00                        | 0.15                        | 0.10                 |
| 25 min.         | 5.10                        | 2.05                        | 2.50                 |
| Difference      | 1.10                        | 1.90                        | 2.40                 |

The incubation mixture was 0.05 mM potassium phosphate buffer (pH 7.2), 0.1 mM Cd²⁺, 1 mm mercaptoethanol, 20 mM L-histidine, and 4 mm [¹⁴C]urocanate and contained 0.016 mg of enzyme and 1 mCi of H₂O in a final volume of 0.2 ml. The reaction was carried out as described in Fig. 6 and under "Materials and Methods." 5 Urocanate incorporation and tritium incorporation into histidine were measured after separation by thin layer chromatography. The specific activity of urocanate after 25 min of incubation was corrected for the increase in concentration and the decrease in radioactive material, and the specific activity of urocanic acid after 25 min of incubation was 0.05 μM.

As argued below, their difference should be far greater than observed.

**DISCUSSION**

Substitution in various positions of the imidazole ring of L histidine yields analogs with substrate properties markedly different from those of the natural substrate for histidine ammonia-lyase. For example, 2-fluorohistidine is a substrate, with Kₘ = 0.02 μM, L-N'-methylhistidine is a weak competitive inhibitor, Kᵢ = 0.1 μM, and L-N'-methylhistidine is neither a substrate nor an inhibitor. In contrast, 4-fluorohistidine is a competitive inhibitor with a Kᵢ lower than the Kₘ of histidine (Fig. 1). The 4-fluoro compound is also a substrate for the enzyme; since its rate of deamination is 30 to 100 fold smaller than that of histidine, this analog provides a useful tool for study of the mechanism of the enzymic reaction.

It is conceivable that the low rate of deamination in the case of the 4-fluoro compound might result from an inhomogeneity in the enzyme preparation, only a small fraction of the population being active with the analog. However, it seems unlikely that the Kₘ of the analog would give such good agreement with its Kᵢ with regard to histidine, if heterogeneity were the case. Such agreement is to be expected, of course, if it is both substrate and inhibitor for the same enzyme. Other results also argue against this notion.

After addition of NH₄HCO₃ the observed pH was 8.1.
The absence of a detectable initial burst of activity in the rapid kinetic experiments (Table I) indicates that the rate-determining step, in the case of 4-fluorohistidine, cannot follow the formation of 4-fluorouracil acid (29) and probably involves C—H or C—N bond cleavage, or both, in a concerted mechanism. The stopped flow data with histidine are less conclusive because the fast reaction rate makes the measurement of zero time absorbance less accurate. However, since the deamination of both substrates is stimulated by metal ions (11–15) and V_max values are reduced by EDTA to a similar extent, even at high pH, formation of product (the α, β elimination reaction) is most likely the common rate-limiting step.

The observation that both substrates show small but reproducible β-deuterium isotope effects, 1.4 to 2.0, suggests that cleavage of a C—H bond determines at least partially the reaction rate in each case (30). However, the small size of the effects, particularly for histidine, indicates that secondary isotope effects should also be considered (31). Thus, in a carbonium ion mechanism, the rate of C—N bond cleavage could be reduced by the two β-2H atoms through hyperconjugation; C—H bond cleavage may also be reduced by the nonleaving 2H. An equilibrium effect, due to the presence of this atom in the urocanate produced, could also be postulated. Secondary isotope effects arising from such causes are usually even smaller than those reported here, particularly for 4-fluorohistidine (31). In the deamination of a mixture of L-[1-3H]histidine and (β-S)-L-2H₄histidine, a small but reproducible isotope discrimination (1.1 to 1.3) was observed. This would be expected to be even larger in the absence of the tritium exchange reaction. A similar isotope discrimination was reported in the deamination of (β-S)-L-3H₄phenylalanine and of (β-S)-L-2H₄tyrosine by phenylalanine ammonia-lyase. Hanson et al. suggested a concerted elimination mechanism. The stopped flow data with histidine are less conclusive because the fast reaction rate makes the measurement of zero time absorbance less accurate. However, since the deamination of both substrates is stimulated by metal ions (11–15) and V_max values are reduced by EDTA to a similar extent, even at high pH, formation of product (the α, β elimination reaction) is most likely the common rate-limiting step.

As shown in Table III, tritium exchange into histidine is somewhat faster than urocanate exchange. If isotope discrimination were taken into account, the difference could be even greater. This result, together with the facts that tritium exchange can be observed under initial rate conditions which do not support significant urocanate exchange (Figs. 5 and 6), and that the rate of tritium exchange is not significantly dependent on the urocanate concentration, suggests that C—H bond cleavage may precede C—N bond cleavage slightly (9). Separation of the steps may not be sufficient to invoke a carbonium mechanism, as postulated by Bright (35) for the deamination of β-methylaspartate. In the latter case, C—N bond cleavage is considered rate-limiting, and a large tritium exchange is inhibited by the elimination product, mesaconate. Alternatively, if the rate of urocanate dissociation is not significantly faster than the rate of the preceding elimination reaction, a difference in the rates of the urocanate and tritium exchanges could also be observed in a concerted mechanism.

It is not clear why 4-fluorohistidine is such a poor substrate for the enzyme. Although the fluorine atom has a marked effect in depressing the basicity of the imidazole ring, it has little effect on that of the primary amino group. The absence of a significant isotope effect on the K₉₊ values of both substrates suggests that both may be essentially dissociation constants. If so, the analog seems to bind to the enzyme more strongly than does histidine. Similarities in isotope effects and in the inhibitory effect of EDTA suggest that the mechanism of the reaction is the same in both cases. Perhaps the differences in V_max result from the inability of the analog to force the enzyme into an active form, as required by the "induced-fit" theory (36).

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