Vol. 87, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1979 Pages 343-348

4-NITRO-L-HISTIDINE AS A SUBSTRATE FOR HISTIDINE AMMONIA-LYASE: THE ROLE OF β-HYDROGEN ACIDITY IN THE RATE-LIMITING STEP

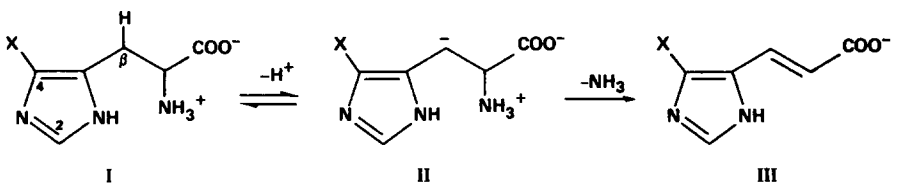
Claude B. Klee*
Laboratory of Biochemical Pharmacology

Kenneth L. Kirk and Louis A. Cohen
Laboratory of Chemistry

National Institute of Arthritis, Metabolism and Digestive Diseases,
National Institutes of Health, Bethesda, Maryland 20014

Received February 1,1979

Summary: The K_m for the interaction of 4-nitro-L-histidine with histidine ammonia-lyase (reduced enzyme, pH 8.0) is comparable to that for L-histidine, while V_{max} is 1/8 that for the natural substrate. With the analog, addition of Cd^{+2} effects a small decrease in K_m but fails to alter V_{max}; the normal deuterium isotope effect for removal of the β-hydrogen (1.5-2.0) is eliminated; and enzyme-catalyzed incorporation of solvent tritium into substrate occurs to a much greater extent than into histidine. Thus, the nitro group increases the acidity of the β-hydrogen and the stability of the conjugate carbanion to such a degree that C-H bond cleavage now precedes rate-limiting C-N bond cleavage.



I II III

X = H, F, NO_2

The detailed mechanism of action of histidine ammonia-lyase--with respect to the roles of the prosthetic group (yet unidentified), a transition metal ion, and a sulfhydryl group--has yet to be elucidated with reasonable certainty (1). We had previously demonstrated (2) that abstraction of a β-hydrogen atom is at least partially involved in the rate-limiting elimination of ammonia from the substrate (I).+ This argument is supported by the demonstration of a small, but reproducible, isotope effect, the absence of an initial burst of activity, and the observation that solvent tritium exchange into histidine is somewhat faster than amino acid regeneration from urocanate.

* Present address: Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

+ The nature of the interaction between the α-amino group and the enzyme's prosthetic group is not specified in the structural formulas.

0006-291X/79/050343-06$01.00/0

4-Fluoro-L-histidine was found to be a strong competitive inhibitor for the enzyme, as well as a weak substrate. The addition of Cd^{+2} has a stimulatory effect, both for histidine and for its 4-fluoro analog; while the enhancement effect of Cd^{+2} on K_m is ca. 40% greater for fluorohistidine than for histidine, its effect on V_{max} is almost 4-fold as great for the analog. This result suggests that the metal ion plays a somewhat more critical role in the removal of ammonia from fluorohistidine, and fits a pattern which can be rationalized on electronic grounds. The carbanion formed by β-proton abstraction (II), whether it have the lifetime of an intermediate or a transition state, is coupled to the substituent at C-4 both by induction and resonance. There are indications (3,4) that the electron-releasing resonance effect of fluorine should surpass its inductive effect in such a system, with the result that the fluorine atom at C-4 will decrease the acidity of the β-hydrogen atom relative to that of histidine. As a logical test for this interpretation, we have examined the action of histidine ammonia-lyase on 4-nitro-L-histidine; in the latter compound, the resonance and inductive effects of the nitro group operate in the same direction and should produce a significant enhancement of β-hydrogen acidity (5).

MATERIALS AND METHODS:

4-Nitro-L-histidine was prepared as previously described (6). A sample of this compound was stored in 1 N NaOD for 2 days at 60°; at this point, the NMR spectrum indicated complete exchange of the β-hydrogens. The solution was acidified to pH 3 and the crystalline, deuterated amino acid was isolated by filtration. Exposure to alkali did not effect racemization at the α-carbon atom, since the deuterated product was totally transformed to nitrourocanate by the enzyme. 4-Nitrourocanic acid was obtained by enzymatic deamination of 4-nitrohistidine. The final reaction mixture was acidified to pH 1 and the crystalline product was separated by filtration, mp 255-263°; m/e 169.

Histidine ammonia-lyase from Pseudomonas ATCC 11299b was prepared as previously described (2,7). The enzyme was assayed spectrophotometrically at 277 nm and 25°, with histidine as substrate (8). The UV absorption spectra of 4-nitrohistidine and 4-nitrourocanate show large pH-dependent changes between pH 7.4 and 9 (Table I); accordingly, formation of 4-nitrourocanate was followed at different wavelengths depending on pH. The wavelength was chosen to provide maximum UV difference spectra and low absorption of the substrate: 400 nm at pH 8.9-9; 260 nm at pH 8.0; and 370 nm at pH 7.4. UV difference spectra were corrected for the effect of $CdCl_2$ on the spectrum of 4-nitrohistidine; the metal ion had no effect on the spectrum of 4-nitrourocanate. The UV spectrum of 4-nitrourocanate is affected by the presence of thiols, and 2-mercaptoethylamine could not be used directly in the assay mixtures. For these studies, the enzyme was reduced and freed of thiol prior to activity measurements (2). Kinetic constants were determined as described (2).

RESULTS AND DISCUSSION:

With histidine ammonia-lyase at pH 8.0, the K_m for 4-nitro-L-histidine is comparable to that of L-histidine, and the analog is deaminated at 1/8 the

Table 1. Absorption Spectral Data for 4-Nitrohistidine and 4-Nitrourocanate.

Medium	4-Nitrohistidine		4-Nitrourocanate	
	λ_{max}, nm	$\varepsilon_m \times 10^{-3}$	λ_{max}, nm	$\varepsilon_m \times 10^{-3}$
Tris·HCl (0.1 M, pH 9.0)	342	5.58	378	8.68
Tris·HCl (0.1 M, pH 8.0)	315	5.55	345	8.40
K_2HPO_4 (0.05 M, pH 7.4)	312	6.11	342	9.18

Table 2. Urocanate Formation from Histidine and its Analogs.

Substrate	K_m (mM)			V_{max}		
	pH 8.8	pH 8.0	pH 7.4	pH 8.8	pH 8.0	pH 7.4
Histidine	2.8	1.9	2.0	22.80	12.50	9.30
4-Fluorohistidine	1.4	0.8	1.0	0.18	0.13	0.06
4-Nitrohistidine	4.2	2.1	0.7	1.70	1.60	0.94

Reactions were performed in 0.1 M Tris-HCl (pH 8.8 and 8.0) and 0.05 M potassium phosphate (pH 7.4), in the presence of 0.1 mM $CdCl_2$. Incubation, at 25°, was started by addition of the substrate; the concentration of reduced enzyme was 10 µg per ml. At high substrate concentrations (0.26-3 mM), reactions were performed in 2 mm pathlength cuvettes and the enzyme concentration was raised to 30 µg per ml. V_{max} is expressed in µmoles product/min/mg enzyme.

rate of the natural substrate (Table II). The value of pK_2 for the nitro compound (imidazole → imidazole anion) is ca. 9.5; thus, the marked increase in K_m with increasing pH may reflect inferior binding (or nonbinding) of the anionic species.

The lyase exists in both an oxidized and a more active reduced form. Reduction liberates one sulfhydryl group per subunit, improving both K_m and V_{max} for deamination in the presence of added Cd^{+2} (7); with the oxidized enzyme, addition of the metal ion appears to increase V_{max} alone. The sulfhydryl group is presumed necessary, therefore, to promote a metal-ion dependent step in catalysis. It has been suggested that the metal ion serves to facilitate removal of the β-proton in the rate-limiting step (9). Significantly different results were found with 4-nitro-L-histidine as substrate (10). With the oxidized enzyme, K_m and V_{max} are the same in the presence of EDTA or Cd^{+2}

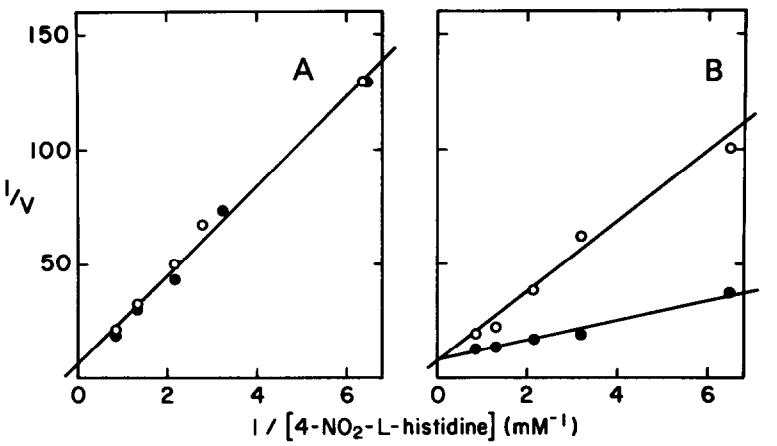


Figure 1. Effect of Metal ion on the deamination of 4-nitro-L-histidine. The reactions were carried out in 0.05 M HEPES-NaOH buffer (pH 7.7) containing 0.1 mM $CdCl_2$ (•) or 1 mM EDTA (o). The enzyme concentration was 20 µg/ml: A, oxidized enzyme; B, reduced enzyme. The velocity is expressed as the increase in absorbance at 260 nm/min.

(Fig. 1A); with the reduced enzyme Cd^{+2} effects a small increase in the overall rate of deamination (Fig. 1B), but this enhancement vanishes at saturating concentrations of substrate and is derived, therefore, from a reduction in K_m. Thus, it would appear that the 4-nitro group has either eliminated the dependence on metal ion for proton abstraction or, more likely, has enhanced the acidity of the β-proton to such an extent that carbanion formation now precedes the rate-limiting step.

Replacement of the β-hydrogens of histidine and of 4-fluorohistidine with deuterium results in modest reductions in V_{max}, with isotope effects of 1.5 and 2.0, respectively (2). This trend is consistent with our argument that the β-hydrogen of the fluoro compound is less acidic than that of histidine. No isotope effect is found for the 4-nitro analog (Fig. 2), providing further evidence for a change in rate-limiting step with this substrate.

The enzyme-catalyzed incorporation of solvent tritium into the β position of histidine and of 4-fluorohistidine demonstrates the reversibility of the lyase reaction (2). With 4-nitrohistidine, the rate of tritium incorporation relative to product formation is 2-3 times as great as for the other substrates (Fig. 3). This result is also consistent with a change in rate-limiting step, and implies a significant degree of stability for the carbanion species in the nitro case.

The ability of the nitro group to stabilize a carbanion by resonance is well-established (11), and is responsible for the acidities of nitromethane

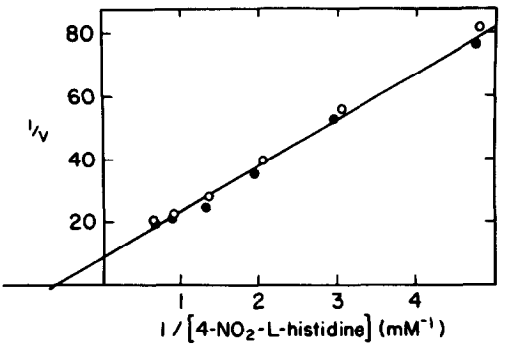


Figure 2. Deuterium isotope effect on the rate of deamination of 4-nitro-L-histidine. Assays were performed at 25° in 0.05 M HEPES-NaOH buffer (pH 7.6) containing 1 mM EDTA. The reaction was started by addition of oxidized histidine ammonia-lyase (20 µg/ml). The concentration of substrate was measured spectrophotometrically at 312 nm or, after conversion to 4-nitro-urocanate, at 342 nm. The velocity is expressed as the increase in absorbance at 260 nm/min: 4-nitro-L-histidine (•), K_m = 1.27 ± 0.27 mM, V_{max} = 0.71 ± 0.09; β-D_2-4-nitro-L-histidine (o), K_m = 1.36 ± 0.18 mM, V_{max} = 0.69 ± 0.06.

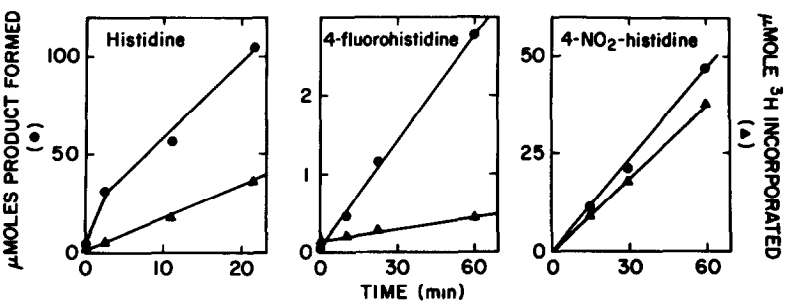


Figure 3. Rates of incorporation of solvent tritium into L-histidine, 4-fluoro-L-histidine, and 4-nitro-L-histidine during the histidine ammonia-lyase reaction. The incubation mixture contained 0.05 M HEPES-NaOH buffer (pH 8), 1 mM mercaptoethylamine, 0.1 mM $CdCl_2$, 4.5 mCi of ^3H_2O, and 32 µg/ml of enzyme. The mixtures were incubated for 10 min at 25° prior to addition of substrate (final concentration 30 mM). At the indicated times, aliquots were removed and incorporation of tritium from water was measured as previously described (2). The formation of 4-nitrourocanate was measured at 400 nm after appropriate dilution in 0.05 M Tris-HCl buffer (pH 9). After repeated lyophilizations of the incubation mixture, 70% of the residual radioactivity was associated with 4-nitrohistidine by thin-layer chromatography. Similar demonstrations for the other substrates were described previously (2).

(pK 10.2) and p-nitrotoluene (pK 28.7) (5). The same stabilization is available to the carbanion of 4-nitrohistidine, since isotope exchange of the β-hydrogens is readily achieved in 1 N aqueous base (see Materials and Methods). Indeed, the facility of this exchange in the presence of the

Table 3. Effect of Metal Ion on Lyase Activity.

Substrate	Enzyme	Activity [a] +0.1 mM Cd^{+2}	Activity [a] + 1 mM EDTA
Histidine (1.25 mM)	oxidized	4.74	0.56
Histidine (1.25 mM)	reduced	7.81	0.56
Nitrohistidine (1.30 mM)	oxidized	0.31	0.29
Nitrohistidine (1.30 mM)	reduced	0.57	0.36

[a]Expressed as μmoles product/min/mg enzyme at pH 7.7.

imidazolate anion ($t_{1/2}$ = 1 hr at 60°) indicates a remarkable degree of acidity for the β-hydrogens, with an estimated pK < 24. The three criteria --metal ioncatalysis, deuterium isotope effect, and tritium exchange--are consistent in relating the kinetic acidity of the β-hydrogen in histidine to the electronic effect of a substituent at C-4 of the ring. As that acidity increases, the rate-limiting step progresses from concerted C-H/C-N bond-breaking (2) to C-N cleavage alone. Examination of other 4-substituted histidines is in progress, in the hope of demonstrating a linear free energy relationship throughout the series.

REFERENCES

1. Hanson, K. R., and Havir, E. A. (1972) in The Enzymes (Boyer, P. D., ed), 3rd Ed, Vol 7, pp 75-166, Academic Press, New York.
2. Klee, C. B., Kirk, K. L., Cohen, L. A., and McPhie, P. (1975) J. Biol. Chem., 250, 5033-5040.
3. Streitweiser, Jr., A., and Koch, H. F. (1964) J. Am. Chem. Soc., 86, 404-409.
4. Adolph, H. G., and Kamlet, M. J. (1966) J. Am. Chem. Soc., 88, 4761-4763.
5. Weiss, C. (1972) Z. Chem., 12, 193-194.
6. Tantz, W., Teitel, S., and Brossi, A. (1973) J. Med. Chem., 16, 705-707.
7. Klee, C. B. (1972) J. Biol. Chem. 247, 1398-1406.
8. Mehler, A. H., and Tabor, H. (1953) J. Biol. Chem., 201, 775-784.
9. Givot, I. L., Mildvan, A. S., and Abeles, R. H. (1970) Fed. Proc., 29, 1590.
10. Klee, C. B., Kirk, K. L., McPhie, P., and Cohen, L. A. (1974) Fed. Proc., 33, 1318.
11. Cram, D. J. (1965) Fundamentals of Carbanion Chemistry, pp 52-55, Academic Press, New York.