Neuberger, A., Gottshalk, A., Marshall, R. D., and Spiro, R. G. (1972), in Glycoproteins, Gottshalk, A., Ed., 2nd ed, Elsevier, Amsterdam, pp 450–490.
Rothfield, L., and Romeo, D. (1971), *Bacteriol. Rev. 35*, 14.
Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C., and Bauman, A. (1969), *Methods Enzymol. 14*, 272.
Sentandreu, R., and Lampen, J. O. (1972), *FEBS Lett. 27*, 331.
Sharma, C. B., Babczinski, P., Lehle, L., and Tanner, W. (1974), *Eur. J. Biochem. 46*, 35.
Tanner, W. (1969), *Biochem. Biophys. Res. Commun. 35*, 144.
Vogel, H. J. (1964), *Am. Nat. 98*, 435.
Waechter, C. J., Lucas, J. J., and Lennarz, W. J. (1973), *J. Biol. Chem. 248*, 7570.
Wrathall, C. R. and Tatum, E. L. (1973), *J. Gen. Microbiol. 78*, 139.

Histidine Ammonia-lyase from Rat Liver. Purification, Properties, and Inhibition by Substrate Analogues†

Larry M. Brand‡ and Alfred E. Harper*

ABSTRACT: Histidine ammonia-lyase (EC 4.3.1.3) from rat liver was purified more than 250-fold to near homogeneity. Electrophoretic determinations indicated a native molecular weight of approximately 200 000. The enzyme has a pH optimum of approximately pH 8.5. The minimum K_m for L-histidine was 0.5 mM at pH 9.0. The Michaelis constant in the physiological pH range was, however, more than 2.0 mM. D-α-hydrazinoimidazolylpropionic acid was found to be a potent competitive inhibitor of liver histidine ammonia-lyase (K_{is} = 75 μM); the L enantiomer of this compound was less effective in this regard. The enzyme was also inhibited competitively by L-histidine hydroxamate (K_{is} = 0.4 mM), and to a lesser extent by L-histidinol, D-histidine, and glycine. Failure of a wide variety of other histidine analogues to inhibit the enzyme substantially indicates high specificity of the active site for L-histidine. No alternate substrates were identified for the enzyme. DL-α-Hydrazinophenylpropionic acid, the α-hydrazino analogue of phenylalanine, was similarly shown to be a very potent competitive inhibitor of a mechanistically similar L-phenylalanine ammonia-lyase purified from *Rhodotorula glutinis*.

The properties of histidine ammonia-lyase from rat liver differ significantly from those of the enzyme from *Pseudomonas fluorescens* which has been studied most extensively to date.

Histidine ammonia-lyase (EC 4.3.1.3), the first enzyme in the major catabolic pathway of histidine metabolism, has been studied from both bacterial and mammalian sources. In mammals, this enzyme is located primarily in liver and epidermis (Zannoni and LaDu, 1963) and has been of some interest since the characterization of a human metabolic disease, histidinemia, which results from the hereditary absence of the enzyme. It is probable that the liver and epidermal enzyme species in mammals are identical in structure since studies of both mice (Kacser et al., 1973) and humans (Zannoni and LaDu, 1963) displaying genetic deficiency of liver histidine ammonia-lyase (histidinemia) have shown the skin enzyme to be lacking as well. In view of the apparent identity of the liver enzyme with the epidermal enzyme, which in humans is assayed clinically as a means of diagnosing histidinemia (LaDu, 1971), and because of the relative difficulty of extracting enzyme sufficient for characterization from epidermal tissue, the molecular and kinetic properties of the mammalian liver enzyme are of particular interest.

To date, the histidine ammonia-lyases of eukaryotes have been studied relatively little; however, the enzyme from bacterial sources has been purified and studied extensively in a number of laboratories (Magasanik et al., 1971; Rechler, 1969; Klee, 1970; Frankfater and Fridovich, 1970). Histidine ammonia-lyase from *Pseudomonas fluorescens* has been shown to exist as a tetramer with a molecular weight of 213 000, composed of apparently identical subunits (Rechler, 1969). The enzyme has been found to contain a strongly electrophilic modified amino acid side chain, referred to as "dehydroalanine" (Givot et al., 1969), but as yet not fully characterized, which participates in the deamination reaction, apparently in a manner analogous to the pyridoxal phosphate cofactor of some other amino acid deaminases, since the enzyme is irreversibly inactivated by hydrazines, borohydride, cyanide, and bisulfite (Smith et al., 1967). Cornell and Villee (1968) have partially purified the enzyme from rat liver and reported it to have an apparent requirement for divalent cations similar to that of the *Pseudomonas* enzyme. The rat liver enzyme has also been shown to have a "dehydroalanine" residue in its active site (Givot and Abeles, 1970). Okamura et al. (1974) have recently reported purification of the rat liver enzyme to homogeneity and determined its molecular weight to be 190 000. It was the purpose of the present investigation to determine some of the molecular and kinetic properties of mammalian histidine ammonia-lyase, particularly with regard to the identification and characterization of inhibitors of this enzyme.

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received September 2, 1975.* This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by Grant No. AM 10748, National Institutes of Health, United States Public Health Service.

‡ Present address: Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154.

Materials and Methods

Female albino rats (375–450 g) were obtained from the Holtzman Company, Madison, Wisconsin, and fed Wayne Lab Blox until used. All chemicals used were of reagent grade purity.

Synthesis of DL-α-Hydrazinoisocaproic Acid. This compound was prepared from DL-α-bromoisocaproic acid by treatment with 95% hydrazine, following essentially the procedure of Niedrich and Grupe (1965) for preparation of L-α-hydrazinoisocaproic acid. The product, after crystallization from water, was obtained in 30% yield, and was shown to be homogeneous by ascending paper chromatography in 1-butanol–acetic acid–water (12:3:5 by volume): mp 199–203 °C; literature (L-enantiomer) 195–200 °C (Niedrich and Grupe, 1965).

Synthesis of DL-α-Hydrazinoimidazolylpropionic Acid Hydrochloride. A modification of the procedure of Sletzinger et al. (1968) was utilized. DL-α-Bromoimidazolylproprionic acid monohydrate was prepared by the procedure of Yankeelov and Jolley (1972) in 60% yield, mp 106–108 °C. The product yielded a single Pauly positive spot after ascending paper chromatography in 1-butanol–acetic acid–water (12:3:5, by volume), R_f 0.50. Values obtained from the literature (Yankeelov and Jolley, 1972) are: mp 108–111 °C; R_f 0.53 (in above solvent). To 18.0 g of DL-α-bromoimidazolylpropionic acid in 60 ml of water was added dropwise 30 ml of 95% hydrazine, and the resulting solution was heated in a sealed flask at 58–60 °C for 2 h. After removal of most of the solvent in a rotary evaporator at 46–55 °C, the resulting light brown oil was chilled in ice and 100 ml of ice-cold concentrated HCl was added dropwise with stirring. During this process, a total of 150 ml of methanol was added in portions to resuspend the salt mass. After filtration and washing of the precipitate with methanol, the filtrate was alternately evaporated to a yellow oil, resuspended in 30 ml of methanol, chilled, and filtered until no further salt was recovered. After a final evaporation to dryness, the oil was resuspended in 25 ml of water and again evaporated to dryess. The resulting thick, yellow oil was dissolved in 90 ml of methanol, and a solution of tributylamine–methanol (1:2 by volume) was added dropwise until no further precipitate formed. Filtration, washing with ice-cold methanol, and drying in vacuo yielded 9.0 g of the product, DL-α-hydrazinoimidazolylpropionic acid hydrochloride (48% yield): mp 186–187 °C dec (softening 140 °C); literature value mp 184 °C dec (softening 143 °C; Sletzinger et al., 1968). Paper chromatographic behavior in a number of solvent systems was identical with that of commercially obtained D-α-hydrazinoimidazolylpropionic acid.

Synthesis of DL-α-Hydrazinophenylpropionic Acid. The hydrazone of phenylpyruvic acid was prepared and reduced to the corresponding hydrazine with sodium amalgam by a procedure exactly analogous to that of Glamkowski et al. (1967) for the preparation of DL-α-hydrazinoindolylpropionic acid from indolylpyruvic acid. The hydrazine salt of phenylpyruvate hydrazone, prepared in 63% yield, mp 125–128 °C, was dissolved in water to give a 10% solution, weight to volume, to which was added 2.3% NaHg (2 g per mol of solution). The mixture was stirred under a constant N_2 stream for 24 h. The aqueous phase was then decanted and extracted twice with one-half volume of ether, and the product was precipitated by adjustment of the pH to 5.0 with acetic acid. After filtration and washing with water, ethanol, and ether, DL-α-hydrazinophenylpropionic acid was recovered in 40% yield (25% yield overall): mp 184–190 °C (softening 164–166 °C); literature value (L enantiomer) mp 187 °C (Glamkowski et al., 1967).

Assay of Histidine Ammonia-lyase. Enzyme activity was assayed at 24 °C by a modification of the procedure of Tabor and Mehler (1955). Except where noted otherwise, reaction mixtures contained initially 50 mM L-histidine, 7.0 mM reduced glutathione, and 10 mM sodium pyrophosphate, in a volume of 1.5 ml. The pH of the assay system was 9.0. The reaction was initiated by the addition of 0.05 ml of enzyme solution, and urocanic acid formation was monitored continuously at 277 nm, using 18.8 mM^{-1} cm^{-1} as the extinction coefficient for urocanic acid (Mehler and Tabor, 1953). Enzyme units represent nanomoles of urocanic acid formed per minute.

Assay of Phenylalanine Ammonia-lyase (R. glutinis). This enzyme was obtained in semipurified form (specific activity: 0.94 unit/mg of protein) from P-L Biochemicals, Inc., Milwaukee, Wisconsin, and was used without further purification. Enzyme units represent the deamination of 1 μmol of L-phenylalanine to cinnamate per minute at 30 °C in the standard assay. In the present study, assays were conducted at 24 °C by the method of Hodgins (1971). Except where noted otherwise, reaction mixtures contained 0.2–1.0 mM L-phenylalanine, 0.1 M Tris-HCl buffer (pH 8.5), and enzyme in a final volume of 2.1 ml. The reaction was started by the addition of enzyme, and the formation of cinnamic acid was monitored continuously at 290 nm, using 10.0 mM^{-1} cm^{-1} as the extinction coefficient for cinnamic acid (Zucker, 1965).

Protein Determination. Protein was assayed by the method of Lowry et al. (1951), using crystalline bovine serum albumin as standard. Samples containing Tris buffer were appropriately diluted (Ji, 1973) such that interference with the Lowry assay was negligible.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out at 0 to 4 °C at pH 8.8 by the method of Davis (1964), substituting 0.0005% riboflavin in place of persulfate in order to preserve enzyme activity. The enzyme sample applied to the gel contained 0.13 M dithiothreitol. Gel staining was carried out in 0.5% Coomassie brilliant blue dissolved in methyl alcohol–acetic acid–water (5:1:5) and destaining was in 7.5% acetic acid.

Kinetic Studies. Values for K_m and V_{max} from individual double reciprocal plots were determined using the computer program of Cleland (1967) to perform a weighted fit of the kinetic data to eq 1:

$$v = VA/(K_m + A) \quad (1)$$

For inhibition studies, the data were first plotted by hand to determine the type of inhibition, and the values for the kinetic constants determined by a computer fit, using the programs of Cleland (1967), to either linear competitive inhibition (eq 2) or linear noncompetitive inhibition (eq 3).

$$v = \frac{VA}{K_m(1 + I/K_{is}) + A} \quad (2)$$

$$V = \frac{VA}{K_m(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (3)$$

The lines drawn through the experimentally determined points in double reciprocal plots are those defined by the computer fit.

Results

Purification of Histidine Ammonia-lyase. In a typical preparation, animals were killed by decapitation, and their

Table I: Purification of Rat Liver Histidine Ammonia-lyase.

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Recovery (%)	Sp. Act. (units/mg)	Purification
Crude	365	7150	54 000	100	7.55	1.00
65 °C Heat	330	2050	43 900	81	21.4	2.82
First $(NH_4)_2SO_4$	10.5	252	37 700	70	150	19.7
DEAE-Sephadex	11.5	62	20 850	39	336	44.2
Second $(NH_4)_2SO_4$	2.0	21	17 480	32	832	110
QAE-Sephadex	4.8	5.2	9 940	18	1910	253

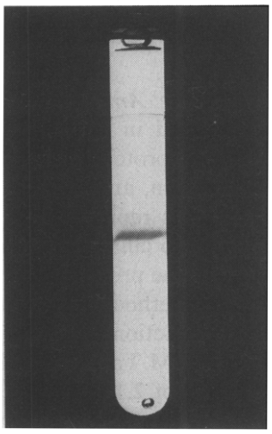

FIGURE 1: Polyacrylamide (6.4%) gel electrophoresis of purified rat liver histidine ammonia-lyase (20 μg of protein) after QAE-Sephadex chromatography. Gel was stained with Coomassie blue. For details of experimental conditions, see Materials and Methods.

livers were excised as quickly as possible and placed in chilled 0.9% sodium chloride. After trimming of extraneous tissue and mincing, the livers were homogenized in 4 volumes of ice-chilled 0.25 M sucrose using a Potter Elvehjem homogenizing apparatus. The homogenate was then centrifuged for 50 min at 40 000g, and the resulting supernatant removed by aspiration. The pH of the supernatant was adjusted to 6.3–6.4 with 1.0 N sodium hydroxide. The preparation was then heated with gentle stirring to 65 °C in a 75 °C water bath, held at this temperature for 5 min, and then rapidly chilled to 0–4 °C in an ice bath. Precipitated protein was removed by centrifugation for 15 min at 25 000g. Saturated ammonium sulfate (0–4 °C) was added to the ice-chilled supernatant, with stirring to give a final saturation of 38%. The pH was adjusted to 7.0 with 1.0 N NaOH and, after stirring at 0–4 °C for 15 min further, the precipitate was removed by centrifugation for 20 min at 25 000g. To the resulting supernatant was added, with stirring, additional ammonium sulfate solution to achieve a final saturation of 50%. The pH was adjusted to 7.0 and, after 15 min additional stirring, the suspension was centrifuged as before. The resulting red-orange precipitate was dissolved in 10 ml of 0.1 M Tris-HCl buffer, pH 7.6, and used immediately in the following step since the enzyme activity decreased as much as 20% after freezing and thawing at this step. The resuspended enzyme preparation was applied to a 2.5 × 30 cm DEAE-Sephadex column equilibrated at 0–4 °C with 0.1 M sodium chloride in 0.1 M Tris-Cl (pH 7.6) and was eluted with the same buffer. Fractions of 4.4 ml were collected. Fractions containing histidine ammonia-lyase were recovered from the column in a single protein peak, eluting just after a red-colored protein which appeared in the first fractions. Samples of this preparation used for kinetic studies were dialyzed overnight against 200 volumes of 20 mM Tris-Cl (pH 7.6) at 0–4 °C. Fractions containing the highest specific enzyme activity were pooled. To this preparation was added, with stirring, 2.0 mg/ml of dithiothreitol. After 10 min, saturated ammonium sulfate solution (0–4 °C) was added dropwise to a saturation of 40%. After removal of the orange-colored precipitate by centrifugation at 40 000g for 10 min, saturated ammonium sulfate solution was again added to a final saturation of 50%. After stirring at 0–4 °C for 10 min, the white precipitate which contained the enzyme was removed by centrifugation at 40 000g for 10 min and resuspended in 2.0 ml of 0.1 M imidazolium chloride buffer, pH 6.8. The resuspended enzyme from the above procedure (1.5 ml) was applied to a 1.5 × 20 cm QAE Sephadex column equilibrated with 0.1 M imidazole buffer, pH 6.80. The column was eluted with a linear gradient of sodium chloride from 0 to 0.5 M in the same buffer. Nearly all of the enzyme was recovered from a single peak near the end of the gradient. The activity of the enzyme was found to deteriorate rapidly when frozen for more than a week in the imidazole buffer. Therefore, fractions containing the highest histidine ammonia-lyase specific activity were generally pooled, saturated ammonium sulfate solution was added to give a final saturation of 65%, and the enzyme was recovered by centrifugation for 15 min at 40 000g and resuspended in 0.1 M Tris-HCl buffer, pH 7.6. Histidine ammonia-lyase thus prepared was then frozen, stored at −20 °C, and used within 2 weeks, during which time the activity was not significantly diminished.

A typical purification scheme is summarized in Table I.

Purity of the Enzyme. Analytical polyacrylamide gel electrophoresis indicated the presence of only one prominent band (Figure 1). Fractionation of the gel into 1.2-mm slices and incubation of each for 1.5 h in 1.0 ml of standard histidine ammonia-lyase assay medium at 37 °C demonstrated that enzyme activity was associated only with this band. The molecular weight of histidine ammonia-lyase was estimated by comparing the rate of migration of the enzyme with those of standard proteins in polyacrylamide gels of different acrylamide concentration, according to the method of Hedrick and Smith (1968). This procedure yielded a value of approximately 203 000 for the native enzyme, in general agreement with the value of 190 000 reported recently by an ultracentrifugal determination (Okamura et al., 1974).

pH Dependence. In the presence of the buffer salts indicated, the kinetic behavior of the enzyme was examined at various pH values, and K_m and V_{max} were determined (Figure 2). The pH optimum for enzymatic activity was found to occur at approximately 8.5, while a minimum K_m value of 0.45 mM was observed at pH 9.0. K_m was increased substantially in the physiological pH range, K_m at pH 7.2 being 2.1 mM, while catalytic activity as reflected in V_{max} drops off steeply below pH 8.0; the enzyme was essentially inac-

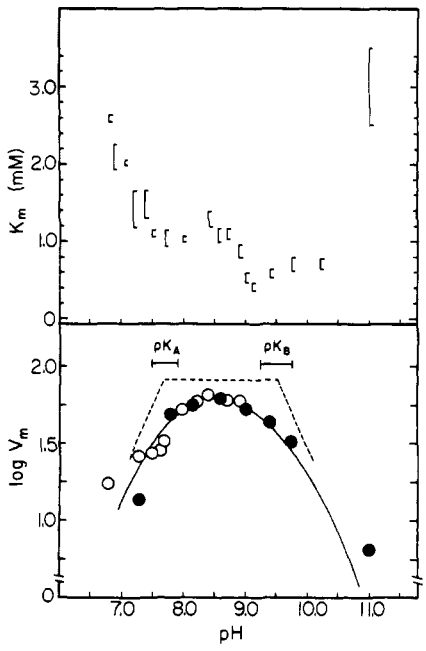

FIGURE 2: The effect of pH on the kinetic parameters of rat liver histidine ammonia-lyase. All reaction mixtures contained: reduced glutathione, 7.0 mM; L-histidine; and either 10 mM sodium pyrophosphate (●) or 10 mM sodium phosphate (○) as buffer in a 1.5 ml volume at 24 °C, to which was added 0.05 ml of enzyme purified to the DEAE-Sephadex step. Values for K_m (indicated by vertical standard error bars) and V_m were calculated from the experimental data by a computer method described in Materials and Methods. The values for pK_a and pK_b, indicated by horizontal standard error bars, were calculated from a weighted computer fit (Cleland, 1967) of the data obtained using pyrophosphate buffer as described in the text. The curve drawn through the experimental points is that determined by the computer fit. Asymptotes of the curve are indicated by dashed lines.

tive below pH 6.5. It is uncertain whether the irregular, apparently biphasic dependence of the Michaelis constant on pH is the result of experimental variance or an indication of the presence in the enzyme preparation of two forms having different affinities for the substrate. In Figure 2, the parabolic curve drawn through the experimental data points in the plot of log V_{max} as a function of pH was calculated by a computerized regression method (Cleland, 1967) from the expression

$$V_{max} = V'_{max}/(1 + [H^+]/K_a + K_b/[H^+])$$

where V'_{max} is the asymptotic maximum velocity if all the (rate-limiting) enzyme-substrate complex is in the most catalytically active ionization state, and K_a and K_b are functions of the true dissociation constants of the involved ionizable groups. From the computer fit of the experimental data to the above equation, values were extracted for pK_a and pK_b of 7.7 ± 0.20 and 9.5 ± 0.25, respectively.

Interaction of Histidine Ammonia-lyase with Substrate Analogues. A number of histidine derivatives and analogues were tested for their ability to inhibit the enzyme in vitro (Table II). The standard pH 9.0 assay system was used for this purpose, but 0.45 mM histidine was substituted for the usual saturating substrate concentration, in order to facilitate detection of competitive inhibition. Of the compounds tested, only L-histidine hydroxamate and D-α-hydrazinoimidazolylpropionic acid were relatively strong inhibitors; D-histidine, L-histidinol, and glycine were also effective inhibitors, but only at much higher concentrations. In contrast, L-1-methylhistidine, *N*-acetyl-L-histidine, L-histidine methyl ester, L-alanine, and DL-pyrazolyl-3-alanine were not inhibitory even when present at 5.0 mM. Interfering substrate activity of histidine analogues with the enzyme was assessed by attempting to observe enzyme-dependent-changes in the uv spectra of each alanine derivative in Table II over the range 260–325 nm, following a 2-h incubation under the standard enzyme assay conditions. None was found for any of the substrate analogues tested.

Interaction with the enzyme of these four imidazole compounds was studied further by kinetic analysis. In all four cases, inhibition was linear competitive. The results of these studies are summarized in Table III. Because the "unnatural" D-α-hydrazino analogue of the L-histidine substrate proved so potent as inhibitor (K_{is} = 0.075 mM), it was an-

Table II: Inhibition of Rat Liver Histidine Ammonia-lyase by Substrate Analogues.

Analogue	Inhibition (%) 5.0 mM Analogue[a]	1.0 mM Analogue[a]
L-Histidine hydroxamate	93	60
DL-Alanine hydroxamate	5	0
D-α-Hydrazinoimidazolyl-propionic acid	92	69
DL-α-Hydrazinoisocaproic acid	30	10
DL-α-Hydrazinophenylpropionic acid	0	0
L-Histidinol	73	31
DL-Alaninol	31	12
D-Histidine	65	27
Imidazolylpropionic acid	37	25
Histamine	35	15
Glycine	42	23
Imidazolylpyruvic acid		21
DL-α-Methylhistidine	29	12
L-3-Methylhistidine	25	6
DL-(2-Thiazolyl)-3-alanine	31	8
DL-(1,2,4-Triazolyl)-3-alanine	21	8
Imidazole	21	10
L-β-Imidazolyllactic acid	17	4

[a] DL compounds were present at 10.0 or 2.0 mM.

Table III: Interaction of L-Histidine and Competitive Inhibitors with Rat Liver Histidine Ammonia-lyase.

Substrate/Inhibitor	K_m(app)[a] (mM)	K_{is}(app)[a] (mM)	K_{is}/K_m
L-Histidine	0.51 ± 0.06		
L-Histidinol		2.71 ± 0.39	5.3
L-Histidine hydroxamate		0.42 ± 0.05	0.82
D-Histidine		1.98 ± 0.19	3.9
D-α-Hydrazinoimidazolyl-propionic acid		0.075 ± 0.004	0.15
DL-α-Hydrazinoimidazolyl-propionic acid		0.22 ± 0.03	0.48
L-Histidine (pH 7.0)	3.85 ± 0.82		
D-α-Hydrazinoimidazolyl-propionic acid (pH 7.0)		0.11 ± 0.02	0.029

[a] Values given are means ± standard errors calculated by the computer method of Cleland (1967).

Table IV: Interaction of L-Phenylalanine and Competitive Inhibitors with *Rhodotorula glutinis* Phenylalanine Ammonia-lyase.

Substrate/Inhibitor	K_m(app)[a] (mM)	K_{is}(app)[a] (mM)	K_{is}/K_m
L-Phenylalanine	0.40 ± 0.02		
Hydrazine dihydrochloride		3.54 ± 0.15	8.9
β-Phenylethylhydrazine		0.24 ± 0.01	0.59
DL-α-Hydrazinophenylpropionic acid		0.024 ± 0.001	0.045
DL-α-Hydrazinoimidazolylpropionic acid		5.75 ± 0.42	14.6

[a] Values given are the means ± standard errors calculated by the computer method of Cleland (1967).

ticipated that the L enantiomer would be even more effective in this regard. Kinetic studies of histidine ammonia-lyase inhibition by DL-α-hydrazinoimidazolylpropionate demonstrated the expected linear competitive inhibition by the latter; however, from the observed K_{is} value of 0.22 mM (Table III), it appears that the L enantiomer is no more effective an inhibitor than the D enantiomer; indeed, the former appeared to be relatively inactive as an inhibitor. Incubation of the purified enzyme in the standard assay buffer for up to 2 h in the presence of 10 mM D- or DL-α-hydrazinoimidazolylpropionate did not produce any resultant loss of activity.

The effect of pH on the inhibition of the enzyme by D-α-hydrazinoimidazolylpropionic acid was also investigated; since the acid dissociation constants of monoalkylhydrazines are in general at least ten times as large as those of the analogous monoalkylamines (Millar and Springell, 1966), it is predictable that with decreasing pH this very nucleophilic substrate analogue should compete more effectively with L-histidine for the electrophilic (Givot and Abeles, 1970) enzyme active site. Potentiometric titration of DL-α-hydrazinoimidazolylpropionic acid indicated carboxyl and imidazole ionization constants virtually identical with those of L-histidine, but a pK value for α-hydrazino ionization of approximately 7.4, compared with a value of 8.95 for the α-amino ionization of L-histidine. Kinetic studies of the behavior of this inhibitor at pH 7.0 demonstrated that, while K_m for L-histidine was several-fold higher than in the standard pH 9.0 assay system, the K_{is} for the D-α-hydrazino analogue was increased by only about 50% (Table III).

Interaction of Yeast Phenylalanine Ammonia-lyase with Substrate Analogues Structurally Related to Hydrazine. The observation that the D-α-hydrazino analogue of histidine was a potent competitive inhibitor of rat liver L-histidine ammonia-lyase suggested that similar dehydroalanine-containing enzymes (Hanson and Havir, 1972) might also be sensitive to this class of compounds. L-Phenylalanine ammonia-lyase purified from *Rhodotorula glutinis*, previously shown to catalyze a reaction mechanistically analogous to that of L-histidine ammonia-lyase (Hodgins, 1971), was subjected to kinetic analysis in the presence of either DL-α-hydrazinoimidazolylpropionic acid or a number of structurally related compounds. The α-hydrazino analogue of histidine proved to be a relatively weak inhibitor of the linear competitive type; however, as anticipated, the DL-α-hydrazino analogue of the substrate, DL-α-hydrazinophenylpropionic acid, was a very potent linear competitive inhibitor of this enzyme (Table IV). Neither of these compounds were substrates of the enzyme. β-Phenylethylhydrazine was expectedly less effective as a competitive inhibitor (Table IV), indicating the importance of the carboxyl moiety in substrate binding; hydrazine alone was even less effective in this regard. Inhibition of phenylalanine ammonia-lyase by DL-α-hydrazinoimidazolylpropionate can apparently be accounted for entirely by the presence of the α-hydrazino group since this effect is no greater than that obtained with hydrazine alone (Table IV). Likewise, DL-α-hydrazinophenylpropionic acid even at 10 mM did not produce detectable inhibition of histidine ammonia-lyase from rat liver (Table II).

Discussion

In a recent study, histidine ammonia-lyase from rat liver was reported to have a molecular weight of about 190 000 (Okamura et al., 1974). The molecular weight value of approximately 200 000 found in the present study confirms this observation. Soutar and Hassal (1969) have determined the native and subunit molecular weights of the enzyme from *Pseudomonas testosteroni* to be 200 000 and 33 000–36 000, respectively, while Rechler (1969) has reported the native and subunit weights of another pseudomonad histidine ammonia-lyase to be 214 000 and 35 000, respectively. On the other hand, Klee (1970), while reporting a comparable native molecular weight for the *Ps. fluorescens* enzyme of 213 000, has determined the subunit to be a 55 000 molecular weight species which is susceptible to partial hydrolysis under relatively mild conditions to a 38 000 molecular weight species; this enzyme, therefore, appears to be a tetramer. It is conceivable that the earlier reports of smaller subunit sizes indicating hexameric structures for other Pseudomonad histidine ammonia-lyases may thus be the result of hydrolysis artifacts. Havir and Hanson (1973) have suggested the existence of a labile peptide chain linkage in the mechanistically similar phenylalanine ammonia-lyases obtained from plant sources; similar disagreement presently exists concerning the subunit structure of these enzymes (Havir and Hanson, 1973). Preliminary reports of the quaternary structure of rat liver histidine ammonia-lyase have suggested this enzyme to be composed of either six (Brand and Harper, 1975) or three (Lamartiniere and Feigelson, 1975) apparently identical subunits; these conflicting values suggest the possibility that the native subunit of the rat liver enzyme, like the bacterial enzyme, may be subject to inadvertent cleavage to a smaller species by unusually mild conditions analogous to those described by Klee (1970). Determination of the true subunit structure of rat liver histidine ammonia-lyase will be necessary to establish the presence of a labile peptide bond.

The kinetic properties of the rat liver enzyme differ substantially from those of the *Ps. fluorescens* enzyme. Histidine ammonia-lyase from rat liver, like the bacterial enzyme (Peterkovsky, 1962), displays a relatively sharp V_{max} maximum at pH 8.4–8.6 (Figure 2). The pH dependence of the rat liver enzyme V_{max} reflected decreases in catalytic activity due to the protonation of an ionizable group having pK = 7.7 and deprotonation of an ionizable group having pK = 9.5 (Figure 2); for the *Ps. fluorescens* enzyme, pK values of 7.9 and 10.2 were obtained for the analogous ionizations (Frankfater and Fridovich, 1970). Although no attempt was made in either case to identify these groups, it is possible that the same two types of ionizable amino acid

side chains are involved in the enzyme from both species since the established presence of "dehydroalanine" in the active centers of both enzymes (Givot and Abeles, 1970; Hodgins, 1971) suggests a common catalytic mechanism. The K_m of the rat liver enzyme for L-histidine is a minimum value of 0.5 mM at pH 9.0, and increases progressively at higher or lower pH. In contrast, the K_m of the *Ps. fluorescens* enzyme was found to remain at a minimum plateau value between pH 6.5 and 8.0 and increase progressively to higher values at increased pH (Frankfater and Fridovich, 1970; Peterkovsky, 1962). Thus the substrate binding sites of the two enzyme types appear to differ in part by the presence of an ionizable group in the liver enzyme having a pK between 7.0 and 9.0, protonation of which results in a pronounced decrease in the affinity of the active site for the substrate.

It is not unexpected that the enzyme has a relatively high pH optimum, regardless of source, since the $\alpha\beta$-deamination reaction is known to be base catalyzed (Hanson and Havir, 1972). It is, however, surprising that the K_m value for the rat liver enzyme increases from a minimum value of 0.5 mM, approximately twice the fasting liver histidine concentration (Herbert et al., 1966), to more than 2.0 mM at the physiological pH of 7.2–7.4. Assuming that the kinetic properties of the enzyme in vivo are similar to those observed in vitro, this characteristic may be of regulatory importance to the animal since a high K_m enzyme would tend to limit the rate of degradation of irreplaceable histidine, normally present in low concentrations in liver, while retaining the capacity for much greater rates of histidine catabolism if a high concentration of this amino acid occurs, e.g., after ingestion of a large amount of protein. Rat liver histidine ammonia-lyase synthesis is known to be altered by a variety of dietary and hormonal effectors (Schirmer and Harper, 1970; Lee and Harper, 1971; Neufeld et al., 1971); however, the response to these is relatively slow. It is thus possible that histidine degradation in rat liver may be controlled primarily by substrate availability.

The rat liver enzyme exhibits a strict specificity for L-histidine as a substrate. None of the histidine analogues tested as substrates displayed any detectable activity as such. Indeed, few of these compounds were even effective as competitive inhibitors of the enzyme (Table II). Givot et al. (1969) likewise failed to observe alternate substrates for the *Ps. fluorescens* enzyme. Among the compounds relatively inactive as inhibitors were the histidine isosteres DL-(2-thiazolyl)-3-alanine and DL-(1,2,4-triazolyl)-3-alanine, suggesting that the substrate binding site of the rat liver enzyme has an absolute requirement for an unmodified imidazolyl moiety. Other investigators (Shibatani et al., 1975) have similarly failed to demonstrate substrate activity of these compounds with the enzyme from *Achromobacter liquidum*. Although L-*N*-methyl- and 3-*N*-methyl-L-histidine were observed to be weak inhibitors, Klee et al. (1975) have reported that 4-nitro-L-histidine and 4-fluoro-L-histidine are both substrates and competitive inhibitors of the *Ps. fluorescens* enzyme.

The results also indicate that other enzyme–substrate interactions important in histidine binding involve the substituents of the α-carbon. Consistent with the postulated mechanism of the enzyme, involving formation of an amino-enzyme intermediate through abstraction of the α-amino group by an enzyme "dehydroalanine" residue (Magasanik et al., 1971; Peterkovsky, 1962), imidazolylpropionic acid, *N*-acetyl-L-histidine, and imidazole were relatively ineffective as competitive inhibitors. The presence of hydrophilic groups on the α-carbon also appears important for binding; thus D-histidine, L-histidinol, L-histidine hydroxamate, and D-α-hydrazinoimidazolylpropionic acid were relatively strong inhibitors, while L-histidine methyl ester and DL-α-methylhistidine were not (Table III).

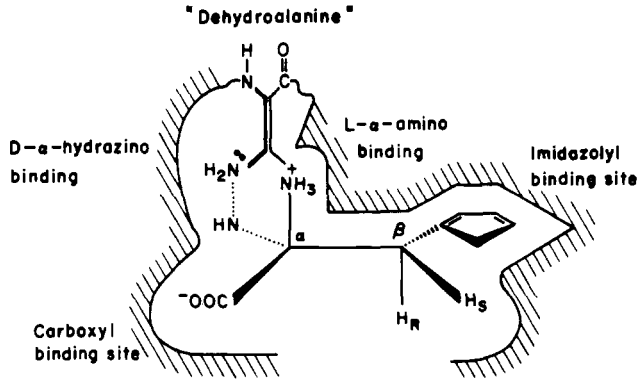

FIGURE 3: Model depicting proposed mechanism of binding to rat liver histidine ammonia-lyase of L-histidine (solid lines) or D-α-hydrazinoimidazolylpropionic acid (dotted lines). Structures of the latter compounds are superimposed for comparison, omitting the α-proton. Stereochemistry of the β-carbon protons is indicated. The enzyme "dehydroalanine" residue as drawn is not intended to represent the actual structure of this moiety, which in reality is probably more complex.

These findings indicate additionally that proper α-carbon stereochemistry is relatively more important for catalysis than for binding in this case. L-Histidine hydroxamate, having an anionic carboxyl substitution, was a much more potent inhibitor (K_{is} = 0.4 mM) than L-histidinol (K_{is} = 2.7 mM), as might be expected. Similarly, D-α-hydrazinoimidazolylpropionic acid (K_{is} = 0.075 mM) was a much stronger inhibitor than D-histidine (K_{is} = 2.0 mM), presumably in part because of the more nucleophilic character of its α-carbon substituent. The inhibitory character of the former is exaggerated at pH 7.0 (Table III), due to the lower pK_a of the α-hydrazino group relative to the α-amino group of the substrate, and its consequent greater affinity for the electrophilic "dehydroalanine" residue in the active site. The explanation for the somewhat unexpected observation that D-α-hydrazinoimidazolylpropionic acid is apparently a much more effective competitive inhibitor than L-α-hydrazinoimidazolylpropionic acid is uncertain. It is possible that the α-amino binding site of the enzyme cannot accommodate groups as bulky as an α-hydrazino moiety, but that an α-hydrazino (or α-amino) group in the D configuration may have access to an alternate compatible binding region in the enzyme active site. An α-hydrazino group, by virtue of its greater linear dimensions, might thus be brought into close proximity with the highly reactive, electrophilic "dehydroalanine" residue in the enzyme active site. A conceptual model of such a phenomenon is depicted in Figure 3. The existence of such an alternate binding region for hydrophilic α-carbon substituents could explain the observation that, with respect to potency as inhibitors of rat liver L-histidine ammonia-lyase, D-α-hydrazinoimidazolylpropionic acid $\gg$ D-histidine $>$ imidazolylpropionic acid (Table II); the opposite result might be expected if such a binding region did not exist.

It is of interest that D-histidine was a much better competitive inhibitor of the rat liver enzyme in the present study than it is of the *Ps. fluorescens* enzyme (Magasanik et al., 1971); differences in the mechanism of substrate binding

are, however, not unexpected in view of the above mentioned differences in pH dependency of the K's of the two enzymes. Similarly, although L-histidine hydroxamate was a potent competitive inhibitor of the liver enzyme (Tables II and III), it has been shown to be a weak inhibitor of histidine ammonia-lyase from *Achromobacter liquidum* (Shibatani et al., 1975); L-histidine methyl ester and L-β-imidazolyllactic acid, very poor inhibitors of rat liver histidine ammonia-lyase (Table II) are, however, powerful competitive inhibitors of the *Achromobacter* enzyme (Shibatani et al., 1975). Cornell and Crivaro (1972) have presented data demonstrating a high correlation between the K_i values of some nonimidazole competitive inhibitors of rat liver histidine ammonia-lyase and the stability constants of the Zn^{2+} complexes of these compounds. Whether the competitive inhibition of the rat liver enzyme observed for the various histidine analogues is due to specific binding or a nonspecific interaction with enzyme-bound Zn^{2+} is not certain since the affinities of these compounds for zinc have not been determined. From the results presented in Table II, it is clear that the imidazolyl moiety is particularly important in binding since L-alanine, DL-alaninol, DL-alanine hydroxamate, and DL-α-hydrazinoisocaproic acid are relatively poor inhibitors of the enzyme. It seems unlikely that the structural similarities to L-histidine of the potent competitive inhibitors characterized in this study are superfluous to their binding to the enzyme.

The two histidine analogues found to be most potent as competitive inhibitors of rat liver histidine ammonia-lyase, L-histidine hydroxamate and D-α-hydrazinoimidazolylpropionic acid, have also been shown to be strong inhibitors of the pyridoxal phosphate dependent mammalian histidine decarboxylase (Smissman and Warner, 1972; Levine et al., 1965), while DL-α-methylhistidine, a powerful inhibitor of histidine decarboxylase (Kahlson et al., 1963), had little effect on the activity of rat liver histidine ammonia-lyase (Table II). Thus, both similarities and differences exist in the substrate binding regions of these two enzymes which catalyze different reactions although having identical substrate requirements and possessing similar electrophilic catalytic groups.

The observed inhibition of *R. glutinis* phenylalanine ammonia-lyase by DL-α-hydrazinophenylpropionic acid (Table IV) is of considerable interest. This finding indicates that highly specific, competitive inhibition of "dehydroalanine"-dependent amino acid ammonia-lyases by the α-hydrazino analogues of their substrates may be a general phenomenon; whether the D enantiomer is the more active inhibitor in this case as it is in the case of rat liver histidine ammonia-lyase inhibition remains to be determined. Phenylalanine ammonia-lyase, not found in any mammalian species, is widely distributed among yeasts and higher plant species; the central role of this enzyme in directing carbon into plant lignification and other phenolic biosyntheses (Rubery and Northcote, 1968) and its induction by a variety of factors, especially light (Zucker, 1965), has been studied extensively. Because DL-α-hydrazinophenylpropionate is among the most potent specific inhibitors yet characterized for phenylalanine ammonia-lyase from any source (K_{is} = 24 μM), it might serve as the basis for the design of a useful experimental inhibitor of this enzyme in vivo in plants. Hodgins (1971) has reported DL-β-(2-hydroxyphenyl)alanine to be an equally effective competitive inhibitor of the *R. glutinis* enzyme (K_{is} = 27 μM); it is conceivable that the α-hydrazino derivative of this or a related compound would display a very high affinity for the substrate binding site of the enzyme.

Histidine ammonia-lyase from rat liver, although similar in superficial respects to the enzyme from some bacterial sources, differs substantially in its structural and kinetic properties from the enzyme isolated from *Ps. fluorescens* and *Anchromobacter liquidum*. The enzyme from epidermis, being the same gene product as the liver enzyme (Kaiser et al., 1973), probably has similar properties. Further studies on the properties of "dehydroalanine"-dependent amino acid ammonia-lyases from both eukaryote and prokaryote sources are necessary to determine the precise nature and mechanism of action of the moieties involved in catalysis.

Acknowledgment

The authors are indebted to Dr. W. W. Cleland and Dr. Michael Schimerlik for their comments and generous assistance in the computer treatment of the kinetic data.

References

Brand, L. M., and Harper, A. E. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 509.
Cleland, W. W. (1967), *Adv. Enzymol. 29,* 1.
Cornell, N. W., and Crivaro, K. E. (1972), *Anal. Biochem. 47,* 203.
Cornell, N. W., and Villee, C. A. (1968), *Biochim. Biophys. Acta 167,* 172.
Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.
Frankfater, A., and Fridovich, I. (1970), *Biochim. Biophys. Acta 206,* 457.
Givot, I. L., and Abeles, R. H. (1970), *J. Biol. Chem. 245,* 3271.
Givot, I. L., Smith, T. A., and Abeles, R. H. (1969), *J. Biol. Chem. 244,* 6341.
Glamkowski, E. J., Gal, G., Sletzinger, M., Porter, C. C., and Watson, L. S. (1967), *J. Med. Chem. 10,* 852.
Hanson, K. R., and Havir, E. (1972), *Enzymes, 3rd Ed. 7,* 79.
Havir, E. A., and Hanson, K. R. (1973), *Biochemistry 12,* 1583.
Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126,* 155.
Herbert, J. D., Coulson, R. A., and Hernandez, T. (1966), *Comp. Biochem. Physiol. 17,* 583.
Hodgins, D. S. (1971), *J. Biol. Chem. 246,* 2977.
Ji, T. H. (1973), *Anal. Biochem. 52,* 517.
Kahlson, G., Rosengren, E., and Thunberg, R. (1963), *J. Physiol. 169,* 467.
Kacser, H., Bulfield, G., and Wallace, M. E. (1973), *Nature (London) 244,* 77.
Klee, C. B. (1970), *J. Biol. Chem. 245,* 3143.
Klee, C. B., Kirk, K. L., Cohen, L. A., and McPhie, P. (1975), *J. Biol. Chem. 250,* 5033–5040.
LaDu, B. N. (1971), *Methods Enzymology, 17B,* 891.
Lamartiniere, C., and Feigelson, M. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 641.
Lee, S. C., and Harper, A. E. (1971), *Biochim. Biophys. Acta 244,* 135.
Levine, R. J., Sats, T. L., and Sjoerdsma, A. (1965), *Biochem. Pharmacol. 14,* 139.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Magasanik, K. B., Kominskas, E., and Kimhi, Y. (1971),

Methods Enzymology 17B, 47.
Mehler, A. H., and Tabor, H. (1953), *J. Biol. Chem. 201,* 775.
Millar, I. T., and Springell, H. D. (1966), Sidgwick's Organic Chemistry of Nitrogen, 3rd ed, Oxford, Clarendon Press, p 505.
Neufeld, E., Harell, A., and Chayen, R. (1971), *Biochim. Biophys. Acta 237,* 465.
Niedrich, H., and Grupe, R. (1965), *J. Prakt. Chem. 27,* 108.
Okamura, H., Nishida, T., and Nakagawa, H. (1974), *J. Biochem. 75,* 139.
Peterkovsky, A. (1962), *J. Biol. Chem. 237,* 787.
Rechler, M. M. (1969), *J. Biol. Chem. 244,* 551.
Rubery, P. H., and Northcote, D. H. (1968), *Nature (London) 219,* 1230.
Schirmer, M. D., and Harper, A. E. (1970), *J. Biol. Chem. 245,* 1204.
Shibatani, T., Kakimoto, T., and Chibata, I. (1975), *Eur. J. Biochem. 55,* 263.
Sletzinger, M., Firestone, R. A., Reinhold, D. F., Rooney, C. S., and Nicholson, W. H. (1968), *J. Med. Chem. 11,* 261.
Smissman, E. E., and Warner, J. D. (1972), *J. Med. Chem. 15,* 681.
Smith, T. A., Cordelle, F. H., and Abeles, R. H. (1967), *Arch. Biochem. Biophys. 120,* 724.
Soutar, A. K., and Hassal, H. (1969), *Biochem. J. 114,* 79P.
Tabor, H., and Mehler, A. H. (1955), *Methods Enzymol. 2,* 228.
Yankeelov, J. A., and Jolley, C. J. (1972), *Biochemistry 11,* 159.
Zannoni, V. G., and LaDu, B. J. (1963), *Biochem. J. 88,* 160.
Zucker, M. (1965), *Plant Physiol. 40,* 779.

Stimulation of Ascites Tumor RNA Polymerase II by Protein Kinase†

Michael E. Dahmus

ABSTRACT: The activity of purified RNA polymerase II from Novikoff ascites tumor cells is stimulated 5–7-fold by a purified protein factor. This protein factor, designated HLF_2, has extensive protein kinase activity and catalyzes the incorporation of γ-^{32}P from ATP into protein under normal RNA polymerase assay conditions. Protein phosphorylation is totally dependent on the presence of HLF_2 and is stimulated 2–3-fold by the presence of highly purified RNA polymerase II. The purification procedure developed for the isolation of the polymerase stimulatory factor resulted in a 4000-fold purification of a protein kinase. Chromatography on carboxymethylcellulose, phosphocellulose, and Sephadex G-100 did not resolve polymerase stimulatory activity from protein kinase activity: Adenylylimidodiphosphate (AMP-PNP), an inhibitor of protein kinases, inhibited the stimulatory activity of purified factor by 80%. The heat denaturation profile of protein kinase was paralleled by the loss of polymerase stimulatory activity. Concentrations of $(NH_4)_2SO_4$ which are known to inhibit polymerase stimulation (Lee and Dahmus, 1973) also inhibit protein kinase activity. The protein kinase activity associated with stimulatory factor catalyzes the phosphorylation of casein or phosvitin but does not catalyze the phosphorylation of basic proteins such as protamine or histone. The protein kinase is not stimulated by cyclic 3′,5′-AMP or -GMP over a concentration range of 10^{-6}–10^{-4} M. Furthermore, protein kinase activity is not inhibited by either the regulatory subunit of rabbit muscle protein kinase or by the heat-stable inhibitor of cyclic 3′,5′-AMP-dependent protein kinases. Protein kinase activity is stimulated by KCl or NH_4Cl and is inhibited by $MnCl_2$. The apparent K_m values, determined in the presence of 4 mM Mg^{2+}, are 0.02 mM for ATP, and 4.1 mM for GTP.

Alterations in gene activity, in a number of instances, have been observed to closely correlate with changes in the phosphorylation of nuclear proteins (Kleinsmith et al., 1966; Langan, 1971; Ahmed, 1971; Jungmann and Schweppe, 1972; Johnson et al., 1974; Karn et al., 1974; Kleinsmith, 1975). Indeed phosphorylated nonhistone chromosomal proteins have been reported to stimulate RNA synthesis in cell-free systems using purified DNA templates (Teng et al., 1971; Shea and Kleinsmith, 1973; Kostraba et al., 1975). This particular class of nonhistone phosphoproteins binds to DNA and stimulates RNA synthesis only in the presence of homologous DNA. The modification of nonhistone chromosomal proteins by phosphorylation is thought to influence their interaction with DNA and thereby lead to alterations in gene activity. It has also been suggested that transcription may in part be regulated by the direct phosphorylation of nuclear RNA polymerase. Jungmann et al. (1974) have reported the stimulation of both calf RNA polymerase I and II, present in partially purified extracts, by the addition of a cAMP-dependent protein kinase from calf ovary cytosol. Martelo and Hirsch (1974) have also reported the stimulation of isolated rat liver polymerase I by a nuclear protein kinase.

Kish and Kleinsmith (1974) have reported the fractionation of nuclear protein kinases from beef liver into 12 distinct fractions. The results of Ahmed and Wilson (1975) also suggest the presence of multiple protein kinases associated with

† From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received October 17, 1975.* This work was supported by the National Institutes of Health (HD 04899).