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Specificity of Lysine : N^6-Hydroxylase: A Hypothesis for a Reactive Substrate Intermediate in the Catalytic Mechanism

L. MARRONE,[1] S. SIEMANN,[1] M. BEECROFT, AND T. VISWANATHA[2]

Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1 Canada

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The recombinant cytoplasmic preparation of lysine : N^6-hydroxlase catalyzes the conversion of L-lysine to its N^6-hydroxy derivative when supplemented with the cofactors NADPH and FAD. A number of lysine analogs reflecting minor alterations in the inherent structural features of the amino acid as well compounds with relatively high affinity for lysine binding domains in other proteins were examined for their ability to serve as substrates of lysine : N^6-hydroxylase. These studies have revealed that the enzyme does not tolerate any change in the structural features of L-lysine, its preferred substrate, with the exception of the replacement of the $C_\gamma H_2$-methylene group by sulfur, as in (*S*)-2-aminoethyl-L-cysteine. L-Norleucine is a potent inhibitor of the enzyme while L-norvaline and L-α-aminobutyric acid do not exhibit such effect, indicating the importance of a C_4 hydrophobic side chain for effective interaction with the enzyme. Among the *N*-alkyl amides of hydrophobic amino acids, only L-norleucine methylamide and L-α-aminobutyric acid ethylamide serve as moderate inhibitors of lysine : N^6-hydroxylase. Based on the enzyme's stringent substrate specificity, a mechanism involving the conversion of L-lysine to 2-aminocaprolactam prior to its oxygenation by the 4*a*-peroxyflavin intermediate in the catalytic cycle is proposed.

INTRODUCTION

Lysine : N^6-hydroxylase catalyzes the conversion of L-lysine into its N^6-hydroxy derivative, the initial step in the biosynthesis of the siderophore aerobactin (*1–4*). The wild-type enzyme is membrane associated, a feature that has precluded the elucidation of the molecular basis for the catalytic mechanism of the protein (*4, 5*). However, recombinant cytoplasmic preparations of lysine : N^6-hydroxylase, *r*IucD,[3] have been developed, and these forms require NADPH as well as FAD for their

[1] L. Marrone and S. Siemann contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: (519) 746-0435. E-mail: tviswan@chemistry.watstar.uwaterloo.ca.

[3] Abbreviations used: DCC, *N*,*N*′-dicyclohexyl carbodiimide; DCU, *N*,*N*′-dicyclohexyl urea; dec., decomposition; DTT, dithiothreitol; ESMS, electrospray mass spectrometry; FAD, flavin adenine dinucleotide (oxidized form); G-6-P, glucose-6-phosphate; G-6-P deH_2, glucose-6-phosphate dehydrogenase; HAbuNHEt, α-aminobutyric acid ethylamide; HNleNH_2, norleucineamide; HNleNHMe, norleucine methylamide; HNvaNHMe, norvaline methylamide; NADPH, nicotinamide adenine dinucleotide phosphate (reduced from); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); nd, not determined; *r*IucD, recombinant cytoplasmic lysine : N^6-hydroxylase; t-Boc, *N*-*tert*-butoxycarbonyl; *t*-BocAbuNHEt, *t*-Boc-protected α-aminobutyric acid ethylamide; *t*-BocNleNHMe, *t*-Boc-protected norleucine methylamide; *t*-BocNvaNHMe, *t*-Boc-protected norvaline methylamide.

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catalytic function (*6, 7*). Preliminary studies have also indicated that the recombinant forms of the enzyme are specific for L-lysine and its analog (*S*)-2-aminoethyl-L-cysteine serving as the preferred substrates (*6, 7*). Current investigations concern a systematic study of various substrate analogs to serve as either a substrate or an inhibitor with the aim of gaining an insight into the details of the mechanism operative in the catalytic process mediated by *r*IucD.

MATERIALS AND METHODS

General

NMR (^{1}H) spectra were recorded on a Bruker AM-250 spectrometer in either $CDCl_3$ or D_2O with tetramethylsilane as an internal standard (δ = 0 ppm). Mass spectra were recorded with a VG/Quattro II triple quadrupole mass spectrometer using electrospray ionization and THF or H_2O as the solvent. Ultraviolet–visible spectra were recorded on a Beckman DU 640 spectrophotometer. Melting points were determined in open capillaries on a Mel-Temp apparatus (Laboratory Devices, U.S.A.) and are not corrected. Thin-layer chromatography was performed on precoated silica gel sheets (TLC aluminum sheets, silica gel $60F_{254}$, precoated sheets, 20 × 20 cm, layer thickness 0.2 mm by Merck, Darmstadt, Germany) using an appropriate solvent system. The location of the components was established by exposing the plates to ninhydrin as follows: the sheets were sprayed with ninhydrin reagent (0.1% w/v in ethanol) and dried at 80°C. Compounds containing free amino groups appeared as colored spots with characteristic R_f values. In the case of *t*-Boc-protected amino acids the sheets were exposed to trifluoroacetic acid vapor for 1 min, dried if necessary, and finally developed with ninhydrin, as described above.

Materials

p-Aminobenzamidine dihydrochloride, benzamidine hydrochloride, DTT, ethylamine (70% aqueous solution), FAD, G-6-P, G-6-P deH_2, *N*-hydroxysuccinimide, L-lysine, methylamine hydrochloride, β-$NADP^+$ sodium salt, β-NADPH tetrasodium salt, ninhydrin, DL-norleucine, and L(+)-norleucine were purchased from Sigma Chemical Company (St. Louis, MO). *Trans*-4,5-dehydro-L-lysine, L-norleucine methylester, and all *t*-Boc-protected amino acids were obtained from Bachem Bioscience Inc. (Philadelphia, PA). Trifluoroacetic acid, iodine, and α-naphthylamine were purchased from BDH Chemical Company (Toronto, ON, Canada). 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide method-*p*-toluenesulfonate, hydrochloric acid (4 M) in 1,4-dioxane, and sulfanilic acid were obtained from Aldrich (Milwaukee, WI). Dowex 50W-X8 (200–400 mesh, H^+ form) was purchased from Bio-Rad Laboratories (Richmond, CA). DCC was purchased from Fluka Chemie AG (Buchs, Switzerland). All inorganic salts and organic solvents were purchased from J. T. Baker Chemical Company (Phillipsburg, NJ). α-*N*-Methyl-L-lysine was kindly provided by Prof. N. L. Benoiton (Department of Biochemistry, University of Ottawa, Canada).

The isolation and purification of *r*IucD was achieved by employing procedures documented in the literature (*6, 7*). The concentration of the enzyme was determined spectrophotometrically using a molar extinction coefficient of 6.75×10^4 M^{-1} cm^{-1} at 280 nm (*8*).

Determination of Lysine: N^6-Hydroxlyase Activity (Hydroxylamine Assay)

The protocol for the determination of lysine: N^6-hydroxylase activity was similar to that described previously (*9*). The assay mixture, in a final volume of 5.0 ml, consisted of 100 mM potassium phosphate buffer (pH 7.0), DTT (400 μM), L-lysine (1 mM), FAD (40 μM), $NADP^+$ (160 μM), G-6-P (800 μM), G-6-P deH_2 (1 unit), and enzyme (100 nM). Following incubation at 37°C for 15 min, the reaction was stopped by adding 2.5 ml of a slurry of Dowex 50W-X8 resin (200–400 mesh, H^+-form). After the termination of the reaction, the entire mixture was transferred into a glass column (1.2 × 25 cm), the resin washed with 40 ml HCl (0.2 M), and the product eluted with 20 ml HCl (6 M). The eluate was then taken to dryness under reduced pressure. The resulting residue was dissolved in 5.0 ml of distilled water, and an aliquot (4.5 ml) was finally used for N^6-hydroxylysine determination (*10*) by the sequential addition of the following reagents: sodium acetate (0.5 ml; 48%, w/v), sulfanilic acid (0.5 ml; 1% w/w in acetic acid (25% v/v)), and iodine (0.2 ml; 1.3% w/v in glacial acetic acid). After standing for 5 min at room temperature, the excess iodine was removed by treatment with sodium thiosulfate (0.2 ml; 0.1 M), followed by the addition of α-naphthylamine (0.1 ml; 0.6% w/v in acetic acid (30%, v/v)). The absorbance at 520 nm was recorded after standing at room temperature for 20 min.

The effect of desired compounds on the catalytic function of *r*IucD was determined by their inclusion in the assay at a final concentration of 1 mM.

NADPH Oxidation

The assay mixture, in a final volume of 3.0 ml, consisted of NADPH (200 μM), FAD (40 μM), potassium phosphate buffer (100 mM, pH 7.0), DTT (400 μM), and *r*IucD (370 nM). The decrease in NADPH concentration was monitored spectrophotometrically at 340 nm at 23°C.

Synthesis of t-Boc L-Amino Acid Alkylamides

The procedure concerning the first step in the synthesis of *t*-Boc L-amino acid alkylamides (*t*-BocAbuNHEt, *t*-BocNvaNHMe, and *t*-BocNleNHMe), the conversion of a *t*-Boc amino acid into its *N*-hydroxysuccinimide ester, was adapted from the literature (*11, 12*). The *t*-Boc-protected L-amino acid (1 mmol) and *N*-hydroxysuccinimide (2 mmol) were dissolved in 5 ml THF, and solid DCC (1.1 mmol) was added slowly to the solution at −5°C. The mixture was stirred at −5°C for 1 h. After 2 h of stirring at room temperature, insoluble DCU was removed by filtration, and the filtrate was stored overnight at −20°C and filtered again. The purity of the activated esters was established by thin-layer chromatography using CH_2Cl_2/methanol (9/1), and ESMS. The filtrate containing the *N*-hydroxysuccinimide ester was cooled to 0°C. In the case of methyl amides, methylamine hydrochloride (1.4

mmol) was dissolved in 2 ml H_2O, the pH adjusted to 11.0 with KOH, and slowly added to the filtrate. In the synthesis of *t*-BocAbuNHEt, 100 μl (approx. 1.2 mmol) ethylamine (70% in H_2O) was diluted to 2 ml with H_2O and slowly added to the filtrate. The reaction mixture was stirred for 2 min at 0°C, 15 min at room temperature, and finally taken to dryness under reduced pressure. The residue was dissolved in a minimal amount of ethanol and treated with water to facilitate the precipitation of DCU that could not be removed from the preparation in the previous step. After the removal of the insoluble material, the filtrate was taken to dryness, and the residue was suspended in H_2O. The *t*-Boc amino acid amides were extracted with ethylacetate, and the organic phase was subsequently washed with H_2O and saturated NaCl solution. After drying over anhydrous Na_2SO_4, the organic solvent was filtered and taken to dryness under reduced pressure. The purity of the amides was established by thin-layer chromatography employing CH_2Cl_2/methanol (9/1), ESMS, and ^{1}H NMR (250 MHz, $CDCl_3$). *t*-BocAbuNHEt: yield: 70%; R_f = 0.58; ^{1}H NMR ($CDCl_3$): δ 0.93 (t, 3 H, $C_\gamma H_3$), 1.13 (t, 3 H, CH_3CH_2N), 1.44 (s, 9 H, $(CH_3)_3C$), 1.75 (m, 2 H, $C_\beta H_2$), 3.30 (m, 2 H, CH_2N), 3.93 (m, 1 H, $C_\alpha H$), 5.0 (band, 1 H, NH), 6.0 (band, 1 H, NH); ESMS: 231.1 (Calcd MH^+ 231.3). *t*-BocNvaNHMe: yield: 70%; R_f = 0.52; ^{1}H NMR ($CDCl_3$): δ 0.93 (t, 3 H, $C_\delta H_3$), 1.3–1.5 (m, 2 H, $C_\gamma H_2$), 1.44 (s, 9 H, $(CH_3)_3C$), 1.8 (m, 2 H, $C_\beta H_2$), 2.81 (d, 3 H, CH_3N), 4.04 (m, 1 H, $C_\alpha H$), 5.0 (band, 1 H, NH), 6.1 (band, 1 H, NH); ESMS: 231.1 (Calcd MH^+ 231.3). *t*-BocNleNHMe: yield: 72%; R_f = 0.60; ^{1}H NMR ($CDCl_3$): δ 0.9 (t, 3 H, $C_\varepsilon H_3$), 1.2–1.5 (m, 4 H, $C_\gamma H_2 C_\delta H_2$), 1.5 (s, 9 H, $(CH_3)_3C$), 1.9 (m, 2 H, $C_\beta H_2$), 2.85 (d, 3 H, CH_3N), 4.1 (m, 1 H, $C_\alpha H$), 5.0 (band, 1 H, NH), 6.1 (band, 1 H, NH); ESMS: 245.1 (Calcd MH^+ 245.3).

Deprotection of t-Boc L-Amino Acid Amides

The residue containing the *t*-Boc-protected amino acid amides was dissolved in 4.0 M HCl in 1,4-dioxane under nitrogen atmosphere at −5°C. After stirring for 30 min at room temperature, the reaction mixture was taken to dryness under reduced pressure. Recrystallization was achieved from ethanol/THF. In the case of HAbuNHEt, however, attempts to crystallize the compound have been unsuccessful. The purity of the deprotected amides was established by thin-layer chromatography using *n*-butanol/acetic acid/H_2O (4/1/2), ESMS, and ^{1}H NMR (250 MHz, D_2O). HAbuNHEt: yield: 90%; R_f = 0.48; ^{1}H NMR (D_2O): δ 0.81 (t, 3 H, $C_\gamma H_3$), 0.97 (t, 3 H, CH_3CH_2N), 1.72 (m, 2 H, $C_\beta H_2$), 3.1 (m, 2 H, CH_2N), 3.7 (t, 1 H, $C_\alpha H$); ESMS: 131.1 (Calcd MH^+ 131.2). HNvaNHMe: yield: 92%; mp 190–191°C; R_f = 0.48; ^{1}H NMR (D_2O): δ 0.77 (t, 3 H, $C_\delta H_3$), 1.2 (m, 2 H, $C_\gamma H_2$), 1.63 (m, 2 H, $C_\beta H_2$), 2.62 (s, 3 H, CH_3N), 3.76 (t, 1 H, $C_\alpha H$); ESMS: 131.1 (Calcd MH^+ 131.2). HNleNHMe: yield: 93%; mp 210–211°C; R_f = 0.54; ^{1}H NMR (D_2O): δ 0.72 (t, 3 H, $C_\varepsilon H_3$), 1.1–1.3 (m, 4 H, $C_\gamma H_2 C_\delta H_2$), 1.68 (m, 2 H, $C_\beta H_2$), 2.62 (s, 3 H, CH_3N), 3.76 (t, 1 H, $C_\alpha H$); ESMS: 145.1 (Calcd MH^+ 145.2).

Synthesis of L-Norleucine Amide

L-Norleucine amide was prepared according to the procedure employed for the synthesis of L-leucine amide (*13*). L-Norleucine methylester (764 mg, 4.2 mmol)

was added to methanol, saturated with NH_3 at −78°C. The methanolic ammonia solution was prepared by adding approximately 75 ml cold anhydrous methanol (−78°C) to liquid NH_3, which had been condensed previously into a 250-ml round-bottom flask on a dry ice/ethanol bath. After the addition of the L-norleucine methylester, the dry ice/ethanol bath was removed, and the mixture was allowed to reach room temperature. After 24 h, the reaction was terminated by evaporating the solvent under reduced pressure. The amide was finally crystallized from methanol/THF. The purity of the compound was established by thin-layer chromatography using *n*-butanol/acetic acid/H_2O (4/1/2), ESMS, and ^{1}H NMR (250 MHz, D_2O). Yield: 85%; mp 200–205°C dec.; R_f = 0.58; ^{1}H NMR (D_2O): δ 0.72 (t, 3 H, $C_\varepsilon H_3$), 1.1–1.3 (m, 4 H, $C_\gamma H_2 C_\delta H_2$), 1.7 (m, 2 H, $C_\beta H_2$), 3.83 (t, 1 H, $C_\alpha H$); ESMS: 131.0 (Calcd MH^+ 131.2).

Synthesis of S-Propargyl-L-Cysteine

The protocol for the preparation of *S*-propargyl-L-cysteine was similar to that documented for the synthesis of *S*-alkyl-L-cysteine (*14*).

L-Cysteine hydrochloride (1 g, 5.72 mmol) was dissolved in an aqueous solution of $Ba(OH)_2$ (33.3 ml, 0.2 M), and approximately 20 ml of ethanol (absolute) was added. After the addition of propargyl bromide (1.7 ml, 19.1 mmol), the mixture was stirred overnight under nitrogen atmosphere. The barium ions were removed quantitatively with the aid of sulfuric acid (15 ml, 10%), followed by the addition of absolute ethanol (3–4 vol). After standing overnight at 4°C, *S*-propargyl-L-cysteine was recovered as a precipitate. The purity of the compound was established by thin-layer chromatography using *n*-butanol/acetic acid/H_2O (4/1/5) as well as ^{1}H NMR (250 MHz, D_2O). Yield: 85%; mp 250°C dec.; R_f = 0.44; ^{1}H NMR (D_2O): δ 2.97 (t, 1 H, J = 2.4, HC≡C), 3.43 (dd, 1 H, J^1 = 7.8, J^2 = 14.8, $C_\beta H_2$), 3.59 (dd, 1 H, J^1 = 4.2, J^1 = 14.8, $C_\beta H_2$), 3.66 (d, 2 H, J = 2.4, C≡C−CH_2), 4.27 (dd, 1 H, J^1 = 4.2, J^2 = 7.8, $C_\alpha H$).

Synthesis of (S)-2-Aminoethyl-DL-Homocysteine

(*S*)-2-Aminoethyl-DL-homocysteine was prepared from DL-homocysteine according to the procedure employed for the synthesis of (*S*)-2-aminoethylcysteine (*15*). DL-Homocysteine was generated by treatment of DL-homocysteine thiolactone hydrochloride (Sigma) with KOH (1 M). Yield: 64%; ^{1}H NMR (250 MHz, D_2O): δ 2.00 (m, 2 H, $C_\beta H_2$), 2.55 (t, 2 H, $C_\gamma H_2$), 2.74 (t, 2 H, $CH_2CH_2NH_3^+$), 3.08 (t, 2 H, $CH_2NH_3^+$), 3.71 (t, 1 H, CαH).

RESULTS

Effect of Substrate Analogs on the Catalytic Function of rIucD

The influence of various compounds serving as analogs of L-lysine was investigated in order to gain an insight into the structure–function relationship of lysine : N^6-hydroxylase. Most of the compounds chosen for these studies share certain structural

features inherent in L-lysine, which consists of a positively charged amino group (A) at the α-carbon as well as a negatively charged carboxyl group (B) at the same position as indicated in Fig. 1. Furthermore, L-lysine is characterized by a hydrophobic carbon side chain composed of four methylene groups (C_n), and a positively charged amino group at the end of this chain (D). Other compounds were chosen in view of their documented ability to interact with lysine binding domains in other proteins. This latter class includes: (i) tranexamic acid (*trans*-4-(aminomethyl)-cyclohexanecarboxylic acid) and ε-aminocaproic acid, components known to bind lysine specific kringles in plasminogen (*16*); and (ii) benzamidine as well as *p*-aminobenzamidine, which serve as inhibitors of trypsin (*17*), a protease with remarkable specificity for lysine (and arginine) substrates (*18*).

As indicated in Table 1, lysine : N^6-hydroxylase appears to be stringently specific with regard to its substrate. However, (*S*)-2-aminoethyl-L-cysteine, an analog containing the same structural features (A, B, C_n, and D) as the preferred substrate L-lysine, was found to be hydroxylated by the enzyme in significant yields. The substitution of the methylene group at the γ-position by a sulfur atom still provides the necessary flexibility and hydrophobicity of the side chain. Neither (*S*)-2-aminoethyl-DL-homocysteine (one additional methylene group), nor L-ethionine and *S*-propargyl-L-cysteine, which are devoid of the ε-amino function present in lysine, was found to serve as a potent substrate/inhibitor of *r*IucD. The importance of the presence of an unmodified ε-amino group gains further support from the observation that neither ε-*N*-methyl-L-lysine nor ε-*N*-acetyl-L-lysine is capable of being hydroxylated by the enzyme. However, methylation of L-lysine at the α-amino function (A) allows the analog to promote NADPH-oxidation as well as to serve as a substrate of lysine : N^6-hydroxylase, although only 20% as effective as the preferred substrate. These observations show that a free, unmodified α-amino group is not absolutely necessary for substrate recognition by *r*IucD, whereas the modification of the ε-amino function leads to a total loss of the ability to serve as a substrate,

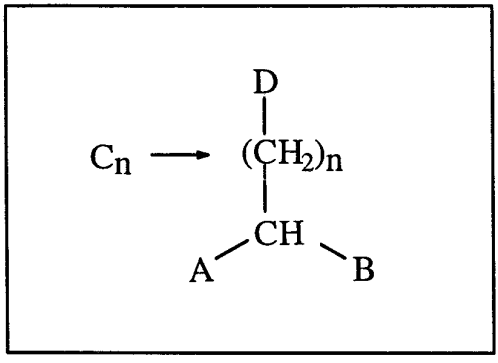

FIG. 1. Structural features inherent in L-lysine. A and D represent the positively charged amino group at the α and ε carbon, respectively. B symbolizes the negatively charged α-carboxyl function. C_n represents the hydrophobic side chain composed of four methylene groups ($n = 4$).

TABLE 1
Influence of Various Effectors on the Enzymatic Activity of *r*IucD

Effector[a]	Structural features[b] A	B	C_n	D	Lysine : N^6-hydroxylase activity (%)[c] +L-Lysine	−L-Lysine
None					100	0
(*S*)-2-Aminoethyl-L-cysteine	NH_3^+	CO_2^-	4[d]	NH_3^+	105	100
α-*N*-Methyl-L-lysine	$NH_2^+CH_3$	CO_2^-	4	NH_3^+	103	21
D-Lysine	NH_3^+	CO_2^-	4	NH_3^+	100	0
ε-*N*-Methyl-L-lysine	NH_3^+	CO_2^-	4	$NH_2^+CH_3$	93	0
ε-*N*-Acetyl-L-lysine	NH_3^+	CO_2^-	4	$NHCOCH_3$	100	0
DL-*α*-Aminocaprylic acid	NH_3^+	CO_2^-	4	CH_3	100 (2 mM)	0
L-Ethionine	NH_3^+	CO_2^-	4[e]	CH_3	85	nd
trans-4,5-Dehydro-L-lysine	NH_3^+	CO_2^-	4[f]	NH_3^+	100	0
6-Aminocaproic acid	H	CO_2^-	4	NH_3^+	100	0
L-Lysineamide	NH_3^+	$CONH_2$	4	NH_3^+	100 (2 mM)	0
L-Lysinemethylester	NH_3^+	CO_2CH_3	4	NH_3^+	100	0
1,6-Diaminohexane	NH_3^+	H	4	NH_3^+	100	0
(*S*)-Aminoethyl-DL-homocysteine	NH_3^+	CO_2^-	5[b]	NH_3^+	80	0
α,*ε*-Diaminopimelic acid	NH_3^+	CO_2^-	3	$CH(NH_3^+)(CO_2^-)$	95	0
L-Ornithine	NH_3^+	CO_2^-	3	NH_3^+	100	0
L-Arginine	NH_3^+	CO_2^-	3	$NHC(NH_2)_2^+$	100	0
S-Propargyl-L-cysteine	NH_3^+	CO_2^-	3[a]	C≡CH	100	nd
2,4-Diaminobutyric acid	NH_3^+	CO_2^-	2	NH_3^+	100 (2 mM)	0

[a] All compounds listed below were purchased from Sigma Chem. Co., except *α*-*N*-methyl-L-lysine, trans-4,5-dehydro-L-lysine, (*S*)-aminoethyl-DL-homocysteine, and *S*-propargyl-L-cysteine (see Materials and Methods).
[b] The structural features of the effectors (A, B, C_n, D) are illustrated in Fig. 1. The subscript *n* indicates the number of methylene groups present in the hydrophobic side chain of these compounds.
[c] The enzymatic activity of *r*IucD was determined as described under Materials and Methods. The concentration of the effectors was 1 mM unless further specified.
[d] $C_\gamma H_2$ replaced by sulfur.
[e] $C_\delta H_2$ replaced by sulfur.
[f] $C_\gamma H_2 - C_\delta H_2$ replaced by $C_\gamma H = C_\delta H$ (*trans*-conformation).

suggesting the detrimental nature of any type of modification at this location. Even the presence of a carboxyl group in the vicinity of the *ε*-amino group, as in the case of *α*,*ε*-diaminopimelic acid, renders the compound incapable of interacting with the enzyme.

Alteration in the site B of the substrate, either by deletion of the carboxy group (as in 1,6-diaminohexane) or by its modification (as in lysine amide or lysine methylester), results in a total loss of the ability to serve as a substrate.

The importance of the flexible hydrophobic backbone consisting of four methylene groups is documented by the observation that analogs with identical structural features (A, B, and D) but a change in the number of methylene groups in the side chain (as in L-2,4-diaminobutyric acid, L-ornithine, and (*S*)-2-aminoethyl-DL-homocysteine) do not serve as substrates/inhibitors of *r*IucD to any significant extent. Hence, a C_4 chain length appears to be essential for a compound to function as a substrate, with a replacement of the methylene group at the *γ*-position by sulfur being tolerated ((*S*)-2-aminoethyl-L-cysteine). Furthermore, a decrease in the flexibility of the hydrophobic side chain, as in the case of *trans*-4,5-dehydro-L-

lysine, leads to the total loss of the ability both to promote NADPH-oxidation and to undergo hydroxylation. Moreover, none of the other compounds (benzamidine, *p*-aminobenzamidine, and tranexamic acid) was utilized as a substrate by lysine : N^6-hydroxylase, providing further support for the essential role of a flexible hydrophobic backbone. It is pertinent to note that the small extent of inhibition observed in a few instances would appear to fall in the range of the experimental error of the assays, based on either lysine : N^6-hydroxlation or NADPH-oxidation.

L-Norleucine, a compound characterized by its C_4 hydrophobic side chain, was found to be the best effector of *r*IucD, inhibiting both NADPH-oxidation and lysine : N^6-hydroxylation. L-Norleucine analogs, such as L-α-aminobutyric acid, L-norvaline, and DL-α-aminocaprylic acid, which differ in the number of methylene groups present in the hydrophobic side chain, failed to exert any adverse effect on the reaction mediated by *r*IucD (Tables 1 and 2), providing further evidence for the stringent specificity of the enzyme for a C_4 backbone.

N-Alkylamides of L-Norleucine and Analogs

Since a C_4 hydrophobic side chain (C_n) plays a vital role in the binding of substrate(s) or effector(s) to lysine : N^6-hydroxylase, experiments were undertaken to determine if a provision of alkyl side chains on the carboxyl side of norleucine and its analogs would result in a promotion of their ability to serve as substrates/inhibitors of the enzyme. Hence, alkyl amides of norleucine, norvaline, and α-aminobutyric acid were synthesized, and their influence on *r*IucD-mediated lysine : N^6-hydroxylation was investigated. Since little can be found in the literature on the synthesis of methyl and ethylamides of hydrophobic amino acids, a few general comments are appropriate concerning the limitations and problems in achieving the synthesis of the *N*-alkyl amides prior to the discussion of their effect

TABLE 2
Influence of Norleucine and Its Analogs on the Catalytic Function of *r*IucD[a]

Effector	Relative activity of lysine : N^6-hydroxylation (%)	Relative activity of NADPH oxidation (%)
None	100	100
L(+)-Norleucine	50	18
L-Norvaline	100	100
L-α-Aminobutyric acid	100	100
DL-Norleucine	75	nd
L-Norleucine methylester	100	100
HAbuNHEt	87	82
HNvaNHMe	100	100
HNleNHMe	78	35
$HNleNH_2$	100	62

[a] The relative activity of lysine : N^6-hydroxylation and NADPH-oxidation was determined as described under Materials and Methods. The concentration of the desired compound was 1 mM in the case of lysine : N^6-hydroxylation, and 5 mM in NADPH-oxidation experiments.