Substrate Specificity of an Adenohypophyseal Endopeptidase Capable of Hydrolyzing Luteinizing Hormone-Releasing Hormone: Preferential Cleavage of Peptide Bonds Involving the Carboxyl Terminus of Hydrophobic and Basic Amino Acids†

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ABSTRACT: The substrate specificity of a peptidase from anterior pituitaries that is capable of hydrolyzing luteinizing hormone-releasing hormone (LH-RH; <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) at the Tyr^5–Gly^6 peptide bond has been investigated by using inhibitors and model substrates. While trypsin and chymotrypsin inhibitors from plants and animals are without any effect, many microbial protease inhibitors and synthetic peptides containing hydrophobic and basic amino acids inhibit the degradation of radiolabeled LH-RH by this enzyme. The model substrates *N*-acetyl-Phe-Gly-Leu-β-naphthylamide, *N*-acetyl-Leu-Gly-Leu-β-naphthylamide, and N^{α}-benzoyl-Arg-Gly-Leu-β-naphthylamide are hydrolyzed at the X-Gly peptide bonds; *N*-acetyl-Gly-Gly-Leu-β-naphthylamide is not degraded. Hydrolysis of typical amino- and carboxypeptidase substrates was not observed. Degradation of the general protease substrates insulin B chain and denatured hemoglobin also could not be detected. Thus, the enzyme is not LH-RH specific but may be characterized as an endopeptidase that hydrolyzes peptides preferentially at the carboxyl terminus of hydrophobic and basic amino acids.

The neuropeptide luteinizing hormone-releasing hormone (LH-RH; <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)[1] is hydrolyzed by various peptidases from different tissues [for review, see Marks (1978) and Griffiths & Kelly (1979)]. In anterior pituitaries, a pyroglutamate aminopeptidase, a post-proline-cleaving enzyme, and two endopeptidases cleaving internal peptide bonds of LH-RH have been identified (Horsthemke & Bauer, 1981). One of these endopeptidases has been characterized as a nonchymotrypsin-like enzyme that hydrolyzes LH-RH preferentially at the Tyr^5–Gly^6 peptide bond and the resulting N-terminal fragment at the His^2–Trp^3 peptide bond (Horsthemke & Bauer, 1980). The present study has been undertaken to investigate the substrate specificity of this enzyme.

Experimental Procedures

Materials

Whole bovine pituitaries were purchased from Pel Freeze. DE-52-cellulose was obtained from Whatman. Hypatite C was a product from Clarkson Chemical Co., phenyl-Sepharose CL-4B from Pharmacia Fine Chemicals, and Ultrogel AcA 44 from LKB. [*pyroglutamyl*-3,4-^{3}H]LH-RH (40 Ci/mmol) was purchased from New England Nuclear. For scintillation counting, emulsifier scintillator 299 from Packard was used. Hemoglobin and oxidized insulin B chain were obtained from Boehringer, Mannheim, West Germany. Chymostatin and leupeptin were purchased from Peninsula Laboratories; pepstatin, bacitracin, aprotinin, soybean trypsin inhibitor, pancreatic trypsin inhibitor, chicken egg white type IV 0 ovoinhibitor, ovomucoid, *N*-Ac-Phe, *N*-Ac-Leu, *N*-Ac-Gly, and Phe-NH_2 were from Sigma Chemical Co.; Gly, Phe, D-Phe, and silica gel F_{254} plates were from Merck AG, West Germany. Elastatinal, bestatin, and phosphoramidon were generous gifts of Dr. Hamao Umezawa, Microbial Chemistry Research Foundation, Tokyo, Japan. Antipain was kindly provided by Bayer AG, West Germany. All di- and tripeptides as well as N^{α}-Bz-Arg-Gly-Leu-2-NNap were purchased from Bachem AG, Bubendorf, Switzerland.

Methods

Enzyme Purification. The endopeptidase was prepared from bovine anterior pituitaries by a modification of the published procedure (Horsthemke & Bauer, 1980). After ammonium sulfate fractionation (35–65% saturation) and ion-exchange chromatography on DEAE-cellulose (0–120 mM KCl in 1.2 L of 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithioerythritol), the enzyme solution was diluted 4-fold with water containing 1 mM dithioerythritol in order to decrease the phosphate concentration. The solution was then applied to a hypatite C column, which was eluted with 800 mL of a linear gradient (5–75 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithioerythritol). The eluate containing the endopeptidase was mixed with saturated ammonium sulfate solution (20% saturation) and subjected to hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (340 mL of a linear ammonium sulfate gradient in 2.5 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithioerythritol; 20–0% saturation). For concentration of the enzyme solution for gel filtration, it was mixed again with saturated ammonium sulfate solution (20% saturation), applied to a small phenyl-Sepharose CL-4B column, and eluted with 2.5 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithioerythritol. Gel filtration was then performed on Ultrogel AcA 44 in 50 mM potassium phosphate buffer, pH 8.0, containing 0.5 mM dithioerythritol. This modified procedure results in a higher yield of enzyme activity, because it avoids

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[1] Abbreviations: LH-RH, luteinizing hormone-releasing hormone; <Glu, pyroglutamic acid; Suc, succinyl; Ac, acetyl; Bz, benzoyl; 2-NNap, β-naphthylamide. All other abbreviations used follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature. Unless otherwise stated, all optically active amino acids are of the L configuration.

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dialysis or ultrafiltration steps where the adsorption loss is high.

Degradation of [^{3}H]LH-RH and Its Inhibition by Various Amino Acids and Peptides. The test for the enzymatic degradation of [^{3}H]LH-RH has been described previously (Horsthemke & Bauer, 1980). In the present study 100 mM potassium phosphate buffer, pH 8.0, containing 0.5 mM dithioerythritol was used as incubation medium. For determination of the concentrations of the substances leading to 50% inhibition of the rate of degradation of [^{3}H]LH-RH by the endopeptidase (IC_{50}), various amounts of the substances were included in the incubation mixture. Since the degradation of [^{3}H]LH-RH exhibits first-order kinetics, the rate constants were calculated from the equation $k_1 = (1/t)$ ln [100/(100 − H)], where t is the time in minutes and H is the percent hydrolysis at time t.

Synthesis of Peptide Derivatives. The synthesis of Gly-Leu-2-NNap and Phe-Gly-Leu-2-NNap has been described previously (Horsthemke & Bauer, 1980). *N*-Ac-Phe-Gly-Leu-2-NNap, *N*-Ac-Leu-Gly-Leu-2-NNap, and *N*-Ac-Gly-Gly-Leu-2-NNap were synthesized as follows: *N*-Ac-Phe, *N*-Ac-Leu, and *N*-Ac-Gly were coupled with Gly-Leu-2-NNap in dimethylformamide by the *N*,*N*′-dicyclohexylcarbodiimide–1-hydroxybenzotriazole method (Houben & Weyl, 1974). The products were purified by chromatography on silica gel columns with $CHCl_3$–CH_3OH (9:1 v/v) as eluant: *N*-Ac-Phe-Gly-Leu-2-NNap, R_f 0.67 and mp 235–237 °C; *N*-Ac-Leu-Gly-Leu-2-NNap, R_f 0.61 and mp 254–256 °C; *N*-Ac-Gly-Gly-Leu-2-NNap, R_f 0.20 and mp 219–221 °C.

Degradation of Oxidized Insulin B Chain. Oxidized insulin B chain (5 mg) was incubated with 1 mL of the enzyme preparation at 37 °C for 8 h. After the pH was adjusted to 3.0 with acetic acid, the reaction mixture was subjected to ion-exchange chromatography on an Aminex Q-15S column using pyridine/acetate buffers as described (Bauer et al., 1979). For continuous detection of peptides, about 7% of the effluent was separated by stream splitting with the aid of a sampling valve. This separated sample was then treated with *o*-phthaldialdehyde at pH 9.0.

Degradation of [^{14}C]Hemoglobin. Hemoglobin was radioactively labeled as described (Roth et al., 1971; specific activity 0.2 μCi/mg of protein). An aliquot of this preparation (0.2 μCi) was incubated with 200 μL of the enzyme solution in 50 mM potassium phosphate buffer, pH 8.0, containing 0.5 mM dithioerythritol (final volume 1 mL). After 0, 3, and 5 h, aliquots of 200 μL were withdrawn from the reaction mixture and mixed with 400 μL of a 1% bovine serum albumin solution and then with 700 μL of a 10% trichloroacetic acid solution. After 12 h at 4 °C, the samples were centrifuged for 20 min at 4000*g*. Samples of 400 μL of the supernatants were mixed with 10 mL of scintillation cocktail and counted for radioactivity.

Results

For elucidation of the structural elements involved in the binding of inhibitors and substrates to the adenohypophyseal endopeptidase, the relative enzyme-binding affinities of naturally occurring protease inhibitors and synthetic model peptides have been estimated from the inhibitory potency of these substances (IC_{50} values) regarding the degradation of radiolabeled LH-RH by this enzyme.

When aprotinin from lung, soybean trypsin inhibitor, pancreatic trypsin inhibitor, chicken ovoinhibitor, and ovomucoid were included in the [^{3}H]LH-RH degradation assay (up to 1 mg/mL final concentration), we observed inhibition only for the trypsin inhibitor from pancreas (IC_{50} = 13 μg/mL). Since this inhibitor is identical with aprotinin (Anderer & Hörnle, 1966) which has no effect, the inhibition is probably due to an impurity contained in the pancreatic preparation. In contrast to these proteins, all the microbial peptide derivatives listed in Table I inhibit the degradation of [^{3}H]LH-RH by the peptidase, with chymostatin being the most potent inhibitor.

Table I: Effect of Microbial Protease Inhibitors on the Enzymatic Activity[a]

substance[b]	IC_{50} (μM)	substance[b]	IC_{50} (μM)
chymostatin	4	bacitracin	50
pepstatin	18	elastatinal	70
leupeptin	26	bestatin	111
antipain	33	phosphoramidon	479

[a] The enzyme was preincubated with the substances at 0 °C for 10 min prior to addition of [^{3}H]LH-RH. The degradation of the substrate and IC_{50} values have been determined as described under Methods. [b] Chymostatin, *N*-[(1-carboxy-2-phenylethyl)carbamoyl]-α-(2-iminohexahydro-4-pyrimidyl)-Gly-Leu-phenylalaninal; pepstatin, isovaleryl-Val-Val-4-amino-3-hydroxy-6-methylheptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid; leupeptin, *N*-Ac-Leu-Leu-arginal; antipain, (1-carboxy-2-phenylethyl)carbamoyl-Arg-Val-arginal; bacitracin, ┌Ile-Cys-Leu-D-Glu-Ile-Lys-D-Orn-Ile-D-Phe-His-D-Asp-Asn┐; elastatinal, *N*-[(1-carboxyisopentyl)carbamoyl)]-α-(2-iminohexahydro-4-pyrimidyl)-Gly-Gln-alaninal; bestatin, 3-amino-2-hydroxy-4-phenylbutanoyl-Leu; phosphoramidon, *N*-[(α-L-rhamnopyranosyloxy)hydroxyphosphinyl]-Leu-Trp.

Table II: Effect of Amino Acids and Peptides of Different Size on the Enzymatic Activity[a]

substance	IC_{50} (mM)
<Glu-His-Trp-Ser-Tyr↓-Gly-Leu-Arg-Pro-Gly-NH_2	0.19
Gly	>20
Phe	4.5
D-Phe	>20
Ac-Phe	1.2
Gly-Phe	2.6
Gly-Phe-Gly	9.0
Phe-NH_2	3.4
Phe-Gly	19
Phe-Gly-NH_2	2.4
Phe-Gly-Gly	1.8

[a] The enzyme was preincubated with the substances at 0 °C for 10 min prior to addition of [^{3}H]LH-RH. The degradation of the substrate and the IC_{50} values have been determined as described under Methods. The arrow indicates the primary cleavage site of LH-RH by the peptidase.

Unlabeled LH-RH inhibits the degradation of [^{3}H]LH-RH at an IC_{50} value of 0.19 mM (=K_M). To investigate which of the two amino acids adjacent to the primary cleavage site (Tyr^5 or Gly^6) is recognized by the peptidase and whether the enzyme has also exopeptidasic activity, we have tested the inhibition of the [^{3}H]LH-RH degradation by glycine, phenylalanine (which is more readily soluble than tyrosine), and phenylalanine derivatives (Table II). While no inhibition was observed for glycine, phenylalanine has an IC_{50} value of 4.5 mM. The D enantiomer of phenylalanine, however, is inactive. N-Acylation of phenylalanine increases the inhibitory potency, whereas Phe-Gly and Gly-Phe-Gly are much less potent than phenylalanine or Phe-Gly-Gly.

When tripeptides in which phenylalanine was substituted for other amino acids (Table III) were used, it was found that Leu-Gly-Gly and Lys-Gly-Gly are even more effective in inhibiting the degradation of [^{3}H]LH-RH than Phe-Gly-Gly. When the aliphatic amino acids glycine, alanine, valine, and

Table III: Effect of Tripeptides on the Enzymatic Activity[a]

substance	IC_{50} (mM)	substance	IC_{50} (mM)
Leu-Gly-Gly	1.2	Ala-Gly-Gly	8.0
Lys-Gly-Gly	1.4	Gln-Gly-Gly	9.0
Met-Gly-Gly	1.6	His-Gly-Gly	18
Phe-Gly-Gly	1.8	Pro-Gly-Gly	>20
Tyr-Gly-Gly	2.0	Gly-Gly-Gly	>20
Trp-Gly-Gly	3.4	Ser-Gly-Gly	>20
Val-Gly-Gly	3.8	Thr-Gly-Gly	>20
Arg-Gly-Gly	7.0	Glu-Gly-Gly	>20

[a] The enzyme was preincubated with the substances at 0 °C for 10 min prior to addition of [^{3}H]LH-RH. The degradation of the substrate and the IC_{50} values have been determined as described under Methods.

leucine are considered, it is obvious that the inhibitory potency increases with hydrophobicity. Arginine exhibits an affinity between alanine and valine. No inhibition was observed for acidic and neutral hydrophilic amino acids.

To determine whether peptides containing hydrophobic and basic amino acids not only inhibit the degradation of [^{3}H]-LH-RH but also are hydrolyzed by the enzyme, we have investigated the degradation of some model substrates. The tripeptides used in the inhibition tests proved to be not suitable for comparative degradation studies, because they are not hydrolyzed by the enzyme (see Table IV, lines 1 and 2). In a previous report (Horsthemke & Bauer, 1980), we had shown that *N*-Suc-Tyr-Gly-Leu-2-NNap and *N*-Suc-Phe-Gly-Leu-2-NNap are substrates for the peptidase. Therefore, we have synthesized tripeptide derivatives of the general structure (*N*-acyl-) X-Gly-Leu-2-NNap, with X being a neutral hydrophilic, an aromatic, an aliphatic, and a basic amino acid (Table IV). After incubation of these peptides with the enzyme, the reaction mixtures were resolved by thin-layer chromatography on silica gel plates, and the fragments were identified by cochromatography of marker substances (detection limit 50 pmol). While degradation of Phe-Gly-Leu-2-NNap and *N*-acetyl-Gly-Gly-Leu-2-NNap could not be detected, *N*-acetyl-Phe-Gly-Leu-2-NNap, *N*-acetyl-Leu-Gly-Leu-2-NNap, and N^{α}-benzoyl-Arg-Gly-Leu-2-NNap were shown to be hydrolyzed exclusively at the X–Gly peptide bonds. As estimated from the intensity of the spots on the chromatograms, the amount of fragments generated from these three substrates was in the same order of magnitude. Since the oxidized insulin B chain and denatured hemoglobin are generally used as protease substrates, we have also tested these proteins. However, even after long periods of incubation, no fragments could be detected.

Discussion

From anterior pituitaries a peptidase had been extracted that hydrolyzes LH-RH preferentially at the Tyr^5–Gly^6 peptide bond (Horsthemke & Bauer, 1980). It differs from pancreatic chymotrypsin by its higher molecular weight (M_r 83 000) and sensitivity toward functional reagents. Furthermore, it cleaves the LH-RH (1–5) pentapeptide at the His^2–Trp^3 peptide bond but does not hydrolyze the Trp^3–Ser^4 peptide bond of LH-RH or the fluorogenic chymotrypsin substrate glutaryl-Gly-Gly-Phe-2-NNap. As shown in the present study, chymotrypsin and trypsin inhibitors from plants and animals do not affect the adenohypophyseal peptidase, but it is strongly inhibited by chymostatin, a chymotrypsin inhibitor found in actinomycetes. Other microbial protease inhibitors, however, are also effective. These substances are small peptide derivatives containing mainly hydrophobic structures and, in some cases,

Table IV: Degradation of Model Peptides[a]

substrate and cleavage site	identified fragments	R_f
Phe-Gly-Gly	(no hydrolysis)	
Gly-Phe-Gly	(no hydrolysis	
Phe-Gly-Leu-2-NNap	(no hydrolysis)	0.68
↓		
N-Ac-Phe-Gly-Leu-2-NNap		0.66
↓	Gly-Leu-2-NNap	0.49
N-Ac-Leu-Gly-Leu-2-NNap		0.57
↓	Gly-Leu-2-NNap	0.49
N^{α}-Bz-Arg-Gly-Leu-2-NNap		0.00
	Gly-Leu-2-NNap	0.49
N-Ac-Gly-Gly-Leu-2-NNap	(no hydrolysis)	0.18

[a] Phe-Gly-Gly and Gly-Phe-Gly (1 mg each) were incubated with 100 μL of the enzyme preparation at 37 °C for 8 h. The reaction mixtures were then analyzed with the aid of a Durrum D-500 amino acid analyzer. The degradation of the naphthylamide derivatives was studied by thin-layer chromatography. Aliquots of the enzyme preparation (100 μL) were incubated at 37 °C with 0.01 mM substrate in 25 mM potassium phosphate buffer, pH 8.0, containing 0.5 mM dithioerythritol (final volume 200 μL). After 4 h, the reaction mixtures were concentrated in vacuo to 20 μL and spotted on silica gel F_{254} plates. β-Naphthylamine, Leu-2-NNap, Gly-Leu-2-NNap, and not incubated reaction mixtures were used as markers. After development (ascending front 6 cm) in $CHCl_3$–CH_3OH–NH_3 aqueous (90:10:1 v/v/v), the plates were dried. β-Naphthylamine and β-naphthylamides were localized by viewing under UV light. R_f (β-naphthylamine) 0.95; R_f (Leu-2-NNap) 0.87. The arrows indicate the sites of cleavage.

aldehyde groups (see footnote to Table I). While these aldehyde groups are essential functionalities for the inhibition of several proteases (Umezawa, 1976), this possibly does not apply for the adenohypophyseal peptidase, because pepstatin, for example, lacks any aldehyde group. Therefore it seems likely that hydrophobic interactions might be involved in the binding of these inhibitors to the enzyme.

Comparing the inhibitory potencies of several amino acids and synthetic model peptides, it was found that the enzyme stereospecifically recognizes hydrophobic and basic amino acids. Aromatic interactions are possibly not involved in the binding of peptides to the enzyme, because the affinity of phenylalanine, tyrosine, and tryptophan is lower than the affinity of leucine. Concerning the basic amino acids, it may be speculated that the binding of these amino acids is due to the long aliphatic chain between the C_α atom of lysine and arginine and the ω-amino and guanidino group. In agreement with the high enzyme-binding affinities of hydrophobic and basic amino acids, we have found selective hydrolysis of model substrates at the carboxyl terminus of these amino acids. A similar cleavage specificity has been reported for a peptidase designated tonin, which cleaves β-lipotropin and corticotropin at specific arginine and phenylalanine residues (Seidah et al., 1979). It differs, however, from our peptidase by its low molecular weight (M_r 28 700) and its sensitivity toward diisopropyl fluorophosphate (Seidah et al., 1978).

The fact that the enzyme-binding affinity of *N*-acyl-Phe is higher than the affinity of phenylalanine indicated that the adenohypophyseal peptidase might not act as an aminopeptidase. Likewise, the low affinity of Phe-Gly compared to phenylalanine and Phe-Gly-Gly suggested that the enzyme has no carboxypeptidasic activity. These notions are substantiated by the finding that the exopeptidase substrates Gly-Phe-Gly and Phe-Gly-Gly are not hydrolyzed by the enzyme.

Considering these results, the enzyme may be characterized as an endopeptidase that hydrolyzes peptide bonds involving the carboxyl terminus of hydrophobic and basic amino acids.

Despite this broad specificity, substrates are hydrolyzed only at selective peptide bonds. This selectivity may be due to the influence of neighboring groups or to the conformation of the substrate. For example, hydrolysis of LH-RH at the Trp^3–Ser^4 and Leu^7–Arg^8 peptide bonds is not observed, and as shown for various LH-RH analogues (Horsthemke et al., 1981), the rate of hydrolysis of a given peptide bond is strongly influenced by modifications of the substrate even at positions remote from the scissile peptide bond. In addition, we have found that the LH-RH (1–9) nonapeptide is degraded 50 times more rapidly than LH-RH itself (data not shown). Further studies are necessary to characterize the determinants for such effects, which are typical for an endopeptidase with an extended substrate-binding region.

Although the enzyme was discovered when the degradation of LH-RH was investigated, it is obviously not an LH-RH specific peptidase. Therefore, its possible physiological function as a neuropeptide-degrading enzyme warrants further investigations. The determination of the cleavage specificity presented in this report may help to synthesize enzyme-specific substrates and inhibitors suitable for further studies.

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Cytochrome *c* Specific Methylase from Wheat Germ†

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ABSTRACT: The cytochromes *c* of plants (e.g., wheat germ) possess two trimethyllysines, residues 72 and 86. In order to investigate the nature of these methylations, we have purified a cytochrome *c* specific methylase *S*-adenosylmethionine: protein(lysine) *N*-methyltransferase (protein methylase III) from wheat germ 135-fold. The in vitro site of methylation by both the purified enzyme and crude wheat germ extract toward various forms of horse heart cytochrome *c* was localized by two-dimensional peptide mapping, Aminex A-5 column peptide analysis, and CNBr cleavage analysis to be the residue 72 lysine. However, no additional sites, in particular residue 86, were seen to be methylated. Although the enzyme is highly specific toward cytochrome *c* as an in vitro protein substrate, avian cytochromes *c* are seen to be much better substrates than those from mammalian sources. The enzyme possesses an extremely low K_m for apocytochrome *c* (1.21 μM), suggesting that methylation may occur before heme attachment in vivo. Various *S*-adenosyl-L-homocysteine analogues were tested for their inhibitory capability toward the enzyme; it was observed that only the D and L forms of *S*-adenosylhomocysteine are inhibitors while analogues modified in the adenine or homocysteine moieties do not possess inhibitory capability. Results from the Aminex A-5 column chromatography of horse heart cytochrome *c* chymotryptic digest showed the N^{ϵ}-methyl-, N^{ϵ}-dimethyl-, and N^{ϵ}-trimethyllysine forms of the residue 68–74 peptide to elute earlier than the unmethylated form. This result suggests that the methylated peptides are less basic than the unmethylated form.

The N^{ϵ}-methylated lysines have been shown to occur in a wide variety of proteins from many species of organisms (Paik & Kim, 1971, 1975, 1980). In particular, the cytochromes *c* of fungal, plant, and protozoan sources contain residues of trimethyllysine while animal cytochromes *c* lack this amino acid (Paik & Kim, 1980).

Much work has been done in seeking to understand the nature and function of cytochrome *c* methylation, particularly in fungal organisms. The methylases that catalyze the reaction were purified from two fungal sources, *Neurospora crassa* and baker's yeast, and were found to be highly specific in regard to their exclusive recognition of cytochrome *c* as a substrate (Durban et al., 1978; DiMaria et al., 1979). In addition, they exhibit a further specificity in that they recognize only the residue 72 lysine as a site of methylation when horse heart cytochrome *c* is used as a substrate (DiMaria et al., 1979). This observation correlates well with the in vivo occurrence of trimethyllysine at exclusively the residue 72 in the cytochromes *c* of *N. crassa* and baker's yeast (DeLange et al., 1969, 1970).

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