MSH-Release-Inhibiting Factor: Inactivation by Proteolytic Enzymes[1] (36528)

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It was proposed recently that an enzymically controlled degradation of oxytocin in the hypothalamus (1) liberates the terminal tripeptide prolyl-leucylgycinamide, and that this tripeptide is the inhibiting hormone or "factor" (referred to as MSH-R-IF) for the release from the pituitary of the melanocyte-stimulating hormone (2, 3). In subsequent studies Nair, Kastin and Schally (4) isolated this tripeptide from bovine hypothalamic extracts having MSH-R-IF activity. The same workers also investigated the digestion over an extended period of time of a MSH-R-IF concentrate by a number of known exo- and endopeptidases. Inactivation of MSH-R-IF was determined by bioassay, however, and no information was provided regarding the nature of the susceptible peptide bonds.

In the present study, using synthetic MSH-R-IF as substrate, we compare the activity rates and relative specificities of known peptidyl hydrolases as inactivators of the inhibiting factor, and also investigate the effects on MSH-R-IF of crude enzyme preparations from the brain and median eminence of the rat.

Materials and Methods. The crystalline tripeptide, Pro-Leu-Gly-NH_2, used in this study was the same as that employed previously (3). The enzymes were obtained as follows: basic arylamidase (with Arg-β NA as substrate) from rat brain (5); leucineaminopeptidase (LAP, EC 3.4.1.1, from pig heart; P-L Biochemicals, Inc.); aminopeptidase M (from pig heart; Rohm and Haas Co.); thermolysin (from B. thermoproteolyticus, three times recrystallized; Calbiochem); carboxypeptidases A and B (EC 3.4.2.1 and 2, from bovine pancreas; Nutritional Biochemicals Corp., and Sigma Chemical Co.); collagenase (EC 3.4.4.19 from *Clostridium histolyticum*; Schwarz/Mann) and chymotrypsin (EC 3.4.4.5, from bovine pancreas; Schwarz/Mann).

Brain extract was prepared by homogenization of 1 vol freshly dissected tissue from adult male rats in 10 vol of 0.32 *M* sucrose; the homogenate was centrifuged twice at 14,000*g* to remove debris and subcellular particulates, and the resulting supernatant was then centrifuged at 100,000*g* for 30 min to remove the microsomal fraction from the preparation. Median eminence extract was prepared by homogenization of fresh tissue from 10 adult male rats (approx 10 mg of tissue) in 1 ml of 0.32 *M* sucrose; the homogenate was centrifuged at 30,000*g* for 1 hr and the supernatant used.

The incubation mixture consisted of 5 μg of enzyme or tissue protein (unless otherwise stated), and 0.15 mmole of MSH-R-IF dissolved in 0.5 ml of Tris–HCl buffer (10 m*M*, pH 7.6). After incubation at 37° for the designated periods of time, the enzymic digests were stored frozen until each sample was subjected to quantitative amino acid analysis in order to determine the micromoles of Pro, Leu and Gly. Glycinamide was not determined since the ammonia peak interfered on the basic column, but each digestion was tested for the possible release of Gly. When the short-column procedure described earlier (6) was used, Pro eluted 95 min after

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TABLE I. Hydrolysis of Synthetic MSH-Release-Inhibiting Factor by Proteolytic Enzymes.[a]

Enzyme	Incubation time (hr)	Amino acid released (μmoles)			% Breakdown (in terms of Pro liberated)
		Pro	Leu	Gly	
LAP[b] (EC 3.4.1.1)	1	0.06	0.08	0	40
	4	0.14	0.14	0	94
	24	0.14	0.14	Tr	94
	1[c]	—	0.05[c]	—	33[c]
Aminopeptidase M	1	0	0	0	0
	4	0	0	0	0
	24	0.09	0.11	Tr	60
Thermolysin	1	0.04	0.03	0	28
	4	0.08	0.14	0	53
	24	0.13	0.14	0	87
Collagenase (EC 3.4.4.19)	24	0	0	0	0
Carboxypeptidase A (EC 3.4.2.1)	24	0	0	0	0
Carboxypeptidase B (EC 3.4.2.2)	24	0	0	0	0
Chymotrypsin (EC 3.4.4.5)	4	0	0	0	0
Brain arylamidase (Arg-βNA)	2	0.13	0.13	0	90
Brain extract	4	0.12	0.10	0	80
Median eminence extract	4	0.045	0.04	0	30

[a] Results are the mean of 3 experiments: values agreed within 10%. Incubation mixtures contained 0.15 mmole MSH-R-IF, and 5 μg of enzyme or tissue protein. Control samples of the purified enzymes or tissue extracts were incubated in the absence of substrate, and the values given are adjusted accordingly; Tr = trace; — = not determined.

[b] LAP = leucineaminopeptidase.

[c] Incubation with 0.15 μmoles of Leu-NH_2 as substrate.

the start of the chromatogram; the corresponding times for Gly and Leu were 127 and 200 min, respectively. In all cases, appropriate controls were run, using the tissue extracts or purified enzymes incubated under identical conditions but in the absence of substrate, and values given in the data tables are adjusted accordingly. MSH-R-IF itself is known to be stable under these conditions.

Results and Discussion. The tripeptide MSH-R-IF was inactivated by leucineaminopeptidase, aminopeptidase M and thermolysin, confirming the observations by Nair, Kastin and Schally (4). We found that brain arylamidases also degraded MSH-R-IF, but that collagenase, carboxypeptidases A and B and chymotrypsin failed to cleave the tripeptide hormone. It had been observed earlier, in connection with another investigation (7), that chymotrypsin fails to degrade Pro-Leu-Gly-NH_2.

Several interesting points can be made regarding this data from an enzymological point of view. Surprisingly, 40% of the MSH-R-IF was degraded by leucineaminopeptidase within the first hour, and over 90% within 4 hr as determined by the release of Pro (Table I). Leu-NH_2 has generally been regarded as the most common substrate for LAP, although a number of di- and tripeptides (in some cases with COOH-terminal amide substitutions) are hydrolyzed at even higher rates (8, 9). Conversely, the amide bond of a proline residue has been considered relatively stable, although very little specific information on the relative susceptibility to cleavage

of this bond by LAP can be found in the literature. It has been reported that Pro-NH_2 is hydrolyzed by LAP at 1/100 the rate of Leu-NH_2 (8). The rapid cleavage of the Pro-Leu peptide bond in MSH-R-IF in the present study suggests that certain prolyl oligopeptides are excellent substrates for LAP. In fact, the rate of hydrolysis of MSH-R-IF is comparable to that of Leu-NH_2 (Table I).

Aminopeptidase M, prepared from sources similar to those of LAP, can be differentiated from LAP by an increased capacity to cleave the peptide bond at the carboxyl side of alanine residues when alanine is N-terminal (8, 10). As might be expected, hydrolysis of MSH-R-IF by this enzyme was much slower than by LAP, with no detectable metabolites after 1- or 4-hr incubation times. With prolonged incubation, 60% inactivation occurred, and approximately equimolar levels of Pro and Leu, and some free Gly appeared (Table I). It has been reported that Pro, Leu and Gly nitroanilides are poor substrates for aminopeptidase M (8, 10), but no data are available on the relative rate of hydrolysis of tripeptidyl amides.

Thermolysin is an example of a neutral proteinase, and is reported to be specific for peptide bonds where the amino acid contributing the peptide nitrogen has a hydrophobic side chain (11). There are no reported studies on the hydrolysis of tripeptidyl amides with free N-terminal groups by this enzyme; most studies using thermolysin hydrolysis have been done on protected NH_2- terminal di- and tripeptides. In our experiments, incubation of thermolysin with MSH-R-IF led to the appearance of Pro and Leu, though at a less rapid rate than with LAP and without the appearance of any free Gly (Table I). Since most dipeptidyl amides with free N-terminal groups resist hydrolysis by this enzyme and by related neutral proteinases of bacterial origin (11, 12), evidently the specificity of thermolysin for degrading dipeptides does not apply to tripeptides.

Collagenase is another example of a neutral proteinase, but one which has specificity for undenatured collagen and for some prolyl-containing peptides. The present study shows that Pro-Leu-Gly-NH_2 is completely resistant to this enzyme. The absence of any breakdown of MSH-R-IF is consistent with the known specificity of collagenase for the peptide Pro-Leu-Gly while the polymer of this tripeptide, $(Pro\text{-}Leu\text{-}Gly)_m$, is hydrolyzed by collagenase to give H·Gly-Pro-Leu-OH (13).

Basic arylamidase degraded MSH-R-IF by 90% within 2 hr. We recently reported inactivation by this enzyme of angiotensin-II via stepwise release of its N-terminal residues (6), and it is well known that brain arylamidase can hydrolyze N-acylated amino acids and dipeptidyl-arylamides (14). To our knowledge, however, no data were available until now regarding the susceptibility of tripeptidyl amides to cleavage by this enzyme.

Preliminary experiments indicate that extract supernatants of whole rat brain degrade MSH-R-IF more rapidly than comparable preparations of rat median eminence. This is of interest, since median eminence is presumed to be the storage site of MSH-R-IF. It is relevant to note that rat median eminence contains a spectrum of enzymes capable of forming MSH-R-IF from oxytocin (2, 3), as well as those involved in its subsequent inactivation. The sequence of formation and breakdown of MSH-R-IF in the living animal will of course depend on the subcellular location of the hormonal substrates relative to the enzymes. There is also some question of whether an activation process is required *in vivo* for the release of bound substrate or enzyme, before inactivation of the factor can occur.

Summary. Several peptidyl hydrolases with known specificity were tested for their capacity to inactivate the melanocyte-stimulating hormone-release-inhibiting factor (MSH-R-IF). Leucineaminopeptidase, aminopeptidase M, thermolysin, and brain arylamidase degraded this inhibiting hormone, but collagenase, the carboxypeptidases A and B, and chymotrypsin did not. Crude rat tissue extracts were also tested for enzymic activity. Brain extracts were highly active in degrading MSH-R-IF, whereas extracts of the median eminence, a storage site for the factor, were significantly less active.

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