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Significant Differences in the Degradation of Pro-Leu-Gly-NH_2 by Human Serum and that of Other Species[1] (38484)

RODERICH WALTER, A. NEIDLE, AND N. MARKS

Department of Physiology and Biophysics, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029; and New York State Research Institute for Neurochemistry and Drug Addiction, Ward's Island, New York 10035

The tripeptide, H-Pro-Leu-Gly-NH_2 (PLG) was reported by Nair *et al.* to be completely degraded within 1 hr upon incubation with human plasma (1, 2). The major degradation products were identified by these workers as Pro and Leu-Gly-NH_2, thus focusing on the Pro-Leu bond as the site of initial enzymic attack. Independent studies in our laboratory (3) revealed no detectable degradation ot the synthetic tripeptide by human plasma or serum under incubation conditions similar in terms of time, substrate concentration and temperature to those used by Nair *et al.* (2). On the other hand, blood from several other species released varying amounts of products.

Studies on the breakdown of H-Pro-Leu-Gly-NH_2 are thought to be of importance, since this tripeptide has been proposed by us to be enzymatically released from oxytocin by a hypothalamic enzyme system (4) and to be a natural factor which inhibits the release of pituitary melanocyte-stimulating hormone (4). Although confirmatory evidence was presented by Nair *et al.* (5), difficulties have been encountered by other laboratories in reproducing consistently or at all the MSH-release-inhibiting effects of PLG (6–9) and, hence, the identity of the hypothalamic factor needs further confirmation. Extrapituitary effects have been described for PLG including potentiation of the behavioral effects of levodopa, diminution of oxotremorine-induced tremor in mice, reversal of the sedative effects of deserpidine in mice and monkeys, antiparkinsonian activity in man (for summary see Ref. 10), and induction of stereotyped behavior and diminution of locomotor activity in cats (11). Also, H-Pro-Leu-Gly-NH_2 has been subject to conformational investigation by proton NMR (12), carbon-13 NMR (13), X-ray crystallography (14) and conformational energy calculations (15) and the results are in excellent agreement with a compact conformation capable of a β-turn formation.

In view of the differences between the results of Nair *et al.* and those of our own preliminary study on PLG inactivation, we have repeated and extended our investigation of the degradation of this tripeptide by serum of human, rat, chicken, and carp. Moreover, the products released from PLG in serum have been identified. In addition, in light of our earlier studies on the degradation of PLG by rat brain extract, and rat brain arylamidase (16), we compared the type of enzymic activity present in rat serum with that in a rat brain preparation.

Materials and Methods. Substrates and inhibitor. Crystalline H-Pro-Leu-Gly-$NH_2 \cdot \frac{1}{2}H_2O$ and H-Leu-Gly-NH_2 were the same as those described in an earlier study (17). H-Pro-Leu-OH was purchased from Fox Chemical Co. Leucine-, lysine-, and proline-β-naphthylamides were obtained from Cyclo Chemical Co., and puromycin from Nutritional Biochemical Co.

Incubation of peptides with serum. Plasma or serum was prepared from blood samples collected from human, rat, chicken, or carp in the presence or absence of heparin and was used immediately. Protein concentration was determined according to the procedure of Lowry *et al.* (18) using bovine serum albumin as standard. In routine experiments, 50 μg ($\sim$170 nmole) H-Pro-Leu-Gly-NH_2 was dissolved in 0.2 ml 10 m*M* Tris–HCl buffer, pH 7.6, and was added to 0.1–0.2 ml of serum to give a final concentration of 0.4–0.6 m*M* substrate. In some experiments, the concentration of tripeptide was lowered to 0.02 m*M*. Incubations were carried out in

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duplicate at 37° with human, rat and chicken serum, and at room temperature with carp serum, for various lengths of time ranging from 10 min to 24 hr. The reaction was stopped by addition of water to a vol of 1.0 ml followed by precipitation of protein with 30 mg of sulfosalicylic acid and centrifugation at 2000 *g* for 20 min at room temperature. Two sets of controls were used: serum of all species was incubated at the appropriate temperature in the absence ot tripeptide, and tripeptide alone was incubated in Tris buffer. Incubation of the dipeptides H-Leu-Gly-NH_2 or H-Pro-Leu-OH with human or rat serum was carried out in a similar manner to incubation with PLG. H-Leu-Gly-NH_2 (40 μg, ~200 nmole) or H-Pro-Leu-OH (55 μg, ~200 nmole) were dissolved in 0.2 ml Tris–HCl buffer, pH 7.6, and incubated for 1 hr at 37° with 0.6 ml rat or human serum (final substrate concentration 0.25 m*M*). Products in the deproteinized mixture were identified as described below for the tripeptide.

Identification of products. Aliquots of deproteinized supernatant of each control and experimental sample were subjected to quantitative amino acid analysis. The analysis method used was described previously (19) and can accurately detect 1.0 nmole of each amino acid. In brief, samples were applied to a single column (100 × 0.6 cm) of BioRad A6 resin and eluted with the lithium citrate buffer system of Perry *et al.* (20). In this system the following relevant amino acids and peptides are separable and detectable: Pro, Leu, H-Gly-NH_2, Gly, H-Leu-Gly-NH_2. Buffers were passed through a column (15 × 2.0 cm) of Pierce DC-3 resin before use to eliminate ammonia and allow good resolution of the glycinamide peak. Increased sensitivity was gained by using two single-channel Technicon colorimeters with linear output with respect to optical density, one set at 440 nm (for proline detection) and the other at 570 nm. The ninhydrin color yields (compared to norleucine, used as an internal standard) of H-Pro-Leu-Gly-NH_2 and H-Pro-Leu-OH were too low for accurate determination.

Preparation of rat brain homogenate. Rat brain homogenate was prepared as previously described (17). Brain tissue was obtained from young Wistar albino rats of both sexes and homogenized in an Aldrich-type homogenizer for 1 min at 100 rpm in 10 vol of 0.32 *M* sucrose, keeping the temperature at 0–4°.

Measurement of arylamidase activity. Arylamidase activity in rat brain homogenate or in serum was measured using a fluorometric procedure described previously (21), based on the release of β-naphthylamine from the substrates Leu-, Lys-, or Pro-β-naphthylamide. In general, serum containing 10–100 μg protein (in 10 μl), or brain homogenate containing 10 μg protein (in 10 μl), was added to a solution of substrate (10^{-3}, 10^{-4}, or 10^{-5} *M*) in 5 m*M* Tris buffer (pH 7.6) containing 0.5 m*M* dithiothreitol (DTT). The fluorescence of the reaction mixture was read continuously until it stabilized, in a Turner Model III fluorimeter kept at 37°. When puromycin was tested as an inhibitor, it was added to the incubation mixture as a solid, to give a final concentration of 10^{-3} *M*.

Results and Discussion. The rate at which synthetic H-Pro-Leu-Gly-NH_2 was enzymatically degraded by serum, determined on the basis of H-Gly-NH_2 released, varied markedly among the species studied (Table I). The tripeptide was not degraded when incubated with human serum (male, female, or pregnant female) for 1 hr at 37° at a concentration of 125–165 μg/ml (0.4–0.6 m*M*), in contrast to a previous report of 100% breakdown under similar conditions (i.e., 37° for 1 hr at a concentration of 100 μg/ml in human plasma) (1, 2). Some breakdown of PLG is observed after 24 hr incubation (Table I). In line, Redding *et al.* (22) report that even after 24 hr, 70% of the 25% of total radioactivity of urinary PLG excreted by men after injection of labeled PLG is in the form of the intact tripeptide. Lowering the concentration of tripeptide in our study to 0.02 m*M* did not affect the results. In addition, we found no differences in the inactivation of PLG caused by human serum or plasma, as was also the case for all other species tested (results not shown).

When hydrolysis of PLG by serum of other species was measured it was found that, in rat, cleavage was extremely rapid, with 100% of H-Gly-NH_2 released in 1 hr,

TABLE I. DEGRADATION OF H-PRO-LEU-GLY-NH_2 BY SERUM FROM VARIOUS SPECIES.[a]

Species	Incubation time (hr)	Amino acid released in nmoles			Percent breakdown (based on H-Gly-NH_2)
		Pro	Leu	H-Gly-NH_2	
Human					
Female	1	0	0	0	0
	24	12	12	12	7
Pregnant Female	1	0	0	0	0
	24	7	8	8	4
Male	1	0	0	0	0
	24	24	32	28	16
Rat	10 min	20	24	25	15
	1	110	168	170	100
	24	—	—	170	100
Chicken	1	7	9	7	4
	24	116	114	105	60
Carp	1	48	52	—	30
	24	165	165	—	97

[a] Values are the average of three experiments using sera from three different individuals, and results agreed within 10%. All amino acid analyses were run in duplicate. Serum (0.1–0.2 ml) and 170 nmole H-Pro-Leu-Gly-NH_2 were incubated at 37° for the time indicated and products were identified by amino acid analysis (see text for detail). Percent breakdown is based on amount of Gly-NH_2 released (since previous results (3) indicated that no breakdown of this compound occurred) except in the case of carp, where Pro was used.

and 15% released in only 10 min. In the case of rat, lowering the substrate concentration to 0.02 m*M* did affect the rate of cleavage, and approximately 60% of Gly-NH_2 was released after 1 hr; apparently at this concentration the enzyme reaction was not at maximum velocity. Chicken serum was found to cleave PLG at a slower rate, releasing 4% of H-Gly-NH_2 after 1 hr. Among the cold-blooded animals, carp serum released 30% of H-Gly-NH_2 in 1 hr. The inactivation of H-Pro-Leu-Gly-NH_2 by frog serum has been reported previously (23); however degradation was determined on the basis of bioassay results and no products were identified. Since protein content of all serum samples was similar, it is unlikely that it affected the cleavage rates.

Thus, it is apparent that the levels of the specific enzyme(s) responsible for PLG degradation in serum vary among species. From the very small number of species studied to date, it is interesting to note that the serum of a species with a well-defined *pars intermedia* (rat) can rapidly inactivate PLG, while serum of a species in which the *pars intermedia* fails to develop (bird) or regresses during the individual's lifetime (human) does not inactivate this regulatory factor. A tentative explanation has been advanced for the relationship of the anatomical differences between species and the ability of their blood to inactivate PLG (3). This correlation may, however, be fortuitous.

Elucidation of the mechanism of enzymatic inactivation of PLG has received some attention. Degradation products of ^{14}C-PLG were identified, upon incubation with human plasma, as Pro and ^{14}C-Leu-Gly-NH_2 (1, 2).[2] However, we in no case observed the formation or accumulation of H-Leu-Gly-

[2] Accumulation of labeled H-Leu-Gly-NH_2 in the pituitary and pineal glands during *in vivo* distribution studies was also reported (24, 25). Possibly these metabolites are formed in the glands after PLG has reached the tissue or, conversely, these metabolites, formed in the blood, are selectively sequestered by the pituitary and pineal glands.

NH_2, although our incubation conditions were similar to those used in the earlier study (i.e., 37°, 1 hr, 125 μg/ml compared to 100 μg/ml), the amino acid analysis system is as sensitive as the radioactive TLC method (i.e., 1.0 nmole of amino acid is detectable, or less than 1% cleavage) and resolution is excellent. In order to investigate further the mechanism of PLG degradation in serum, we incubated both rat and human serum with H-Leu-Gly-NH_2. As shown in Table II, the breakdown of this peptide was slower in human than in rat serum, but was still highly significant, with 40% degraded by human serum and 90% by rat serum in 1 hr under our conditions. On the basis of these results it is difficult to explain the reported finding that, after *total* breakdown of PLG by human plasma, the only products are Pro and H-Leu-Gly-NH_2 (1, 2); according to our findings, H-Leu-Gly-NH_2 would be degraded rapidly.

When H-Pro-Leu-OH was incubated with both rat and human serum (Table II) the Pro-Leu bond was hydrolyzed in each species at a rate similar to that found for hydrolysis of the H-Leu-Gly-NH_2 bond. The rate-limiting step in cleavage of PLG in rat serum cannot be determined on the basis of these results, nor on the basis of analysis of the breakdown of the tripeptide, since neither free H-Gly-NH_2 nor Pro was observed in the absence of the other amino acids after incubation of H-Pro-Leu-Gly-NH_2 with rat serum. Therefore, we regard the mechanism of PLG breakdown in rat serum as unresolved. The difficulty we encounter in identifying the initial cleavage site of PLG is explained by the rapid cleavage of the transient dipeptide intermediate(s) formed.

TABLE II. HYDROLYSIS OF H-PRO-LEU-OH AND H-LEU-GLY-NH_2 BY SERUM OF RAT AND HUMAN.[a]

	Amino acids released from H-Leu-Gly-NH_2 (nmole)		Percent breakdown
	Leu	H-Gly-NH_2	
Human	82	82	40
Rat	182	180	90
	Amino acids released from H-Pro-Leu-OH (nmole)		
	Pro	Leu	
Human	66	65	33
Rat	210	200	100

[a] The dipeptides (200 nmole in 0.2 ml Tris-HCl buffer, pH 7.6) were incubated at 37° for 1 hr with 0.6 ml serum. Products were identified by amino acid analysis as described in the text. Results are the average of two experiments in duplicate agreeing within 10%.

It appears reasonable to postulate that in brain tissue, in contrast to plasma and serum, the rate-limiting step in H-Pro-Leu-Gly-NH_2 cleavage may be hydrolysis of the Pro-Leu bond. In experiments in which various oxytocin analogs were incubated with a membrane-bound hypothalamic enzyme which can produce MSH-release-inhibiting activity from oxytocin (4), and which is thought to be the responsible enzyme for this reaction *in vivo*, [4-proline]oxytocin failed to produce MSH-release-inhibiting activity, suggesting that the Pro-X bond was only slowly or not at all cleaved by the enzyme. The resistance to cleavage of the Pro-X bond would also explain the accumulation of H-Pro-Leu-Gly-NH_2 in hypothalamic extracts (26).

In addition to the hypothalamic enzyme responsible for PLG formation, there are several brain enzymes which can inactivate the tripeptide, among them a Mn^{2+}-stimulated bovine brain aminopeptidase (27), a pig brain arylamidase (17), and a rat brain arylamidase (16). It was of interest to compare the arylamidase(s) in rat brain with those of rat serum. Homogenate of whole rat brain, as well as rat serum, were incubated with the typical arylamidase substrates, Leu- and Lys-β-naphthylamide, as well as with Pro-β-naphthylamide. The results are shown in Table III; arylamidase activity is apparently present at levels several hundredfold less in serum than in rat brain, although the amounts of total protein are comparable. A characteristic feature of the brain arylamidase is its inhibition by high substrate concentrations. This inhibition by high concentrations of Leu- and Lys-β-naphthylamide is not seen in the rat serum enzyme. Furthermore, hydrolysis of H-Pro-Leu-Gly-NH_2 by serum was unaffected in the present study by 10^{-3} *M* puromycin, a

TABLE III. DEGRADATION OF AMINO ACID NAPHTHYLAMIDES BY RAT AND HUMAN SERA AND RAT BRAIN HOMOGENATES.[a]

Substrate	Concentration (M)	β-Naphthylamine released (nmoles/min/mg protein)		
		Human serum	Rat serum	Rat brain homogenate
Pro-β-naphthylamide	1×10^{-3}	tr.	0.8	95
	1×10^{-4}	0	0	31
	1×10^{-5}	0	0	11
Leu-β-naphthylamide	1×10^{-3}	0.37	0.87	150
	1×10^{-4}	0.26	0.65	260
	1×10^{-5}	0.40	0.28	260
Lys-β-naphthylamide	1×10^{-3}	0.90	0.50	322
	1×10^{-4}	0.80	0.50	365
	1×10^{-5}	0.90	0.32	365

[a] Incubation mixture contained 10–100 μg of serum protein, depending on the substrate used, or 10 μg of brain homogenate protein. Substrate concentration was 1, 0.1, or 0.01 m*M* in 5 m*M* Tris buffer, pH 7.6, containing 0.5 m*M* DTT. Product was determined fluorometrically as previously described (21). Results are the average of three experiments using sera from three different individuals; rat serum and brain homogenate of the same animal were compared. Values agreed within 10%.

concentration which specifically blocks the action of the brain arylamidase (28). Therefore, the arylamidase in rat serum is different from the arylamidase in rat brain which inactivates PLG.

Summary. The acyclic C-terminal tripeptide of oxytocin, H-Pro-Leu-Gly-NH_2, is not degraded upon incubation with human (male, female or pregnant female) plasma or serum for 1 hr at 37°. However, the sera of other species tested, including rat, chicken and carp, degrade this tripeptide 100%, 4% and 30%, respectively, in 1 hr, as determined by quantitative amino acid analysis of released products. Among the species studied there seems to exist a correlation between the anatomic development of the *pars intermedia* and the ability of the serum to hydrolyze H-Pro-Leu-Gly-NH_2, which has been proposed to be a MSH-release-inhibiting factor. The only identified degradation products are Pro, Leu and H-Gly-NH_2, with no detectable levels of H-Leu-Gly-NH_2. The dipeptides H-Leu-Gly-NH_2 and H-Pro-Leu-OH are each cleaved at similar rates in either human or rat serum, although the rate of hydrolysis of both peptides is lower in human than in rat. Thus, it does not appear that the dipeptide, H-Leu-Gly-NH_2, can accumulate as one of the breakdown products of the tripeptide. The arylamidase present in rat serum has different characteristics from the enzyme in rat brain which can degrade H-Pro-Leu-Gly-NH_2.

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