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Glycine-directed peptide amidation: Presence in rat brain of two enzymes that convert p-Glu-His-Pro-Gly-OH into p-Glu-His-Pro-NH_2 (thyrotropin-releasing hormone)

(thyrotropin-releasing hormone biosynthesis/post-translational processing of prohormones)

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ABSTRACT **To study the possibility of glycine-directed amidation in rat brain, we synthesized the substrate p-Glu-His-Pro-Gly-OH. Adult and neonatal rat brain and adult rat pituitary were sonicated, frozen and thawed, and fractionated by gel permeation chromatography, and fractions from each tissue were assayed for enzymatic activity capable of converting this model substrate into thyrotropin-releasing hormone. We report the presence in rat brain and rat pituitary of two enzymes catalyzing conversion of p-Glu-His-Pro-Gly-OH into thyrotropin-releasing hormone. Based on the differing chemical and physical properties of these two enzymes and their differing affinities for a number of p-Glu-His-Pro-aa-OH analogs (in which aa = glycine, β-alanine, γ-butyric acid, and δ-aminovaleric acid), we conclude that there are two distinct enzymatic processes for the terminal amidation of peptides in brain and that COOH-terminal extensions other than glycine are capable of directing COOH-terminal amidation.**

The majority of physiological secretory polypeptides are COOH-terminal amides. Because all protein synthesis within mammalian cells is ribosomal (glutathione being the single known exception), secretory polypeptides are generally believed to be derived from the enzymatic, post-translational processing in the endoplasmic reticulum of considerably larger precursors (1–5). Based on these considerations and other circumstantial evidence (6–10), several authors have postulated that a COOH-terminal glycine may be a recognition "sequence" leading to enzymatic amidation of the daughter polypeptide prior to release. Recently, Bradbury *et al.* (11) have discovered, and others (12) have confirmed, the presence of a glycine-directed amidase in neurosecretory granules prepared from porcine pituitary.

Despite the fact that many neurotransmitter polypeptides in the central nervous system are COOH-terminal amides, glycine-directed COOH-terminal amidation has never been demonstrated in brain. The purpose of this study, therefore, was to determine whether the model peptide p-Glu-His-Pro-Gly-OH could be converted into p-Glu-His-Pro-NH_2 (thyrotropin-releasing hormone; TRH) by proteins present in rat brain. This substrate was chosen because it provided the dual opportunity to search for glycine-directed amidases in brain and to study the possible biosynthetic pathway for TRH, which is currently unknown. Our successful studies form the basis of this report.

METHODS

Chemicals. γ-Aminobutyryl-His-Pro-NH_2 (GABA-His-Pro-NH_2) was synthesized as described (13, 14).

p-Glu-His-Pro-NH_2 (TRH) was purchased from Peninsula Laboratories (San Carlos, CA), and its structure was verified by mass spectrometry (15).

Pyroglutamylhistidylprolyl amino acid analogs (p-Glu-His-Pro-aa-OH): Pyroglutamylhistidine methyl ester (p-Glu-His-OCH_3) was prepared essentially by the methods of Folkers and co-workers (16) and Geiger *et al.* (17) and purified by silica gel chromatography using 20% anhydrous methanol and chloroform as the eluate. p-Glu-His-OCH_3 was saponified in ice-cold 0.1 M NaOH for 5–8 min, the pH was adjusted to 3.9 (theoretical isoelectric point), and the sample was freeze-dried and used without further purification.

Pro-Gly-OCH_3 HCl, Pro-β-Ala-OCH HCl, Pro-GABA-OCH_3 HCl, or Pro-δ-AVa-OCH_3 HCl (δ-AVa, δ-aminovaleric acid) were prepared and coupled to p-Glu-His-OH using methods detailed elsewhere (13). After saponification of the methyl esters, p-Glu-His-Pro-Gly-OH, p-Glu-His-Pro-β-Ala-OH, p-Glu-His-Pro-GABA-OH, and p-Glu-His-Pro-δ-AVa-OH were purified by silica gel column chromatography using acetone/38% acetic acid (60:40).

Analytical. *Mass spectrometry.* Mass fragmentation spectra of solid probe samples were obtained using electron impact ionization in a Finnegan 3300 GC-MS.

Amino acid analysis. Peptides were hydrolyzed in 4 M methane sulfonic acid by the method of Blackburn (18) followed by amino acid analysis on an Aminex HPC (Bio-Rad) column by the post-column derivatization method of Voelter and Zech (19). Alternatively, samples for amino acid analysis by gas chromatography were hydrolyzed in constant boiling HCl and analyzed according to the method of Gehrke and Stalling (20).

Thin layer chromatography. TLC was carried out on silica gel plates using the following three solvent systems: (*i*) solvent system A, methanol/methylene chloride (40:60); (*ii*) solvent system B, methanol/chloroform/38% acetic acid (40:60:20); (*iii*) solvent system C, methanol/chloroform/concentrated ammonium hydroxide (60:40:20). Where appropriate, TLC plates were developed by iodine vapor or by spraying with an aqueous solution of ninhydrin or the Pauly reagent. Sample loads were 10–20 μg for each peptide.

TRH radioimmunoassay. Antibodies were raised to a bovine serum albumin-GABA-His-Pro-NH_2 conjugate by immunization of New Zealand White rabbits by the method of Vitakaitis *et al.* (21). Antibodies developed against this immunogen have a high affinity for TRH (K_a, 2.4×10^{-9} M), do not crossreact with any other substances present in rat brain (K_a for p-Glu-His-Pro-COOH is 4.8×10^{-4} M, and K_a for His-Pro-diketopiperazine is 4×10^{-4} M), and have a crossreactivity for p-Glu-His-Pro-Gly-OH of <0.0005% (14). The lack of affinity of this antiserum for the p-Glu-His-Pro-Gly-OH, when compared to the affinity for this tetrapeptide of antiserum generated by standard techniques, allows the

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Abbreviations: TRH, thyrotropin-releasing hormone; GABA, γ-aminobutyric acid.

accurate measurement of TRH despite the presence of significant quantities of p-Glu-His-Pro-Gly-OH. This RIA is presented in detail elsewhere (14, 22).

Sample preparation. Neonatal rats (Sprague–Dawley 8–10 days old) were decapitated, their brains were removed, and the cerebellum was separated. The tissues were then sonicated in ice-cold 0.05 M Tris·HCl buffer (pH 7.0) followed by one cycle of freeze-thawing. The sonicate was centrifuged at 30,000 × *g* for 30 min at 4°C, and the clear supernatant was applied to a 100 × 3.5 cm column of Sephadex G-100 previously equilibrated with 200 mM sodium chloride/0.05 M Tris buffer, pH 6.5. Fractions of 3.5 ml were collected and assayed for the presence of enzymatic activity capable of converting p-Glu-His-Pro-Gly-OH or other analogs into TRH as described below. The brains and pituitaries from adult rats were collected, processed, and chromatographed in a similar manner. Where indicated, subcellular fractionation was carried out on sucrose gradients by the method of DeRobertis *et al.* (23).

Analysis for the Enzymatic Conversion of p-Glu-His-Pro-aa-OH into TRH. Aliquots (250 μl) from the fractionation of neonatal or adult rat brain or adult rat pituitaries were incubated at 37°C for 5 hr in the presence of 5 μg of p-Glu-His-Pro-Gly-OH (final concentration, 0.05 mM). Where indicated, *N*-ethylmaleimide was added to a final concentration of 0.5 mM. After identification of peaks of enzymatic activity, substrate affinities were determined by adding various concentrations of each of the model substrates. Control incubations contained all ingredients but were frozen immediately instead of incubated. After incubation, the samples were frozen, lyophilized, and extracted with 2.5 ml of absolute methanol. The methanolic extracts were centrifuged, the supernatants were transferred to clean test tubes, and the contents were dried under a stream of nitrogen. The samples were then dissolved in 1 ml of 0.05 M pyridine and applied to 7 × 0.5 cm columns of Dowex 1 (anion exchange resin), which had been extensively washed and previously equilibrated with 0.05 M pyridine. The columns were washed with a further 2.5 ml of 0.05 M pyridine and the eluate was lyophilized. The dried samples were then dissolved in 2 ml of absolute methanol, and the samples were removed for either product verification by TLC or directly for RIA. The overall recovery for internal standards carried through the extraction procedure is between 90% and 95%. The extraction procedure over Dowex also removes >98% of the p-Glu-His-Pro-aa-OH from the final RIA sample.

In other experiments, samples of the fractionation of rat brain and pituitaries were subjected to boiling before incubation.

Verification of Product. To verify the identity of the enzymatic product, samples of the final methanolic extracts were applied to silica gel plates and eluted either with solvent system B or C. After development, the TLC plates were dried, divided into 12 fractions, and each fraction was extracted with absolute methanol. After methanolic extraction, the samples were centrifuged and the supernatant was removed, dried, and assayed for TRH by the RIA described above. Internal standards were carried through the exact same procedures.

RESULTS

After purification, all of the p-Glu-His-Pro-aa-OH analogs chromatographed as single, Pauly-positive, ninhydrin-negative spots on silica gel thin-layer plates using solvent system B. The amino acid composition (molar ratios of amino acid residues) and R_f for each peptide analog are as follows:

(*i*) p-Glu-His-Pro-Gly-OH: R_f, 0.4; Glu (expected, 1.00; observed, 0.98), His (1.00; 0.92), Pro (1.00; 1.14), Gly (1.00; 1.00);

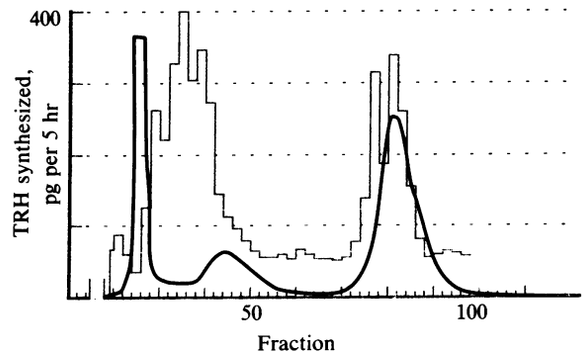

FIG. 1. Formation of TRH from p-Glu-His-Pro-Gly-OH by fractions of adult rat pituitary chromatographed on Sephadex G-100. Solid line in this and other figures represents absorption at 254 nm.

(*ii*) p-His-Glu-Pro-β-Ala-OH: R_f, 0.56; Glu (1.00; 0.93), His (1.00; 1.00), Pro (1.00; 0.95), β-Ala (1.00; 0.98);

(*iii*) p-Glu-His-Pro-GABA-OH: R_f, 0.73; Glu (1.00; 1.07), His (1.00; 1.00), GABA (1.00; 1.01);

(*iv*) p-Glu-His-Pro-δ-Val-OH: R_f, 0.69; Glu (1.00; 0.91), His (1.00; 1.00), Pro (1.00; 1.10), δ-Val (1.00; 1.03).

Incubation of p-Glu-His-Pro-Gly-OH with fractions of rat pituitary obtained after gel chromatography demonstrated two peaks of enzymatic activity capable of converting this tetrapeptide into p-Glu-His-Pro-NH_2 (TRH). The first of these proteins had an apparent molecular size between 60 and 70 kDa (as determined by calibration with standard proteins of known molecular size), whereas the second peak eluted after the salt peak, implying that it was either considerably smaller or was retarded by the polysaccharide matrix of the Sephadex (Fig. 1). Incubation of fractions of rat brains with p-Glu-His-Pro-Gly-OH also gave two peaks of enzymatic activity, although this profile of activity varied slightly from that of rat pituitary. Whereas the second peak of enzymatic activity from rat brain appeared similar to that obtained from adult rat pituitary, the first peak of activity was considerably less and appeared to elute some fractions later than that observed for the pituitary enzymes (Fig. 2). Incubation of known amounts of TRH with each of the fractions obtained from rat brain gave a profile of TRH degradative activity as shown in Fig. 3. This degradative activity significantly overlaps the first peak of amidating activity and implies that the dissimilarity between the profiles of enzymatic activity obtained from rat pituitary and from rat brain is due to enzymatic destruction of TRH product and/or substrate. Based on our previous demonstration that the enzymes catabolizing TRH are dependent on functional sulfhydryl groups (24), fractions of rat brains were again assayed for their ability to convert p-Glu-His-Pro-Gly-OH into TRH

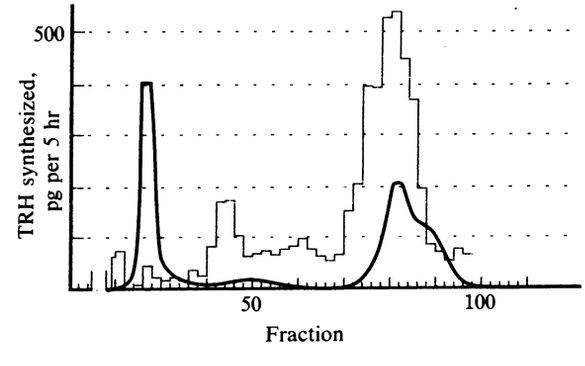

FIG. 2. Formation of TRH from p-Glu-His-Pro-Gly-OH by fractions of neonatal rat brain chromatographed on Sephadex G-100 and assayed in the absence of *N*-ethylmaleimide.

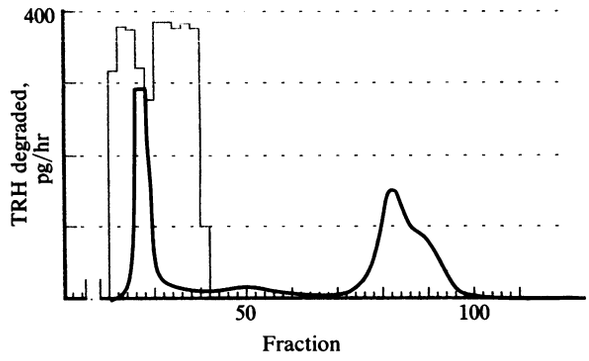

FIG. 3. Degradation of TRH by fractions of neonatal rat brain in the absence of *N*-ethylmaleimide. See legend to Fig. 2 for details.

with 0.5 mM *N*-ethylmaleimide added to the first 60 fractions (Fig. 4). Under these conditions, the majority of the degradative enzymes were inhibited, and the first peak of amidating activity in rat brain was greatly enhanced: its chromatographic profile now similar to that obtained for rat pituitary. Some degradative activity still persisted, however, in the very early chromatographic fractions, which could not be completely inhibited by the addition of *N*-ethylmaleimide. There was no inhibition of the first peak of glycine-directed amidase activity by 0.5 mM *N*-ethylmaleimide.

The authenticity of the product resulting from the incubation of p-Glu-His-Pro-Gly-OH with each of the protein peaks was verified by comparing its chromatographic properties to TRH standards on TLC using two different solvent systems (B and C). In both systems, the product from the two enzymatic reactions comigrated with standards of TRH (Figs. 5 and 6; data from solvent system C not shown). The conversion of p-Glu-His-Pro-Gly-OH into TRH by both the first and second peaks of enzymatic activity was readily inhibited by 5 min of boiling. Furthermore, in no case was TRH found in the fractions of either brains or pituitaries before incubation. This absence of TRH results from the active catabolism of TRH by degradative enzymes that are highly active even during preparation of tissue at 4°C (24).

In further studies, we compared the chemical and physical properties and the substrate specificities of the two enzyme peaks (Table 1). Although both enzymes are metalloproteins and have similar pH optima, the peak 1 enzyme appeared to have an absolute specificity for a COOH-terminal glycine, and its catalytic activity was not dependent on intact hydroxyl or sulfhydryl groups. The second peak of enzymatic activity, however, had a wider substrate specificity with p-Glu-His-Pro-β-Ala-OH having the highest affinity for the enzyme. The catalytic activity of this second enzyme is dependent on intact sulfhydryl and/or hydroxyl groups. Furthermore, the retardation of the peak 2 enzymatic activity by the polysaccharide matrix of Sephadex suggests that this enzyme is either considerably smaller than or more basic than peak 1.

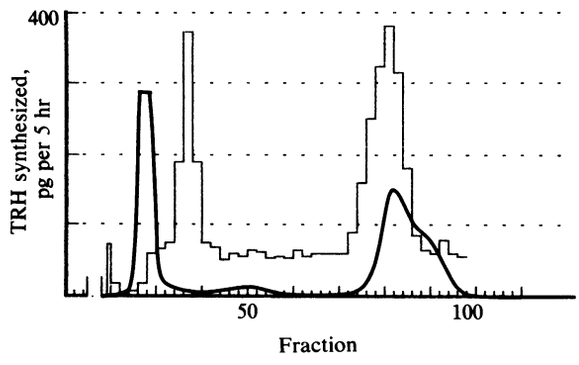

FIG. 4. Formation of TRH from p-Glu-His-Pro-Gly-OH by fractions of neonatal rat brain assayed in the presence of 0.5 mM *N*-ethylmaleimide. See legend to Fig. 2 for details.

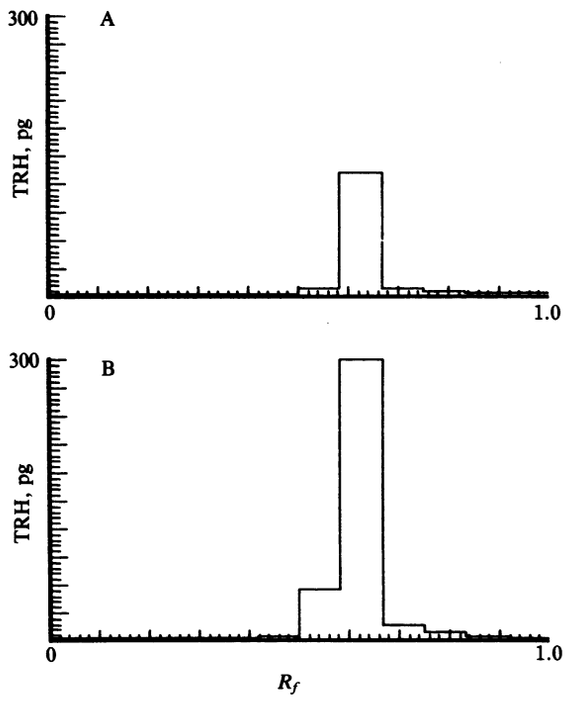

FIG. 5. (*A*) TLC (system B) of product from incubation of fractions 37–41 from neonatal rat brain with p-Glu-His-Pro-Gly-OH. (*B*) Same as *A*, but with added internal standard of authentic TRH.

DISCUSSION

In these studies, we have observed glycine-directed, COOH-terminal amidation of polypeptide sequences in rat brain.

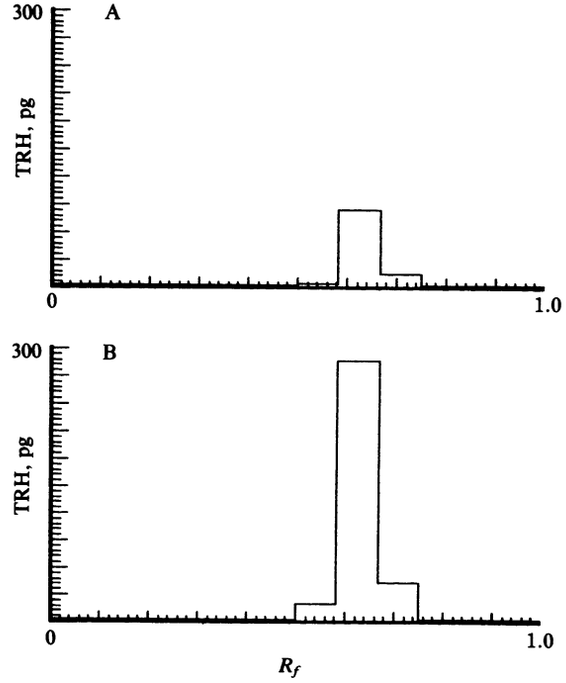

FIG. 6. (*A*) TLC (system B) of product from incubation of fractions 77–87 from neonatal rat brain with p-Glu-His-Pro-Gly-OH. (*B*) Same as *A*, but with added internal standard of authentic TRH.

Table 1. Substrate affinities and chemical and physical properties of brain glycine amidases

	Peak 1	Peak 2
Substrate, K_m		
p-Glu-His-Pro-Gly-OH	30 μM	100 μM
p-Glu-His-Pro-β-Ala-OH	—	50 μM
p-Glu-His-Pro-GABA-OH	—	560 μM
p-Glu-His-Pro-δ-AVa	—	1260 μM
pH optimum	5.9–6.0	6.2–6.3
$PhMeSO_2F$		
2 mM	No effect	Inhibits
10 mM	No effect	Inhibits
N-Ethylmaleimide		
2 mM	No effect	Inhibits
10 mM	Slight inhibition	Inhibits
EDTA		
2 mM	Inhibits	Stimulates
10 mM	Inhibits	± Inhibits
1,10-Phenanthroline		
2 mM	Inhibits	Inhibits
10 mM	Inhibits	Inhibits
Subcellular localization	70% soluble	
Neonatal	30% particulate	100% particulate
Adult	100% particulate	100% particulate

$PhMeSO_2F$, phenylmethylsulfonyl fluoride.

Rat brain and adult rat pituitary both contain two protein peaks capable of enzymatically converting p-Glu-His-Pro-Gly-OH into TRH. Although these observations may represent the initial demonstration of glycine-directed peptide amidation in brain, the original observation of glycine amidation is credited to Bradbury *et al.* (11). In an elegant study, these authors demonstrated the conversion of the model tripeptide, D-Tyr-Val-Gly-OH, to D-Tyr-Val-NH_2 by preparation of secretory granules from bovine pituitary. Although our studies can be viewed as confirming the findings of these authors, our results differ in two significant aspects: First, in both pituitary and brain we find two enzymes catalyzing COOH-terminal amidation, whereas these authors found only one in bovine pituitary. Second, our results demonstrate that COOH-terminal peptide extensions other than glycine may be capable of directing COOH-terminal amidation.

One reason for the apparent failure of the studies by Bradbury *et al.* (11) to detect a second peak of amidating activity may be that they partially purified their enzyme from a preparation of secretory granules, whereas our initial fractions were obtained from whole-cell sonicates. In recent studies, we have observed that during the pelleting of the membrane fraction in isotonic sucrose, the enzymatic activity of peak 2 is lost.

The importance of these observations lies in their furthering our understanding of the processing of precursor hormones into physiologically active daughter peptides. At present, general theory supposes that all hormones are synthesized ribosomally as part of a larger prohormone sequence (25). Synthesis of the prohormone is probably initiated by the synthesis of a 17–31 amino acid long "signal-sequence," which serves as a recognition site for the binding of the nascent prohormone and ribosomes to the endoplasmic reticulum (25). It is also theorized that on binding to the endoplasmic reticulum, the nascent hormone is inserted into the endoplasmic reticulum and the signal-sequence is split from the NH_2 terminus of the prohormone by a signal peptidase (25). Furthermore, processing is believed to take place by the action of "trypsin-like" activity, which fragments the prohormone at sites containing basic amino acid residues (1–5, 25). Subsequently, the basic amino acids are removed, and hormones containing a free COOH-terminus are then ready for release. Until recently, however, the mechanism of COOH-terminal amidation was not understood even though structural analysis of several prohormones suggested the presence of a COOH-terminal glycine extension of the future daughter hormone. Thus, the observations of Bradbury *et al.* (11) and the observations of this report suggest the presence of enzymes in pituitary and brain that are capable of carrying out this important COOH-terminal modification of secretory peptides and hormones. We further predict that future structural analyses of prohormones will discover COOH-terminal extensions of the daughter peptide other than glycine.

The enzyme of Bradbury *et al.* (our enzyme 1) amidates a variety of peptide–glycine substrates (11, 26). Although the mechanism of catalysis by this first enzyme has not been completely elucidated, the work of these authors suggests that the ultimate source for the nitrogen in the terminal carboxamide is glycine itself, the result of the enzymatic process being a COOH-terminally amidated peptide and glyoxylic acid. Based on our observations of differing substrate specificities and chemical properties for the two brain amidases, we suggest that the catalytic mechanisms of the two enzymes may be dissimilar.

It is important to note that, although we have demonstrated the conversion of a model tetrapeptide ending in glycine into TRH by enzymes present in rat brain and pituitary, these observations do not in themselves prove that this is the mechanism for the biosynthesis of TRH *in vivo*. Such a conclusion can only be derived from the demonstration that an endogenous protein, when incubated in the presence of one or the other of these enzymes, gives rise to TRH. Nevertheless, investigations of this possibility will enhance our understanding of the mechanism of the biosynthesis not only of TRH but also of a wide variety of other COOH-terminally amidated proteins.

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