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SUBSTRATE SPECIFICITY OF AN AMIDATING ENZYME IN PORCINE PITUITARY

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A series of tripeptides was synthesised and tested as substrates for an amidating enzyme present in porcine pituitary. The rates of conversion of the tripeptides to the corresponding dipeptide amides were determined by ion exchange chromatography of the radio-iodinated peptides. The experiments showed that the amidating enzyme has a mandatory requirement for glycine in position 3 of the tripeptide substrates; peptides containing lysine, glutamic acid, leucine or alanine in the C-terminal position did not undergo reaction. In studies of the substrate requirements at position 2 of the tripeptides, facile reaction took place with neutral amino acids in this position but much slower reactions occurred with basic or acidic residues. With the neutral substrates the enzyme exhibited an optimum pH value of 6.8; with histidine in position 2 the optimum reaction occurred at a higher pH, consistent with a preference shown by the enzyme for an uncharged amino acid in the penultimate position of the peptide substrate.

It has recently been shown that porcine and bovine pituitary contain an enzyme that can convert the synthetic substrate d-tyrosylvalylglycine to the corresponding dipeptide amide, d-tyrosylvaline amide(1). Of particular interest was the finding that the nitrogen of the amide was derived from the amino nitrogen of the glycine, which suggested that the nature of the C-terminal residue might be important for the activity of the enzyme. In order to investigate the specificity requirements of the amidating enzyme in more detail we have prepared a range of tripeptide substrates and examined their conversion to the corresponding dipeptide amides. In this communication we describe the results obtained with tripeptides in which the amino acid residues at positions 2 and 3 were varied. The results demonstrate that the

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amidating enzyre has a mandatory requirement for glycine in the C-terminal position of the substrate and a strong preference for a neutral amino acid in the penultimate position.

MATERIALS AND METHODS

The tripeptides, d-Tyr Val Gly, d-Tyr Val Ala, d-Tyr Val Glu, d-Tyr Val Lys, d-Tyr Phe Gly, d-Tyr Gly Gly, d-Tyr d-Ala Gly, d-Tyr Glu Gly, d-Tyr Lys Gly and d-Tyr His Gly, and the tetrapeptide d-Tyr Val Gly Lys, were synthesised by conventional solid phase methods(2). The peptides were purified by gel filtration on a column (30 x 1cm) of Sephadex G10 with 50% acetic acid (v/v) as eluent, followed by ion exchange chromatography on a column (30 x 1cm) of SP-Sephadex C25. The neutral tripeptides were eluted with 200mM sodium choride - 50mM sodium phosphate at pH 4.0; the acidic tripeptide, d-Tyr Glu Gly, was chromatographed at pH 3.0 with the same buffer; the basic tripeptides were eluted at pH 5.5 in 500mM phosphate buffered sodium chloride. The peptides were located in the column effluents by measurement of optical density at 280nm. The identity and purity of each peptide was confirmed by amino acid analysis after acid hydrolysis. Radiolabelling of the peptides (10nmole) was carried out with ^{125}I (1mC) using chloramine T and the products were purified by chromatography on SP-Sephadex C25.

Amidation reactions were performed at 37°C by incubating the tripeptide (100µl, 10^5cpm) in 200mM sodium chloride 50mM sodium phosphate pH 7.0 in the presence of 10^{-4}M Cu (II) with enzyme (100µl) obtained by salt precipitation and gel filtration of a porcine pituitary extract(1). Aliquots (20µl) were removed at intervals and the reactions were terminated by addition of 1N-HCl (5µl). The solutions were stored at -20°C and analysed by chromatography on SP-Sephadex C25 (10 x 1cm). The neutral tripeptides were eluted in 200mM sodium chloride-50mM sodium phosphate at pH 5.5; the basic tripeptides were eluted in 500mM sodium chloride-50mM sodium phosphate at pH 5.0; the acidic peptides were eluted in 200mM buffer at pH 4.0 using a 20cm column. The degree of conversion of a tripeptide to the corresponding dipeptide amide was calculated from the proportion of the total radioactivity that appeared in the elution position of the appropriate radio-iodinated dipeptide amide.

RESULTS AND DISCUSSION

Incubation of ^{125}I d-tyrosylvalylglycine with amidating enzyme from porcine pituitary led to progressive conversion of the tripeptide to ^{125}I d-tyrosylvaline amide (Fig.1). Under the conditions employed, the reaction was essentially complete in 5 hours. When the glycine residue at position 3 of the tripeptide was replaced by glutamic acid, lysine or alanine, no conversion could be detected. Thus, on the basis of the

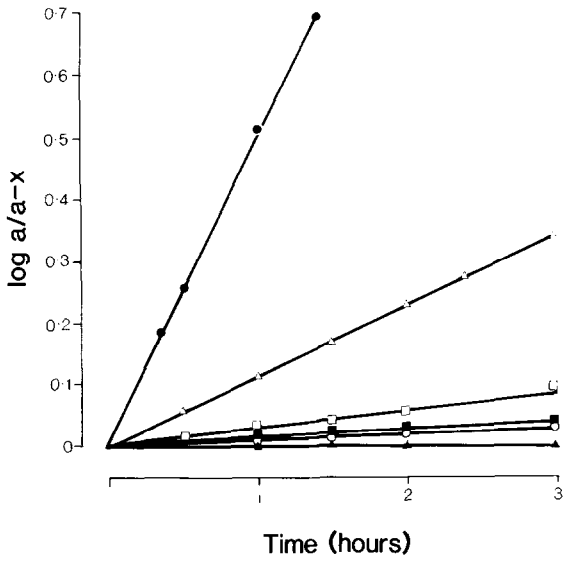

Figure 1. Rate of enzymic conversion of synthetic tripeptides to the corresponding dipeptide amides. △——△, d Tyr Val Gly; □——□, d Tyr Gly Gly; ●——●, d Tyr Phe Gly; ■——■, d Tyr Lys Gly; ○——○, d Tyr Glu Gly; ▲——▲, d Tyr Val Ala. Incubations were carried out at pH 7.0 with amidating enzyme from porcine pituitary as described in the text.

substrates tested, the amidation reaction exhibits a specific requirement for glycine in the C-terminal position. It was notable that the tetrapeptide d-Tyr Val Gly Lys did not undergo significant reaction, which suggests that the glycine carboxyl group is essential for recognition by the enzyme.

These findings with small synthetic substrates are consistent with the observation that the biosynthetic precursors of peptides that terminate in an α-amide group invariably contain glycine immediately adjacent to the peptide sequence (3-6). Moreover, the glycine residue is generally linked to consecutive basic residues which form favourable sites for proteolytic processing(7). In the elaboration of hormone amides *in vivo*, therefore, cleavage of the polypeptide chain of the prohormone followed by removal of the basic residues by a CpAse B-like enzyme leads to exposure of C-terminal glycine, which provides a substrate appropriate for amidation.

In experiments with tripeptide substrates in which position 2 was occupied by phenylalanine or glycine, similar rates of reaction were found to the rates observed with d-tyrosylvalyl-glycine (Fig.1); it can be seen that the phenylalanine containing substrate was the most reactive and the glycine containing substrate the least. This suggests that the pituitary enzyme would be capable of catalysing the formation of the α-amide group of gastrin, oxytocin and vasopressin which terminate in phenylalanine or glycine amide, in addition to being able to form the amide group of α-melanotropin which terminates in valine amide. When the neutral amino acid at position 2 was replaced by glutamic acid or lysine, however, relatively slow reactions were observed. With d-alanine in position 2, no reaction at all took place. When histidine was present in position 2 the amidation reaction proceeded readily at pH 7.0; at lower pH values, however, the reaction took place more slowly than it did with substrates that had neutral amino acids in this position (Fig.2). The optimum pH for conversion of the histidine containing substrate was 0.3 units higher than the optimum pH for the neutral substrates.

The results indicate that the pituitary enzyme would be able to catalyse the amidation of a wide variety of peptides to form the corresponding peptide amides, particularly when the residue undergoing amidation is a neutral amino acid. In this connection it may be significant that at weakly acid pH values the reaction rates observed with the histidine containing substrate were less than those observed with the neutral substrates, a phenomenon probably explained by protonation of the imidazole nitrogen. Furthermore it is notable that all naturally occurring hormone amides seem to contain a neutral amino acid in the C-terminal position.

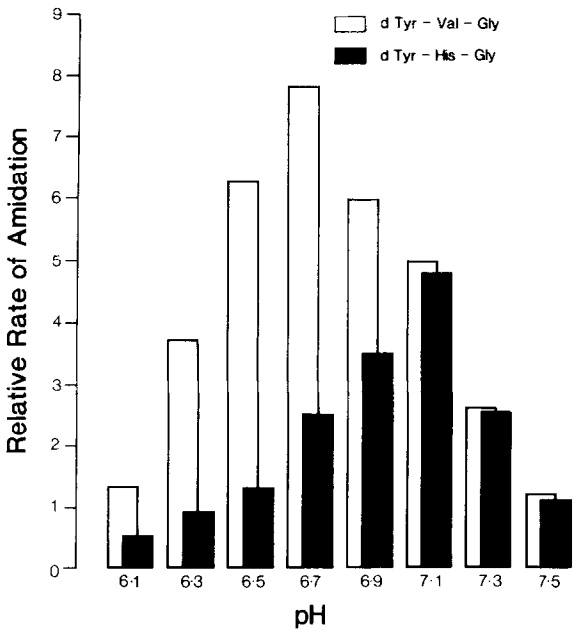

Figure 2. pH dependence of the enzymic conversion of d Tyr Val Gly to d Tyr Val $CONH_2$, and d Tyr His Gly to d Tyr His $CONH_2$.

The present experiments on amidation, taken in conjunction with the results of previous work on the isolation of prohormone fragments(8,9), support a general view of the sequence of reactions that take place during prohormone processing. The initital step appears to involve cleavage of the prohormone on the carboxyl side of consecutive basic residues; trimming of the resulting fragments by carboxypeptidases specific for basic amino acids would then account for the formation of most of the intracellular peptides that have been identified by extraction of tissues or pulse labelling of cells cultured in vitro. In the case where the C-terminal residue of a prohormone fragment is glycine, further reaction can take place which leads to replacement of the glycine by an α-amide group. The mechanism of this reaction involves dehydrogenation and hydrolysis of the glycine containing substrate. The data presented in this communication add to our knowledge of the amidation reaction by demonstrating some

characteristics of the amidating enzyme, particularly in respect of its mandatory requirement for C-terminal glycine and its marked preference for neutral amino acids in the penultimate position of the substrate.

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