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THE AMIDATING ENZYME IN PITUITARY WILL ACCEPT A PEPTIDE WITH C-TERMINAL D-ALANINE AS SUBSTRATE

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A series of tripeptides which terminated in d-alanine, d-serine, d-leucine or l-alanine was synthesized and the peptides tested for their ability to act as substrates for an amidating enzyme present in porcine pituitary. The peptides were allowed to compete with a radiolabelled substrate ^{125}I d-Tyr Phe Gly in the presence of a rate limiting concentration of amidating enzyme and the degree of conversion to ^{125}I d-Tyr Phe amide was determined by ion exchange chromatography. An accelerated procedure was developed for investigating the rates of reaction. The results showed that d-Tyr Phe d-Ala has a significant affinity for the amidating enzyme; no affinity could be demonstrated with d-Tyr Phe l-Ala, d-Tyr Phe d-Ser or d-Tyr Phe d-Leu. Direct evidence that d-Tyr Phe d-Ala can undergo amidation was obtained by incubating the ^{125}I labelled tripeptide with the pituitary enzyme. Amidation took place readily with d-Tyr Phe d-Ala but not with the other tripeptides; thus, while the enzyme is unable to catalyse the conversion of a peptide terminating in l-alanine, it can accept a peptide terminating in d-alanine. The results indicate that the amidating enzyme has a highly compact substrate binding site.

Recent studies which revealed the presence of an amidating enzyme in porcine pituitary utilized a synthetic peptide d-tyrosyl-l-valylglycine as a substrate for monitoring amide forming activity(1,2). It was shown by ^{15}N labelling that the amide nitrogen of the d-Tyr Val amide formed from d-Tyr Val Gly was derived from the amino nitrogen of the glycine residue and by testing a range of tripeptides evidence was obtained that the amidating enzyme has a mandatory requirement for glycine in the C-terminal position of its substrates. Thus the C-terminal residue of the substrate appeared to form an important recognition site for the amidating enzyme. In this communication we present the results of further studies on the specificity of the amidating

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enzyme, using an accelerated procedure for investigating amidation reactions. In particular we report that the enzyme can catalyse the amidation of a peptide that terminates in d-alanine; peptides terminating in d-serine or d-leucine, or in l-alanine, did not undergo amidation. The results demonstrate that the enzyme has a critical specificity for the C-terminal residue of its substrates.

MATERIALS AND METHODS

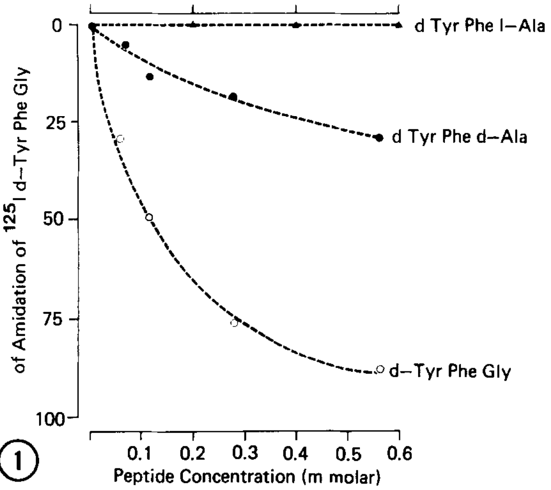
The tripeptides d-Tyr Phe Gly, d-Tyr Phe d-Ala, d-Tyr Phe d-Ser, d-Tyr Phe d-Leu and d-Tyr Phe l-Ala were synthesized by the solid phase method(3) and purified by ion exchange chromotography. Their susceptibility to amidation was tested by studying their ability to compete with a radiolabelled substrate, ^{125}I d-Tyr Phe Gly, which undergoes amidation readily(2). The reaction was carried out in the presence of a rate limiting amount of amidating enzyme and the degree of conversion of the ^{125}I-tripeptide to ^{125}I-dipeptide amide was determined by ion exchange chromatography employing a rapid method for assaying multiple samples.

The peptides were added to a reaction mixture containing ^{125}I d-Tyr Phe Gly (10 nmole of peptide labelled with 1 mC of ^{125}I, 4.10^{5} cpm used in each reaction) and 50 µl of amidating enzyme (from 10 ml of enzyme solution obtained by extracting 50 g porcine pituitary). The enzyme, prepared by homogenization, salt precipitation and gel filtration(1), was present in 200 mM NaCl containing 50 mM sodium phosphate at pH 6.8, with addition of Cu II to a final concentration of 0.1 mM(2,4). The reaction mixture was incubated at 37°C and the degree of conversion of the ^{125}I-tripeptide to ^{125}I-dipeptide amide determined by ion exchange chromatography. Columns of SP Sephadex C25 (6 x 0.5 cm) were suspended in H_2O and prewashed with 0.15 ml of 100 mM HCl and 2 ml of H_2O. Aliquots of the reaction mixture (10 µl) were added to the columns and a series of fractions were collected: (1) 1 ml H_2O, (2,3) 500 µl H_2O, (4-9) 600 µl of 100 mM phosphate at pH 7.0, (10-11) 600 µl of 100 mM phosphate + 200 mM NaCl at pH 7.0, (12-15) 800 µl of 100 mM phosphate + 200 mM CaCl at pH 7.0. Under these conditions, iodide released from the peptide during storage was eluted in fraction 1, unreacted ^{125}I-peptide in fraction 6, and ^{125}I-dipeptide amide in fraction 12. A bank of columns was eluted simultaneously and the fractions were collected manually.

RESULTS AND DISCUSSION

It was observed that peptides terminating in d-Ser, d-Leu or l-Ala, added at a concentration of 0.5 mM, did not significantly decrease the rate of formation of ^{125}I d-Tyr Phe amide from ^{125}I d-Tyr Phe Gly. In contrast, addition of d-Tyr Phe d-Ala or d-Tyr Phe Gly markedly reduced the rate of amidation of the ^{125}I-labelled tripeptide (Figure 1). The glycine containing tripeptide d-Tyr Phe Gly was more effective in this assay than was d-Tyr Phe d-Ala, similar results being obtained with $3.5.10^{-2}$ mM d-Tyr Phe Gly and 3×10^{-1} mM d-Tyr Phe d-Ala. The data indicate that both the glycine and d-alanine containing substrates have affinity for the amidating enzyme, but preference is shown for the substrate with C-terminal glycine.

Direct evidence for the ability of d-Tyr Phe d-Ala to undergo amidation was obtained by incubating the ^{125}I-labelled tripeptide with the pituitary enzyme. Under the conditions used, ^{125}I d-Tyr Phe d-Ala underwent conversion to ^{125}I d-Tyr Phe $CONH_2$ in high yield but it is notable that ^{125}I d-Tyr Phe Gly was converted more rapidly (Figure 2). Experiments with ^{125}I-labelled d-Ser



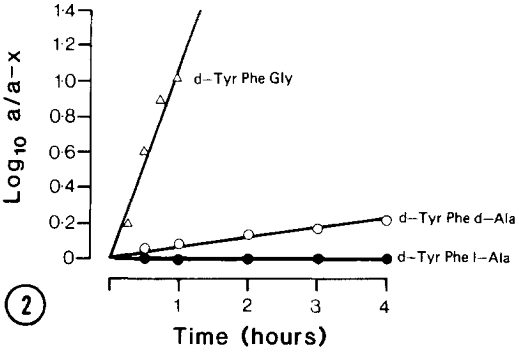


Figure 1. Effect of substrate competition on the amidation of ^{125}I d-tyrosylphenylalanylglycine. Incubation of ^{125}I d-Tyr Phe Gly with unlabelled d-Tyr Phe Gly (o ····· o) or d-Tyr Phe d-Ala (● ····· ●) was carried out with amidating enzyme as described in the text.

Figure 2. Rate of conversion of ^{125}I d-tyrosylphenylalanyl d-alanine and ^{125}I d-tyrosylphenylalanylglycine to ^{125}I d-tyrosylphenylalanine amide. O ——— O, d-Tyr Phe d-Ala; Δ ——— Δ, d-Tyr Phe Gly; ● ——— ●, d-Tyr Phe l-Ala. Incubations were carried out at pH 6.8 with amidating enzyme from porcine pituitary. The experimental conditions are described in the text.

or d-Leu containing peptides gave, as expected, little (< 1% of the glycine containing substrate) or no evidence of amidation.

Both the direct and indirect methods show that a peptide terminating in d-alanine, but not in l-alanine, can serve as a substrate for the amidating enzyme. This indicates that the orientation of the substituent attached to the α-carbon of the terminal residue of the peptide is important for enzyme-substrate interaction. It would also suggest that with glycine only one of the two α-carbon hydrogen bonds is involved in the amidation reaction. Of further interest is the observation that peptides terminating in d-leucine or d-serine were not acceptable as substrates. The substituent attached to the α-carbon of these amino acids would appear to offer steric hindrance to binding.

It may be noted that tripeptides with electrically charged residues such as aspartic acid or lysine at position 2 are relatively poor substrates whereas neutral hydrophobic residues at this position provide good substrates(2). It seems, therefore, that the binding of substrate to the amidating enzyme can also be influenced by the nature of the penultimate residue.

Previous studies have shown that the tetrapeptide d-Tyr Val-Gly-Lys and the tripeptide d-Tyr Val N-methylglycine do not undergo amidation(1). These findings, together with the results of the present study, indicate that the structural elements that are recognized by the amidating enzyme include the ionised carboxyl group, an α-carbon hydrogen bond, and possibly the α-carbon nitrogen linkage of C-terminal glycine or d-alanine (Figure 3). As these structures are disposed adjacently on the C-terminal amino acid of the substrate, the evidence supports a view that the amidating enzyme has a highly compact substrate binding site.

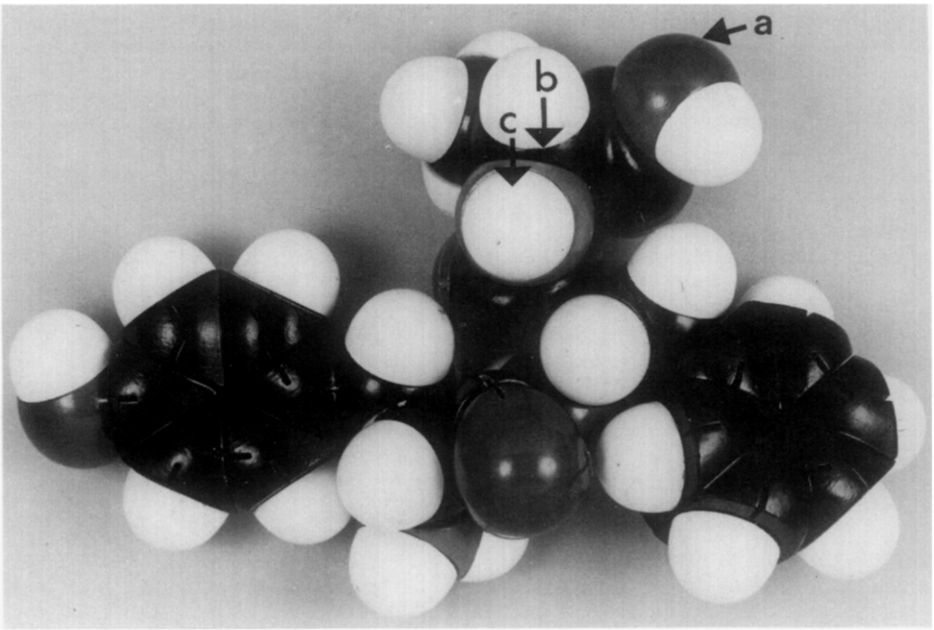


Figure 3. Space filling molecular model of d-tyrosyl-l-phenylalanyl d-alanine. The structural elements included in the binding site of the amidating enzyme are indicated by arrows. They comprise the carboxyl anion (*a*), the α-carbon hydrogen bond (*b*), and the C_2-N linkage (*c*) of the terminal d-alanine residue.

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REFERENCES

1. Bradbury, A.F., Finnie, M.D.A. and Smyth, D.G. (1982) Nature 298, 686-689.
2. Bradbury, A.F. and Smyth, D.G. (1983) Biochem. Biophys. Res. Commun. 112, 372-377.
3. Merrifield, R.B. (1963) J. Amer. Chem. Soc. 85, 2149-2154.
4. Bradbury, A.F. and Smyth, D.G. (1982) In: Peptides 1982, eds. K. Blaha and P. Malon (Walter de Gruyter & Co., Berlin-New York) pp. 381-386.