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SUBSTRATE SPECIFICITY OF HOG PLASMA ANGIOTENSIN-CONVERTING ENZYME

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SUMMARY

The substrate specificity of hog plasma converting enzyme which was free of a second dipeptidylcarboxypeptidase activity was studied by employing a number of peptide analogues related to the C-terminus of the decapeptide substrate, angiotensin I. Tripeptides, even when related to angiotensin I such as Phe–His–Leu, are not substrates. N-terminal-blocked tripeptides and a tetrapeptide, Pro–Phe–His–Leu, are fair to good substrates at high substrate concentrations. Five substrates, the tripeptide to the heptapeptide, derived from the C-terminus of angiotensin I were studied in detail. The Michaelis–Menten constants and maximum velocities were measured. The second-order rate constants which are proportional to the percentage rate of hydrolysis of the substrate when $[S] \ll K_m$ were tabulated for the enzyme reaction. There is little specificity in V. However, there is specificity in K_m but it develops slowly as the length of the amino acid sequence of the polypeptide increases from the C-terminus and approaches angiotensin I.

INTRODUCTION

Plasma angiotensin-converting enzyme which catalyzes the formation of the vasoactive octapeptide angiotensin II from the biologically inactive decapeptide angiotensin I has been found in several species and in several tissues[1–6]. This enzyme releases the dipeptide histidylleucine from the carboxyl terminus of angiotensin I in a hydrolytic reaction. However, a number of simpler substances have also been used as substrates for the converting enzyme. Although the determinants of its specificity have not been delineated we can say in a formal sense that the converting enzyme acts as a dipeptidylcarboxypeptidase, *i.e.* it hydrolyzes the penultimate C-terminus peptide linkage.

Piquilloud *et al.*[7] have used Cbz-Pro–Phe–His–Leu and Cbz-Phe–His–Leu as substrates for horse plasma converting enzyme. Yang *et al.*[8,9] have reported that

Abbreviation: Hip, hippuryl residue.

Hip–Gly–Gly (Hip, hippuryl residue) is a substrate for the converting enzyme from hog plasma and Cushman and Cheung[10] have used Hip–His–Leu as a substrate for the enzyme from dog lung.

We have recently shown that hog plasma contains at least one other enzyme, besides the converting enzyme, that acts as a dipeptidyl carboxypeptidase[11]. This second enzyme does not act on angiotensin I but it does release the C-terminal dipeptide from a number of tripeptides and tetrapeptides including Hip–His–Leu and Hip–Gly–Gly. These two compounds have been used as substrates for assaying the converting enzyme. Fortunately, this second enzyme is not Cl^- dependent so that assays for the converting enzyme based upon measurements with and without Cl^- may be valid. The existence of this second enzyme does point up the possibility that the occurrence of dipeptidylcarboxypeptidases may be widespread[8,9,12,13] and one or more of them may be Cl^- dependent.

In the present study we have collected data to aid us in answering the next questions. What structural features does the converting enzyme recognize in its substrates? Does it recognize a penultimate peptide linkage?, a Phe–His linkage? Does it recognize the amino acid residues near the N-terminus?, *etc.* We have used a number of substrates including a series of five peptides derived from the C-terminus of angiotensin I. The preparation of hog plasma converting enzyme that we used was free of the second dipeptidylcarboxypeptidase.

EXPERIMENTAL

Enzyme preparation

A crude enzyme preparation which was obtained from hog plasma by $(NH_4)_2SO_4$ fractionation[1,14] was subjected to column chromatography (2.2 cm × 75 cm) using Sephadex G-200, (particle size 40–120 μm) equilibrated with 0.05 M sodium phosphate buffer (pH 7.2) to separate the converting enzyme from a second dipeptidylcarboxypeptidase[11].

Enzyme assay

Two methods were used; a radioactivity assay with angiotensin I as the substrate[4,14–16] and a colorimetric assay using the JEOLCO Model JLC-5AH amino acid analyzer[17] with the other substrates. In the radioactivity assay, the activity of the converting enzyme was measured by the formation of radioactive histidylleucine from the substrate, [^{14}C]Leu 10-labeled angiotensin I (ref. 14).

In the second method, the peptide substrates at $1 \cdot 10^{-3}$ M concentration were incubated with 10–50 μg of the enzyme preparation in 50 μl of 0.05 M sodium phosphate buffer (pH 7.3) at 37 °C. After 3 h incubation, the reaction was stopped by adding 10 μl of 50% trichloroacetic acid. The solution was then diluted to 1–2 ml with the sample diluting buffer, sodium acetate (pH 2.2), and applied to the amino acid analyzer. The extent of the enzymic hydrolysis was determined by measuring the amount of the free amino acid or the dipeptide formed as the reaction product.

Inhibition studies

The peptide inhibitors at $1 \cdot 10^{-3}$ M concentration were preincubated with 20 μg of the enzyme preparation in 50 μl of 0.05 M sodium phosphate buffer (pH 7.3) at

37 °C. After 1 h preincubation, 10 μl of radioactive Asp-1-Ile-5-[^{14}C]Leu-10-labeled angiotensin I (4 nC_i, approx. 2.5 nmoles) were added to the reaction mixture, and the incubation was continued for 2 h. The reaction was stopped by adding 10 μl of 50% trichloroacetic acid. The whole solution was then applied to Whatman 3MM paper for high voltage paper electrophoresis at 2500 V for 1 h[14–16]. The degree of inhibition was measured by comparing the amount of His-[^{14}C]Leu formed in the presence and absence of the inhibitors.

Materials

Asp 1-Ile 5-angiotensin I (radioactive and non-radioactive) and its C-terminal analogue peptides, His–Pro–Phe–His–Leu, Pro–Phe–His–Leu, Phe–His–Leu, His–Pro–Phe–Gly–Gly, and Phe–Gly–Gly were synthesized by the solid phase method of Marshall and Merrifield[18] in a manner similar to that of Thampi *et al.*[19], and were purified by gel filtration with Bio-gel P-2 and by ion-exchange column chromatography using Aminex A-5. A pH gradient formed with a pyridine–acetate buffer was used for elution. The homogeneity of these peptides was checked by thin-layer chromatography, high voltage paper electrophoresis and amino acid composition. The hexapeptide and heptapeptide analogues, Ile–His–Pro–Phe–His–Leu and Tyr–Ile–His–Pro–Phe–His–Leu, and Hip–His–Leu were the gifts of Dr John Stewart, Medical Center, University of Colorado. Other peptides were purchased from Cyclo Chemical Co., Fox Chemical Co. and Sigma Chemical Co.

RESULTS AND DISCUSSION

Hog plasma converting enzyme which was free of the second dipeptidylcarboxypeptidase activity was obtained as a partially purified form with a specific activity of 14.6 nmoles/min per mg of protein when angiotensin I was used as the substrate. The enzyme preparation showed no carboxypeptidase or aminopeptidase activity. The enzyme was nearly inactive in the absence of NaCl.

Table I presents the results that were obtained using substrate concentrations of $1 \cdot 10^{-3}$ M except for angiotensin I which was measured by the radioactive assay at $3.8 \cdot 10^{-5}$ M concentration. The product measured is given in parenthesis. In general only a small amount of activity was found in the absence of NaCl. The enzyme does seem to be a carboxypeptidase, *i.e.*, a free carboxyl group is a determinant of specificity, because the methyl ester of His–Pro–Phe–His–Leu is not a substrate whereas the pentapeptide is. Tripeptides, even when related to angiotensin I such as Phe–His–Leu, are not substrates. However the N-blocked tripeptides, even those unrelated to angiotensin I such as Hip–Gly–Gly, are significant substrates. The tetrapeptide Pro–Phe–His–Leu which might be considered a blocked tripeptide is a very good substrate at high substrate concentration ($1 \cdot 10^{-3}$ M).

Interestingly enough, as far as we can discern from the data for the blocked tripeptides and for pentapeptides, His–Leu is not unique as a good leaving group for this enzyme. Thus in comparing Compounds 4 and 6 and 9 and 12 we find that Gly–Gly is released at a rate that is slower but still comparable to the rate at which His–Leu is released. However this question will have to be examined in greater detail.

Five substrates related to angiotensin I were studied in greater detail; the maximum velocities and Michaelis–Menten constants were measured. The kinetic

TABLE I

PEPTIDE HYDROLYSIS BY HOG PLASMA CONVERTING ENZYME PREPARATION

Peptide	*Percent hydrolysis*	
	With 0.1 M NaCl	*No NaCl*
1 Angiotensin I*	33 (His–Leu)	5 (His–Leu)
2 Tyr–Ile–His–Pro–Phe–His–Leu	27 (His–Leu)	3 (His–Leu)
3 Ile–His–Pro–Phe–His–Leu	19 (His–Leu)	0 (His–Leu)
4 His–Pro–Phe–His–Leu	18 (His–Leu)	2 (His–Leu)
5 His–Pro–Phe–His–Leu-OMe	0 (His–Leu-OMe)	0 (His–Leu-OMe)
6 His–Pro–Phe–Gly–Gly	12 (Gly–Gly)	0 (Gly–Gly)
7 Pro–Phe–His–Leu	40 (His–Leu)	8 (His–Leu)
8 Phe–His–Leu	0 (Phe, His–Leu)	0 (Phe, His–Leu)
9 Cbz-Phe–His–Leu	12 (His–Leu)	2 (His–Leu)
10 Hip–His–Leu	13 (His–Leu)	0 (His–Leu)
11 Hip–Gly–Gly	8 (Gly–Gly)	0 (Gly–Gly)
12 Cbz-Phe–Gly–Gly	10 (Gly–Gly)	4 (Gly–Gly)
13 Ac-Ala–Ala–Ala	7 (Ala–Ala)	0 (Ala–Ala)
14 Gly–Ala–Ala	1 (Gly, Ala–Ala)	0 (Ala–Ala)
15 Gly–His–Gly	0 (Gly)	0 (Gly)
16 Ala–Ala–Ala–Ala–Ala	0 (Ala–Ala)	0 (Ala–Ala)

* Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu.

parameters of the substrate analogues were obtained by reciprocal plots of the integrated form of the Michaelis–Menten equation[20]. These results are presented in Table II. These substrates are peptides representing the C-terminal portion of angiotensin I. An interesting observation emerges; the maximum velocity is about the same for all the compounds except the tetrapeptide which has a maximum velocity which is about twice as high as for the other compounds. Thus the normal substrate does not have the highest maximum velocity; but a factor of two is relatively small. There is little specificity in V. However there is specificity in K_m but it develops slowly and more or less step by step as the amino acid sequence approaches angiotensin I. The N-terminal sequence of angiotensin I, Asp–Arg–Val has only a small

TABLE II

Relative percentage rates of hydrolysis were calculated by V/K_m; *i.e.*, when $[S] << K_m$, the percentage rate of hydrolysis is independent of $[S]$ and the Michaelis–Menten equation becomes first order for the rate of hydrolysis: $-\frac{d[S]}{dt}\frac{1}{[S]} = \frac{V}{K_m}$ = constant.

Substrate	K_m *(μM)*	V *(nmoles/min per mg)*	*Relative percentage rate of hydrolysis*
Angiotensin I*	46	14.6	100
Tyr–Ile–His–Pro–Phe–His–Leu	69	14.0	64
Ile–His–Pro–Phe–His–Leu	110	14.0	40
His–Pro–Phe–His–Leu	120	14.2	37
Pro–Phe–His–Leu	630	26.4	13
Hip–His–Leu	540	13.0	7

* Radioactivity measurement.

effect on K_m and almost none on V. Thus the converting enzyme does not have an important recognition site for these N-terminal amino acid residues.

The largest change in K_m occurs with the introduction of histidine to form the pentapeptide substrate and one might therefore suspect that histidine plays an important role in the molecular complementarity between substrate and enzyme. However we have not ruled out that this effect may arise merely by blocking the nitrogen function of proline and thereby eliminating a positive electrical charge at this locus.

We have tabulated V/K_m. This quantity, proportional to the second order rate constant for the reaction of enzyme and substrate, is also proportional to the percentage rate of hydrolysis of the substrate when $[S] \ll K_m$. For an enzyme such as the converting enzyme, which normally acts on very low concentrations of substrate to produce a highly potent product, the percentage rate of hydrolysis is the most pertinent quantity for examining specificity in relationship to physiological function. Again, we note a slow development of specificity as the polypeptide length increases from the C-terminus and approaches angiotensin I. Thus although Pro–Phe–His–Leu has a high V its very unfavorable K_m makes this compound a relatively poor substrate at low substrate concentrations.

The effect of various added substances at $1 \cdot 10^{-3}$ M on the conversion of angiotensin I is given in Table III. The tripeptides are poorly bound as might have been expected since they are not substrates. On the other hand Hip–Gly–Gly is poorer bound than might have been anticipated. We have repeatedly found that His–Leu is not an active inhibitor. This is worth some emphasis because it contrasts with the findings of others[8,9,21]. Others who have found that His–Leu is an inhibitor have assayed the converting enzyme by biologically evaluating the rate of formation of angiotensin II, whereas we have measured the rate of appearance of (His–[^{14}C]-Leu. These apparently opposing findings could be explained if His–Leu were to be involved in a transacylation reaction. Such a reaction might decrease the rate of conversion of angiotensin I to angiotensin II yet would not decrease the rate of release

TABLE III

INHIBITION OF THE CONVERTING ENZYME ACTIVITY BY PEPTIDE ANALOGUES

Inhibitor	*% inhibition*
1-Asp-angiotensin II	58
Phe–His–Leu	16
Phe–Gly–Gly	13
Gly–Pro–Gly	22
Hip–Gly–Gly	18
His–Leu	6
Pro–Phe	9
Cbz-Pro–Phe	24
Cbz-Pro–Phe-OCH_2Ph	48
6-Amino-caproyl-Pro–Phe-OCH_2Ph	80
6-Amino-caproyl-Gly–Pro–Gly–OMe	85
BPF (pentapeptide)*	32
BPF (nonapeptide)**	86

* L-2-pyrrolidone 5-carboyxlic acid-Lys–Trp–Ala–Pro.
** L-2-pyrrolidone 5-carboxylic acid-Trp–Pro–Arg–Pro–Gly–Ile–Pro–Pro.

of His-[^{14}C]Leu. However this is an *ad hoc* suggestion and at present we have no other evidence for a transacylation reaction mediated by the converting enzyme.

A number of substances show marked inhibition but it must be remembered that the concentration of inhibitor is high ($1 \cdot 10^{-3}$ M). One of the bradykininpotentiating peptides (the pentapeptide) does not have impressive inhibitory potency. On the other hand three compounds might be quite potent inhibitors.

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