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COMPARISON OF DIPEPTIDYL CARBOXYPEPTIDASE AND ENDOPEPTIDASE ACTIVITIES IN THE THREE ENKEPHALIN-HYDROLYSING METALLOPEPTIDASES: "ANGIOTENSIN-CONVERTING ENZYME", THERMOLYSIN AND "ENKEPHALINASE"

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Angiotensin-converting enzyme (ACE), thermolysin and "enkephalinase", three metallopeptidases cleaving the Gly^3-Phe^4 amide bond of enkephalins, were compared regarding substrate specificity and effects of butanedione, an arginyl-directed reagent.
The hydrolysis of enkephalins and analogues was more affected by the nature of P1 and P2 residues in the case of thermolysin than in those of ACE or "enkephalinase" ; amidation of the C-terminal carboxylate decreased drastically the hydrolysis by ACE but only marginally by thermolysin and the effect was intermediate for "enkephalinase". With adequate model substrates, the ratio of dipeptidylcarboxypeptidase to tripeptidylcaroxypeptidase (endopeptidase) activities were of 25 for ACE, 3 for "enkephalinase" and only 0.3 for thermolysin. Finally a butanedione treatment increased thermolysin activity, but abolished ACE activity ; it reduced "enkephalinase" activity by 80 % when measured with a free C-terminal carboxylate enkephalin analogue but only slightly with the corresponding amidated derivative. A critical role of an Arg residue in ACE and, to a lesser extent, in "enkephalinase" (but not in thermolysin) is suggested to be responsible for the preferential dipeptidylcarboxypeptidase activity of these two enzymes.

Peptidases are classified according to both their catalytic mechanism and substrate specificity, the latter property namely leading to the distinction of endopeptidases (EC 3.4.2.) and exopeptidases (EC 3.4.1.). That this distinction might not be always clearcut is illustrated in the case of ACE (EC 3.4.15.1), for a long time considered as a strict dipeptidylcarboxypeptidase but whose endopeptidase activity was more recently recognised (1-3).

A similar difficulty is encountered with the "neutral proteinase from rabbit kidney brush border membranes" (EC 3.4.24.11) more often designated "enkephalinase" in view of its functional role (reviewed in 4-7). Because, initially, the renal enzyme was mainly studied using large MW substrates, like the insulin B chain, and was found to be inhibited by Phosphoramidon, a thermolysin

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inhibitor, it was considered as a typical "neutral endopeptidase" (8). In contrast initial studies on "enkephalinase" from cerebral membranes (9) with enkephalins as substrates from which the C-terminal dipeptide is released, underlined its similarities with ACE (4,10). We have presently compared "enkephalinase" to two other metallopeptidases also able to cleave the Gly^3-Phe^4 amide bond of enkephalins i.e. ACE (11) and thermolysin (present results) regarding the hydrolysing of enkephalins or model peptides and the effects of butanedione.

MATERIALS AND METHODS

Source of enzymes : Purified thermolysin (EC 3.4.24.4), obtained from Boeringer (Mannheim) was dissolved in Hepes/NaOH buffer (50 mM, pH 7.4) at a concentration of 3 mg/ml. "Enkephalinase" was purified from rat kidney dose to homogeneity (12,13). ACE was extracted from rabbit lung acetone powder (Sigma) into Tris/HCl buffer 5 mM pH 8.5 containing 0.5 % Triton X 100, and purified by hydroxylapatite (Bio-Rad HT), concanavalin A-sepharose (Pharmacia) and DE 52 (Whatman) chromatography (13) . The purified ACE had a specific activity of 120 nmol/mg protein/min and was completely free of "enkephalinase" or aminopeptidase activity as tested using ^{3}H-(D-Ala^2, Met^5)enkephalinamide, suc-Ala-Ala-Phe (7 amido, 4 methyl) coumarin, and Phe-(7 amido, 4 methyl) coumarin as substrates, respectively.

Determination of enzyme activities : ACE activity was always measured in the presence of 300 mM NaCl, and "enkephalinase" activity in the presence of 0.2 % Triton X 100.

A - **Hydrolysis of labelled substrates** : Thermolysin and "enkephalinase" activities were routinely assayed in 30 min incubations (100 µl) at 37°C in 50 mM pH 7.4 Hepes/NaOH buffer, using 20 nM ^{3}H-(D-Ala^2, Leu^5)enkephalin or ^{3}H-(D-Ala^2, Met^5)enkephalinamide (Amersham) as substrates, the characteristic metabolite ^{3}H-Tyr-D-Ala-Gly being isolated by polystyrene bead chromatography (14). ACE activity was measured using 5 µM ^{14}C-Hip-His-Leu (NEN Chemicals) as substrate and ^{14}C-hippuric acid was extracted into ethyl acetate.

B - **Hydrolysis of unlabelled peptides** : Incubations of peptides (donated by B.P. Roques or purchased from Bachem) in 100 µl 5 mM pH 7.4 Hepes/NaOH buffer and terminated by the addition of 50 µl buffer containing 1 µM Captopril, 10 µM Phosphoramidon, or 1 µM Thiorphan, for ACE, thermolysin or "enkephalinase", respectively. Blanks were obtained by adding inhibitors at the start of incubations. The incubation medium was then heated at 95°C for 5 min and metabolites separated by HPLC on C18 uBondapak (Waters ass.) and quantified by their absorbance at 210 nm. The elution system consisted of 0.2 mM HCl (retention volumes : Tyr-Gly-Gly 17 ml, Tyr-D-Ala-Gly 32 ml, Ala-Pro-Ala 6 ml), in some cases with 3.5 % acetonitrile (retention volumes : Tyr-Ala 2.5 ml, Tyr-Ala-Ala 4 ml).

RESULTS AND DISCUSSION

Low concentrations of Captopril (100 nM), Phosphoramidon (1 µM), and Thiorphan (100 nM) completely abolished the hydrolysis of all substrates by

ACE, thermolysin and "enkephalinase" respectively, indicating that the preparations did not contain any interfering peptidase activity.

All three enzymes cleaved the enkephalins at their Gly^3-Phe^4 amide bond but the hydrolysis by ACE could only be detected in the presence of 300 mM NaCl and its rate with (Met^5)enkephalin as substrate was lower (by two orders of magnitude) than that by thermolysin or "enkephalinase" (table 1).

The substitution of any of the two aminoacids engaged in the scissile amide bond by a D-isomer indeed abolished activity (compounds IV and V, table 1). Hydrolysis of enkephalin derivatives by the three metallopeptidases was differently affected by their C-terminal amidation (Table 1). Whereas amidation of ($D\text{-}Ala^2$, Met^5)enkephalin had little effect on its hydrolysis by thermolysin

TABLE 1
HYDROLYSIS OF ENKEPHALINS AND ANALOGS BY METALLOPEPTIDASES

		RELATIVE HYDROLYSIS RATE BY		
		Angiotensin Converting Enzyme	Thermolysin	"Enkephalinase"
I	Tyr-Gly-Gly-Phe-Met	100	100	100
II	Tyr-Gly-Gly-Phe-Leu	86	85	70
III	Tyr-Gly-Ala-Phe-Met	33	538	60
IV	Tyr-Gly-D-Ala-Phe-Met	< 1	< 1	< 1
V	Tyr-Gly-Gly-D-Phe-Met	< 1	< 1	< 1
VI	Tyr-D-Ala-Gly-Phe-Leu	19	2	30
VII	Tyr-D-Ala-Gly-Phe-D-Leu	< 1	< 0.1	< 1
VIII	Tyr-D-Ala-Gly-Phe-Met	30	3.6	44
IX	Tyr-D-Ala-Gly-Phe-Met-NH_2	< 1	1.2	6

Peptides (10 μM) were incubated for 60 min at 37°C with 60, 5 and 0.5 ng of ACE, thermolysin and "enkephalinase", respectively (except for peptides VI to IX for which the amount of thermolysin was increased to 50 ng) and the release of Tyr-Gly-Gly or Tyr-D-Ala-Gly evaluated. The value 100 corresponds to 0.1, 18 and 32 μmoles/mg protein/min for ACE, thermolysin and "enkephalinase", respectively.

(only 3-fold reduction), it more or less abolished its hydrolysis by ACE and the effect was intermediate with "enkephalinase" (more than 7-fold reduction) (see also ref. 15). Also substitutions of the P1 or P2 residues affected much more their hydrolysis by thermolysin than by ACE or "enkephalinase" (compare compounds II and III as well as compounds II and IV, in table 1). This suggests a major role of subsites S1 and S2 in the bacterial endopeptidase whereas binding of peptides in the active sites of ACE (16) and "enkephalinase" (4) seems mainly governed by their S'1 and S'2 subsites.

The differential role of the C-terminal carboxylate is also clearly illustrated with adequate model peptides (Table 2). Whereas a terminal carboxylate in P'3 decreased hydrolysis rate by 25-fold in the case of ACE (compare compounds I and II) and by 3-fold in the case of "enkephalinase", it actually increased the rate of hydrolysis by thermolysin (compare compounds III and

TABLE 2
COMPARISON OF DIPEPTIDYL CARBOXYPEPTIDASE AND ENDOPEPTIDASE ACTIVITY OF METALLOPEPTIDASES

PEPTIDES	RELATIVE HYDROLYSIS RATE BY		
	Angiotensin Converting Enzyme	Thermolysin	"Enkephalinase"
Ala-Ala▼Ala-Pro	100	-	-
Ala-Ala-Ala-Pro-Ala	4	-	-
Ala-Ala-Tyr-Ala	-	100	100
Ala-Ala-Tyr-Ala-Ala	-	320	31

Peptides (100 μM with ACE, 10 μM otherwise) were incubated for 60 min at 37°C with 50, 10 and 1 ng of ACE, thermolysin and "enkephalinase" respectively. The release of the peptides Ala-Pro, Ala-Pro-Ala, Tyr-Ala and Tyr-Ala-Ala was monitored after HPLC separation, by their absorbance at 210 nm. Means from at least three determinations of hydrolysis rates relative to that of Ala-Ala-Ala-Pro by ACE (1.33 μmol/mg protein/min) or Ala-Ala-Tyr-Ala by thermolysin and "enkephalinase" (6 and 8 μmol/mg protein/min, respectively).

IV). In other words both ACE and "enkephalinase" seem to function better as dipeptidylcarboxypeptidases than as tripeptidylcarboxypeptidases, whereas the reverse seems true for thermolysin. Although ACE was, for a long time, considered as a strict dipeptidylcarboxypeptidase, its tripeptidylcarboxypeptidase activity particularly on substrates with a favourable P'2 prolyl residue, was recently shown (1-3).

Finally the differential role of a critical arginyl residue in the active sites of the three enzymes is illustrated by the effects of a butanedione treatment (Table 3). Whereas the activity of thermolysin towards (DAla^2, Leu^5)enkephalin was enhanced by 40 %, that of "enkephalinase" was decreased by more than 80 % and that of ACE abolished (see also ref. 12,17). Furthermore with the corresponding amidated substrate the butanedione-induced change was similar in the case of thermolysin but much less marked in the case of "enkephalinase" (Table 3). Cristallographic studies with thermolysin have suggested that the role of the Arg 203 residue in its active site was to bind the P'1

TABLE 3
EFFECT OF A BUTANEDIONE TREATMENT ON THE ACTIVITY OF METALLOPEPTIDASES

PEPTIDES	ACTIVITY (% of control) OF		
	Angiotensin Converting Enzyme	Thermolysin	"Enkephalinase"
(D-Ala^2, Leu^5)enkephalin	0	141 ± 16	18 ± 4
(D-Ala^2, Met^5)enkephalinamide	0	140 ± 23	87 ± 8

Treatment with butanedione (10 mM, 1 h at 37°C) and measurement of enzyme activities (30 min incubations at 37°C with 30, 10 and 1 ng of ACE, thermolysin and "enkephalinase", respectively) were performed in 10 mM pH 8 borate buffer. Control activities were 0.031, 0.8 and 2.2 nmol/mg protein/min for the hydrolysis of 20 nM ^{3}H-(D-Ala^2, Leu^5)enkephalin by ACE, thermolysin and "enkephalinase", and 0.09 and 1.1 nmoles/mg protein/min for the hydrolysis of ^{3}H-(D-Ala^2, Met^5)enkephalinamide by thermolysin and "enkephalinase", respectively. The latter substrate was not significantly hydrolysed by ACE (see table 1). Data represent means ± S.E.M. from 4 experiments.

carbonyl group engaged in an amide bond of peptide substrates or inhibitors (18-20). In contrast we have proposed that the role of the critical Arg residue in "enkephalinase", as evidenced by the butanedione treatment, was to salt-link the P'2 free carboxylate of inhibitors like Thiorphan (12), an hypothesis that can now be extended to substrates. Such a binding in "enkephalinase" accounts for the reduced hydrolysis rate of substrates in which this P'2 carboxylate is amidated by either ammoniac (compound IX from table 2) or an aminoacid amino group (table 2). The abolition of ACE activity by butanedione (17) together with the stronger influence of amidation of the P'2 carboxylate of ACE substrates (Tables 1 and 2) suggests a similar but more marked role of the guanidinium group in this enzyme.

In conclusion, whether "enkephalinase" should be considered as mainly an endopeptidase or a dipeptidylcarboxypeptidase seems mostly a question of semantics. However it appears that "enkephalinase" displayed little ressemblance with thermolysin in the various experimental approaches presently used and that, in terms of functional importance of a free carboxylate in P'2 of substrates, it behaved qualitatively like ACE.

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