Biochem. J. (1989) 262, 125–130 (Printed in Great Britain)

Novel activity of angiotensin-converting enzyme

Hydrolysis of cholecystokinin and gastrin analogues with release of the amidated *C*-terminal dipeptide

Phillibert DUBREUIL, Pierre FULCRAND, Marc RODRIGUEZ, Hélène FULCRAND, Jeanine LAUR and Jean MARTINEZ*

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, rue de la Cardonille, 34094 Montpellier Cédex 2, France

ACE (angiotensin-converting enzyme; peptidyl dipeptidase A; EC 3.4.15.1), cleaves *C*-terminal dipeptides from active peptides containing a free *C*-terminus. We investigated the hydrolysis of cholecystokinin-8 [CCK-8; Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2] and of various gastrin analogues by purified rabbit lung ACE. Although these peptides are amidated at their *C*-terminal end, they were metabolized by ACE to several peptide fragments. These fragments were analysed by h.p.l.c., isolated and identified by comparison with synthetic fragments, and by amino acid analysis. The initial and major site of hydrolysis was the penultimate peptide bond, which generated a major product, the *C*-terminal amidated dipeptide Asp-Phe-NH_2. As a secondary cleavage, ACE subsequently released di- or tri-peptides from the *C*-terminal end of the remaining *N*-terminal fragments. The cleavage of CCK-8 and gastrin analogues was inhibited by ACE inhibitors (Captopril and EDTA), but not by other enzyme inhibitors (phosphoramidon, thiorphan, bestatin etc.). Hydrolysis of [Leu^{15}]gastrin-(14–17)-peptide [Boc (t-butoxycarbonyl)-Trp-Leu-Asp-Phe-NH_2] in the presence of ACE was found to be dependent on the chloride-ion concentration. K_m values for the hydrolysis of CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide at an NaCl concentration of 300 mM were respectively 115, 420 and 3280 μM, and the catalytic constants were about 33, 115 and 885 min^{-1}. The k_{cat}/K_m for the reactions at 37 °C was approx. 0.28 $\mu M^{-1} \cdot min^{-1}$, which is approx. 35 times less than that reported for the cleavage of angiotensin I. These results suggest that ACE might be involved in the metabolism *in vivo* of CCK and gastrin short fragments.

INTRODUCTION

Angiotensin-converting enzyme (ACE; peptidyl dipeptidase A; EC 3.4.15.1) is a peptidyl dipeptidase which is distributed throughout the body, including the central nervous system (Patchett & Cordes, 1985). The enzyme catalyses the generation of *C*-terminal dipeptides from a wide variety of substrates, such as angiotensin I (Skeggs *et al.*, 1956; Erdös, 1976), bradykinin (Erdös & Yang, 1967; Yang & Erdös, 1967) and enkephalins (Erdös, 1979). However, some recent results have shown that ACE is able to cleave the penultimate bond of substrates in which the last amino acid is replaced by nitrobenzylamine (Hersh *et al.*, 1983) or to release the *C*-terminal di- or tri-peptide of peptides having an amidated *C*-terminal amino acid residue, such as substance P (Cascieri *et al.*, 1983; Yokosawa *et al.*, 1983; Skidgel *et al.*, 1984; Bunnett *et al.*, 1985), luliberin (luteinizing-hormone-releasing hormone) (Skidgel & Erdös, 1985) or amidated enkephalins (Hooper & Turner, 1987), thus indicating that ACE can also express an endopeptidase activity. Our recent results concerning a specific cleavage of the *C*-terminal tetrapeptide analogue of gastrin, Boc-Trp-Leu-Asp-Phe-NH_2, by a gastric-mucosal-cell-membrane fraction (Dubreuill *et al.*, 1987), led us to study the enzyme system responsible for such endopeptidase activity and prompted us to investigate several enzymes. Because ACE is present in the gastrointestinal tract, especially in the the pancreas and stomach (Cushman & Cheung, 1971), we investigated the action of ACE on peptides of the gastrin family, including cholecystokinin-8 (CCK-8).

EXPERIMENTAL

Materials

Purified rabbit lung ACE (lots 66F-96010 and 117F-9745; activity 2–4 units/mg of protein) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Peptides and peptide derivatives were synthesized in our laboratory, using the conventional methods of solution peptide synthesis. The protocols used in these syntheses have been published elsewhere (Martinez *et al.*, 1985; Fulcand *et al.*, 1988) or are available from J.M. on request. *N*-(α-Rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp (phosphoramidon), bestatin and amastatin were obtained from Sigma. (*S*)-*N*-(3-Mercapto-2*D*-methylpropanoyl)proline (captopril) and (DL-3-mercapto-2-benzylpropanoyl)glycine (thiorphan), were kindly provided by Dr. B. P. Roques, Faculté de Pharmacie, Paris, France. H.p.l.c.-grade acetonitrile was purchased from

Abbreviations used: ACE, angiotensin-converting enzyme; CCK, cholecystokinin; Boc, t-butoxycarbonyl; Z, benzyloxycarbonyl; Tyr(SO_3H), O^4-sulphated tyrosine; Nle, norleucine; Ψ(CH_2-NH) indicates that the peptide bond has been replaced by a CH_2NH bond; IC_{50}, concentration causing 50% inhibition; Hip, hippurate.

* To whom correspondence and reprint requests should be addressed.

Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K. Water for h.p.l.c. was of Milli-Q grade. Trifluoroacetic acid was Sequanal grade from Pierce Chemical Co., Rockford, IL, U.S.A. In our gastrin analogues, methionine has been replaced by leucine. This substitution preserves full biological activity and sensitivity to ACE of the gastrin analogues and also offers the advantage of preventing inactivation via oxidation of the thioether group of methionine.

Methods

Enzymic hydrolyses of CCK-8 and gastrin analogues were analysed by h.p.l.c. Enzyme (0.005 unit) was incubated with substrate (0.1 mM) and buffer in a final volume of 0.1 ml. Buffer was 100 mM-Tris/HCl, pH 7.5, containing 0.3 M-NaCl. When specific inhibitors of ACE were used, they were preincubated with the enzyme for 5–15 min at 37 °C. Reactions were carried out at 37 °C for various times and then stopped by freezing in liquid nitrogen. Samples (20 μl) were stored at −80 °C before h.p.l.c. analysis. In kinetic studies, ACE (0.005 unit) was incubated with CCK-8 and gastrin analogues at ten different substrate concentrations (ranging from 10 to 1000 μM), in a buffer containing 300 mM-NaCl. For determination of kinetic constants, data were plotted according to the Lineweaver–Burk representation and were fitted to the best straight line by linear regression. Correlation coefficients were always better than 0.98.

H.p.l.c. analyses were performed with a Hitachi–Merck apparatus. Peptides were applied to a μBondapak C_{18} reverse-phase column and separated under isocratic conditions using solvent A (0.1% trifluoroacetic acid/acetonitrile, 13:7, v/v) for CCK-8 and gastrin II analogues at 280 nm, solvent B (0.1% trifluoroacetic acid/acetonitrile, 14:11, v/v) for gastrin fragments at 280 nm, and solvent C (0.1% trifluoroacetic acid/acetonitrile, 41:9, v/v) for all runs at 214 nm, at a flow rate of 1 ml/min. Each peptide was identified by comparison with synthetic standards synthesized in our laboratory and by amino acid analysis for the isolated metabolites.

For amino acid analysis, degradation products were collected after separation by h.p.l.c. and freeze-dried. Peptides were hydrolysed at 105 °C for 24 h in 6M-HCl/phenol (99:1, v/v). Acid was removed by freeze-drying, and the remaining products were resuspended in triethylamine/ethanol/water (2:2:1, by vol.) and freeze-dried again. Then, derivatization was performed with phenyl isothiocyanate/triethylamine/ethanol/water (1:1:7:1, by vol.) for 20 min at 20 °C. After freeze-drying, samples were analysed by h.p.l.c. (PICO-TAG, Waters), at 254 nm.

RESULTS

Homogeneous rabbit lung ACE was incubated, at 37 °C, for various periods of time with CCK-8, [Asp^{26}-Tyr(SO$_3$H)^{27}-Met^{28}-Gly^{29}-Trp^{30}-Met^{31}-Asp^{32}-Phe^{33}-NH$_2$], [Leu^{15}]gastrin-(5–17)-peptide (Leu^5-Glu^6-Glu^7-Glu^8-Glu^9-Glu^{10}-Ala^{11}-Tyr^{12}-Gly^{13}-Trp^{14}-Leu^{15}-Asp^{16}-Phe^{17}-NH$_2$), [Leu^{15}]gastrin-(11–17)-peptide (Ala^{11}-Tyr^{12}-Gly^{13}-Trp^{14}-Leu^{15}-Asp^{16}-Phe^{17}-NH$_2$), Z-[Leu^{15}]gastrin II-(1–17)-peptide [Z-Tyr(SO$_3$H)^{12}-Gly^{13}-Trp^{14}-Leu^{15}-Asp^{16}-Phe^{17}-NH$_2$] and Boc-[Leu^{15}]gastrin-(14–17)-peptide (Boc-Trp^{14}-Leu^{15}-Asp^{16}-Phe^{17}-NH$_2$).

CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide were significantly degraded by homogeneous rabbit lung ACE when incubated for 1 h at 37 °C as described in the Experimental section (Table 1; Fig. 1). Degradation of CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide produced several products, as shown by h.p.l.c. (Fig. 1). CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide metabolites were separated, collected, identified by amino acid analysis and compared, by h.p.l.c., with synthetic samples. The major product of hydrolysis was the *C*-terminal dipeptide Asp-Phe-NH$_2$. The initial cleavage of CCK-8 occurred at the Met^{31}–Asp^{32} bond, producing Asp-Phe-NH$_2$ (peak 3) and Asp^{26}-Tyr(SO$_3$H)^{27}-Met^{28}-Gly^{29}-Trp^{30}-Met^{31} (peak 2) (Fig. 2*a*). The peptide Asp^{26}-Tyr(SO$_3$H)^{27}-Met^{28}-Gly^{29}-

Table 1. Degradation rates of gastrin/CCK-family peptides by ACE (0.005 unit) at 37 °C

Results are the means of at least five separate experiments. Abbreviation: ND, not determined.

Peptide		Degradation (%)			
	t (min) ...	15	30	60	180
CCK-8		30±5	50±5	76±4	99
[Leu^{15}]gastrin-(5–17)-peptide		—	—	< 4	< 10
[Leu^{15}]gastrin-(11–17)-peptide		39±7	69±3	86±3	100
Z-[Leu^{15}]gastrin II-(12–17)-peptide		66±5	83±3	93±2	100
Boc-[Leu^{15}]gastrin-(14–17)-peptide		73±3	90±2	98±2	100
Z-Tyr-Gly-Trp-Leu-Ψ(CH$_2$-NH)-Asp-Phe-NH$_2$		—	—	—	< 1
Z-Tyr(SO$_3$H)-Gly-Trp-Leu-Ψ(CH$_2$-NH)-Asp-Phe-NH$_2$		—	—	—	< 1
Boc-Gly-Trp-Leu-Ψ(CH$_2$-NH)-Asp-Phe-NH$_2$		—	—	—	< 1
Z-Tyr(SO$_3$H)-Nle-Gly-Trp-Nle-Ψ(CH$_2$-NH)-Asp-Phe-NH$_2$		—	—	—	< 1
[Leu^{15}]gastrin-(10–17)-peptide		27±5	47±5	54±4	ND
[Leu^{15}]gastrin-(9–17)-peptide		3±1	4±1	6±1	ND
[Leu^{15}]gastrin-(8–17)-peptide		—	—	< 4	< 10
Boc-[Leu^{15}]gastrin-(14–17)-OH		ND	ND	100	—
Boc-[Gly^{15}]gastrin-(14–17)-peptide		—	—	< 1	< 4
Boc-Trp-Leu-Asp-2-phenylethylamide		—	—	—	< 1
Boc-Trp-Leu-Asp-2-phenylethyl ester		—	—	—	< 1

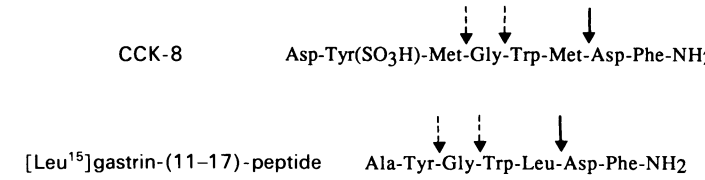

Fig. 1. Hydrolysis of CCK-8 and [Leu^{15}]gastrin-(11–17)-peptide by rabbit lung ACE

Sites of cleavage by ACE are indicated by arrows.

Trp^{30}-Met^{31} was further degraded, mainly between Gly^{29} and Trp^{30} to yield Trp-Met (peak 8) and Asp^{26}-Tyr(SO$_3$H)^{27}-Met^{28}-Gly^{29} (peak 1). Secondary cleavages produced Gly^{29}-Trp^{30}-Met^{31} (peak 7). The compound Trp-Met, oxidized on the methionine residue, was observed as the result of methionine oxidation under the incubation conditions used (peak 5) (Fig. 2*a*). [Leu^{15}]gastrin-(11–17)-peptide was degraded by the same process. After cleavage at the Leu^{15}–Asp^{16} peptide bond, ACE then releases the dipeptide Trp^{14}-Leu^{15} (peak 6) and the tripeptide Ala^{11}-Tyr^{12}-Gly^{13} (peak 1) (Fig. 2*b*). Another minor product of hydrolysis was identified as Gly^{13}-Trp^{14}-Leu^{15} (peak 4). The peptide Ala^{11}-Tyr^{12}-Gly^{13}-Trp^{14}-Leu^{15} could not be detected, suggesting that ACE first cleaves at the bond between Leu^{15} and Asp^{16} to release the *C*-terminal dipeptide Asp-Phe-NH$_2$ (peak 7), and then quickly removes the dipeptide Trp^{14}-Leu^{15}, leaving intact the *N*-terminal tripeptide Ala^{11}-Tyr^{12}-Gly^{13}. The analogue Z-[Leu^{15}]gastrin II-(12–17)-peptide was degraded similarly to Boc-[Leu^{15}]gastrin-(11–17)-peptide. Boc-[Leu^{15}]gastrin-(14–17)-peptide produced only the dipeptide Asp-Phe-NH$_2$ (peak 3) and Boc-Trp-Leu (peak 2) (Fig. 2*c*). [Leu^{15}]Gastrin-(5–17)-peptide was not hydrolysed to any significant extent (less than 4%) after 1 h incubation with rabbit lung ACE (Table 1).

In order to determine if the sites of cleavage of CCK-8 and [Leu^{15}]gastrin-(11–17)-peptide between Gly and Trp could be due to another endopeptidase activity, and to evaluate the role, if any, of the sulphate ester on the enzymic stability of gastrin II, we tested the behaviour of a pseudopeptide analogue of CCK, Z-Tyr(SO$_3$H)^{27}-Nle^{28}-Gly^{29}-Trp^{30}-Nle^{31}-Ψ(CH$_2$-NH)-Asp^{32}-Phe^{33}-NH$_2$, and that of the pseudopeptide gastrin analogues Z-Tyr^{12}-Gly^{13}-Trp^{14}-Leu^{15}-Ψ(CH$_2$-NH)-Asp^{16}-Phe^{17}-NH$_2$, Z-Tyr^{12}(SO$_3$H)-Gly^{13}-Trp^{14}-Leu^{15}-Ψ(CH$_2$-NH)-Asp^{16}-Phe^{17}-NH$_2$ and Boc-Gly^{13}-Trp^{14}-Leu^{15}-Ψ(CH$_2$-NH)-Asp^{16}-Phe^{17}-NH$_2$, when incubated with ACE at 37 °C. In these compounds, the peptide bond between Leu and Asp has replaced by a non-enzymically-cleavable bond. The enzyme was unable to degrade these pseudopeptides, even after a 3 h incubation (Table 1).

Because [Leu^{15}]gastrin-(5–17)-peptide was barely degraded by ACE, whereas Boc-[Leu^{15}]gastrin-(11–17)-peptide was completely hydrolysed, we investigated the degradation of a series of *C*-terminal gastrin analogues by elongating [Leu^{15}]gastrin-(11–17)-peptide at the *N*-terminal end by glutamic acid residues. The peptides [Leu^{15}]gastrin-(10–17)-peptide (Glu^{10}-Ala^{11}-Tyr^{12}-Gly^{13}-Trp^{14}-Leu^{15}-Asp^{16}-Phe^{17}-NH$_2$), [Leu^{15}]gastrin-(9–13)-peptide (Glu^{9}-Glu^{10}-Ala^{11}-Tyr^{12}-Gly^{13}-Trp^{14}-Leu^{15}-Asp^{16}-Phe^{17}-NH$_2$), [Leu^{15}]gastrin-(8–17)-peptide (Glu^{8}-Glu^{9}-Glu^{10}-Ala^{11}-Tyr^{12}-Gly^{13}-Trp^{14}-Leu^{15}-Asp^{16}-Phe^{17}-NH$_2$) were incubated with ACE at 37 °C as described in the Experimental section. Although hydrolysis of these peptides decreases as the peptide length increases, the main and initial cleavage always occurred between Leu^{15} and Asp^{16}, producing the dipeptide fragment Asp-Phe-NH$_2$. Hydrolysis of [Leu^{15}]gastrin-(10–17)-peptide and [Leu^{15}]gastrin (9–17)-peptide proceeded respectively only by about 55 and 6%. Degradation of [Leu^{15}]gastrin-(8–17)-peptide was less than 4% under the conditions after a 1 h incubation (Table 1).

In order to evaluate the importance of the *C*-terminal amide function, the peptide Boc-Trp-Leu-Asp-Phe-OH was incubated in the presence of ACE as described above. This peptide was completely hydrolysed in 1 h to produce Boc-Trp-Leu and Asp-Phe-OH (Table 1). When tetrapeptide gastrin analogues lacking the *C*-terminal amide function, i.e. Boc-Trp-Leu-Asp-2-phenylethylamide or Boc-Trp-Leu-Asp-2-phenylethyl ester (Martinez *et al.*, 1986*a*,*b*) were incubated with ACE, they remained completely stable. Interestingly, the tetrapeptide Boc-Trp-Gly-Asp-Phe-NH$_2$, in which the leucine residue has been replaced by glycine, was not hydrolysed after a 3 h incubation at 37 °C in the presence of ACE (Table 1). This compound was further demonstrated to act as a gastrin antagonist (Dubreuil *et al.*, 1989).

In the absence of chloride ions, conditions that are known to inactive ACE (Igic *et al.*, 1973; Bünning & Riordan, 1983), the rate of total Boc-[Leu^{15}]gastrin-(14–17)-peptide degradation was only 1% of that measured in the presence of 300 mM-NaCl (Table 2). When NaCl was added at 75 mM, it increased to 52% of the optimal rate established at 300 mM-NaCl.

The hydrolysis of CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide was inhibited by captopril, a specific inhibitor of ACE (Ondetti *et al.*, 1977), with IC_{50} values of about 50, 50 and 10 nM respectively. Inhibitors of neutral endopeptidase 24.11 (EC 3.4.24.11), aminopeptidase, thiol proteinases, acidic proteinases and serine proteinases (i.e. phosphoramidon and thiorphan, bestatin and amastatin, *p*-chloromercuribenzoate, pepstatin A and phenylmethanesulphonyl fluoride respectively), were ineffective (Table 3).

In order to measure the kinetics constants of CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide hydrolysis by ACE, studies were carried out at 300 mM-NaCl. CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide were completely degraded after 3 h (Table 1). The catalytic-centre activities ($k_{cat.}$), for CCK-8, [Leu^{15}]gastrin-(11–17)-peptide and Boc-[Leu^{15}]gastrin-(14–17)-peptide with ACE were 33 ± 3, 115 ± 5 and 885 ± 11 min^{-1} respectively, compared with that of angiotensin I (792–810 min^{-1}) (Das & Soffer, 1975; Cascieri *et al.*, 1983), whereas K_m values were 115 ± 9, 420 ± 15 and 3280 ± 25 μM respectively (70–90 μM for angiotensin I).

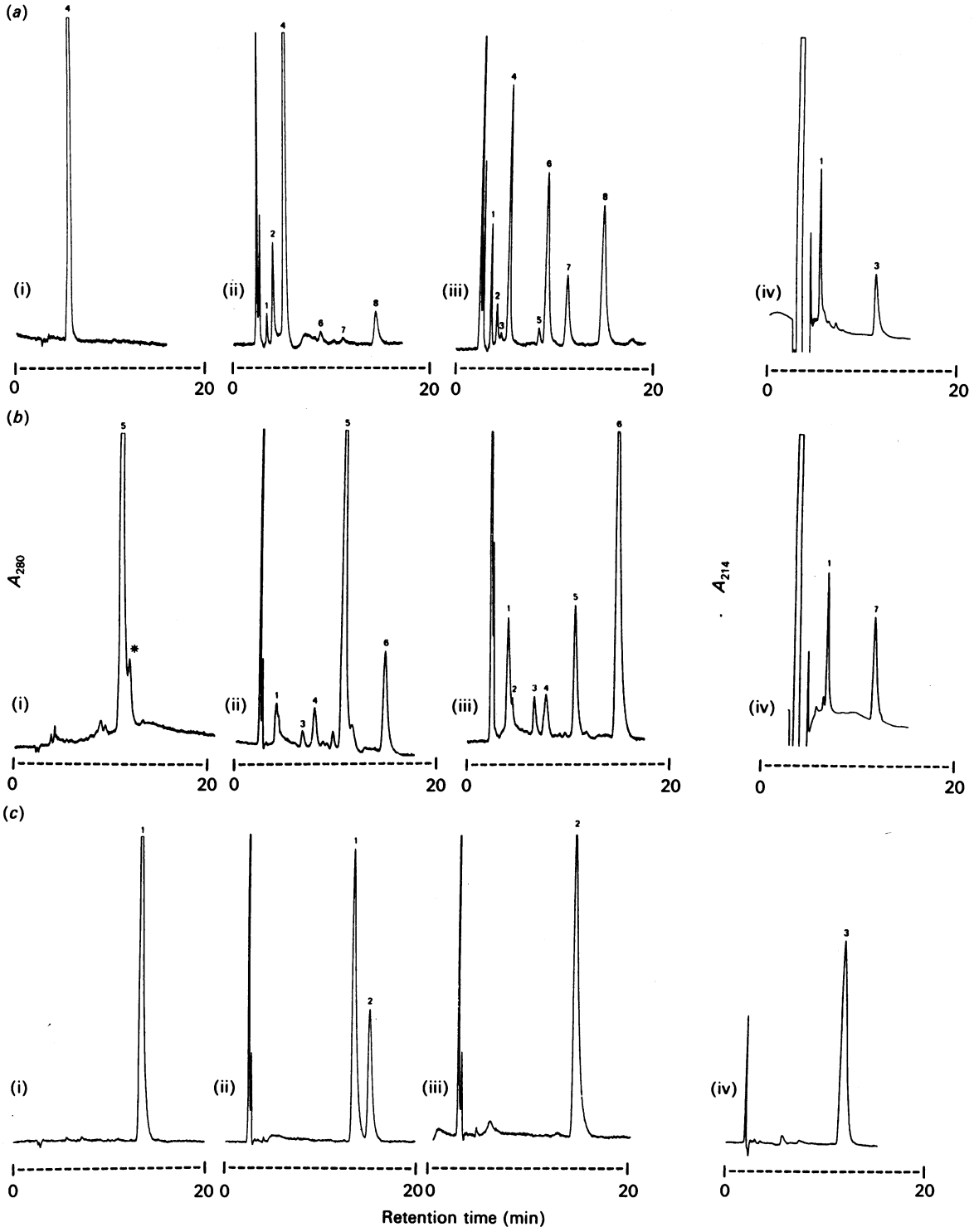

Fig. 2. H.p.l.c. analysis of the cleavage products of CCK-8 (*a*), [Leu^{15}]gastrin-(11–17)-peptide (*b*) and Boc-[Leu^{15}]gastrin-(14–17)-peptide (*c*) by homogeneous rabbit lung ACE

ACE (0.005 unit), was incubated at 37 °C with 0.1 mM-CCK-8 or gastrin analogues, in the presence of 0.3 M-NaCl. Degradation products were separated by h.p.l.c. as described in the Experimental section. (i) Standards; *, succinimide; (ii) h.p.l.c. pattern after 7 min incubation with ACE at 280 nm; (iii) h.p.l.c. pattern after 60 min incubation with ACE at 280 nm; (iv) h.p.l.c. pattern after 60 min incubation with ACE at 214 nm. Numbered peaks correspond to products which were identified by amino acid analysis and comparison, by h.p.l.c., with synthetic samples. (*a*) Asp^{26}-Tyr(SO$_3$H)^{27}-Met^{28}-Gly^{29} (peak 1), Asp^{26}-Tyr(SO$_3$H)^{27}-Met^{28}-Gly^{29}-Trp^{30}-Met^{31} (peak 2), Asp-Phe-NH$_2$ (peak 3), CCK-8 (peak 4), Trp^{30}-Met^{31} oxidized (peak 6), Gly^{29}-Trp^{30}-Met^{31} (peak 7) and Trp^{30}-Met^{31} (peak 8). (*b*) Ala^{11}-Tyr^{12}-Gly^{13} (peak 1), Tyr^{12}-Gly^{13} (peak 2), Gly^{13}-Trp^{14} (peak 3), Gly^{13}-Trp^{14}-Leu^{15} (peak 4), [Leu^{15}]gastrin-(11–17)-peptide (peak 5), Trp^{14}-Leu^{15} (peak 6), Asp-Phe-NH$_2$ (peak 7). (*c*) Boc-[Leu^{15}]gastrin-(14–17)-peptide (peak 1), Boc-Trp-Leu (peak 2) and Asp-Phe-NH$_2$ (peak 3). At least five separate experiments have been performed in each series, consisting of hydrolysis of CCK-8, [Leu^{15}]gastrin-(11–17)-peptide or [Leu^{15}]gastrin-(14–17)-peptide by ACE.

Table 2. Effect of NaCl on Boc-[Leu^{15}]gastrin-(14–17)-peptide hydrolysis by rabbit lung ACE

Rabbit lung ACE was dialysed against chloride-free 0.1 M-sodium phosphate buffer, pH 7.5, for 48 h before the enzyme assays. The assays were carried out in 0.05 M-phosphate buffer (pH 7.5)/0.3 M-NaCl Boc-[Leu^{15}]gastrin-(14–17)-peptide (10^{-4} M) was incubated for 60 min at 37 °C. Activity is expressed as a percentage of the activity of purified rabbit lung ACE incubated with 0.3 M-NaCl on the same substrate.

[NaCl] (mM)	Activity (%)
None	< 1 %
2.22	10
8.9	16
17.75	26
35.35	37
75	52
150	91
300	100

Table 3. Inhibition of hydrolysis of Boc-[Leu^{15}]gastrin-(14–17)-peptide by rabbit lung ACE

Inhibitors were preincubated with enzyme for 5–15 min at 37 °C, then substrate was added to initiate the reaction.

Inhibitor	Molarity (mM)	[NaCl] (0.3 M)	Activity (%)
None	—	+	100
None	—	—	1
1,10-Phenanthroline	10^{-3}	+	10
EDTA	10^{-3}	+	20
Captopril	10^{-6}	+	3
Phosphoramidon	10^{-5}	+	70
Thiorphan	10^{-6}	+	60
Bestatin	10^{-4}	+	80
Amastatin	10^{-4}	+	90
p-Chloromercuribenzoate	10^{-4}	+	99
Pepstatin A	10^{-4}	+	99
Phenylmethanesulphonyl fluoride	10^{-4}	+	99

DISCUSSION

Although it is known that ACE cleaves *C*-terminal dipeptides from active peptides containing a free *C*-terminus, it has already been shown that ACE also inactivates *C*-terminally amidated peptides, such as substance P and luliberin.

In the present study we have shown that CCK-8, and sulphated and non-sulphated gastrin fragments, which are amidated at the *C*-terminal end, were cleaved by rabbit lung ACE at the peptide bond between Met (or Leu) and Asp to release specifically the *C*-terminal dipeptide Asp-Phe-NH_2. Secondary cleavage sites were observed (Gly–Trp and Met–Gly) and were attributed to degradation of the resulting fragments after release of the dipeptide Asp-Phe-NH_2. Analogues of gastrin or CCK-8 in which the peptide bond between leucine (or norleucine in the CCK analogue) and aspartic acid was replaced by a non-enzymically cleavable bond (e.g. CH_2–NH) were completely stable when incubated at 37 °C for 3 h in the presence of ACE (Table 1). These results, along with experiments performed at different times (Fig. 1), suggest that the initial cleavage of CCK-8 and gastrin analogues occurs at the Leu–Asp (or Met–Asp) bond.

Unexpectedly, when the peptide chain was elongated at the *N*-terminus from the alanine residue of [Leu^{15}]gastrin-(11–17)-peptide (Ala-Tyr-Gly-Trp-Leu-Asp-Phe-NH_2) with glutamic acid residues, sensitivity to ACE decreases as the chain length increases (Table 1). In fact, [Leu^{15}]gastrin-(5–17)-peptide was not hydrolysed to any significant extent when incubated with rabbit lung ACE (less than 4 % after a 1 h incubation). These results can probably be correlated and explained with those of Peggion *et al.* (1985), who showed that the environment of the biologically important *C*-terminal sequence Trp-Leu-Asp-Phe-NH_2 is affected by chain elongation. Incorporation of glutamic acid residues induces a structural change, co-operative in nature, that also involves the *C*-terminal tetrapeptide. In fact, the final folded structure at the *C*-terminus is almost reached in the fragment with a sequence of four glutamic acid residues. A different explanation could be found in the fact that ACE usually hydrolyses substrates of relatively short chain length (Dorer *et al.*, 1974). Another important result arose from the observation that all the analogues of tetragastrin lacking the *C*-terminal amide function, i.e. Boc-Trp-Leu-Asp-2-phenylethylamide or Boc-Trp-Leu-Asp-2-phenylethyl ester, which behave as gastrin antagonists (Martinez *et al.*, 1986*a*,*b*), were not cleaved by ACE. These results clearly indicate the importance of the *C*-terminal amide function and, more precisely, of the carbonyl group, for interaction with ACE. The crucial role of the side chain of residue in position 11 is also pointed out by the fact that the peptide Boc-Trp-Gly-Asp-Phe-NH_2, in which leucine in position 11 has been replaced by glycine, is not degraded when incubated with ACE. On the other hand, sulphation of tyrosine, which occurs in CCK-8 and in gastrin II, does not influence the ACE activity at all, as indicated by degradation of CCK-8 and of Z-[Leu^{15}]gastrin II-(12–17)-peptide (Table 1).

The possibility that hydrolysis of these CCK/gastrin-family peptides is due to a contaminant in our ACE preparation is ruled out by the fact that the hydroysis of CCK-8, and gastrin derivatives, especially concerning generation of Asp-Phe-NH_2, is completely inhibited by captopril, a specific ACE inhibitor (Ondetti *et al.*, 1977). Furthermore, the presence of NaCl was essential for hydrolysis of Boc-[Leu^{15}]gastrin-(14–17)-peptide, as in the case of the hydrolysis of a typical synthetic ACE substrate Hip-His-Leu (Yokosawa *et al.*, 1983). ACE requires chloride ions for optimal activity with most of the peptide substrates (Igic *et al.*, 1973; Bünning & Riordan, 1983), such as angiotensin I, which is not cleaved in the absence of chloride ions and requires 200 mM-NaCl for optimal activity.

Gastrin and CCK-8 are also degraded by endopeptidase 24.11, but differently from ACE. The primary site of cleavage takes place at the Asp–Phe-NH_2 bond (Bunnett *et al.*, 1988; Deschodt-Lanckman *et al.*, 1988). Further degradation occurs mainly at the Gly–Trp bond. *In vivo*, enzymes, not surprisingly, inactivate both peptides. It is possible, therefore, that endopeptidase 24.11 may be involved in the enzymic inactivation of gastrin and CCK-8 *in vivo*.

Some authors have already shown that Asp-Phe-NH_2 could be identified in extracts of rat cortex and hypothalamus (McDermott *et al.*, 1983), and of synaptic plasma membranes from pig cortex (Durieux *et al.*, 1984, 1986). We have already reported that Boc-[Leu[15]]gastrin-(14–17)-peptide was hydrolysed by a membrane fraction from rat gastric mucosa, releasing the main products Boc-Trp-Leu and Asp-Phe-NH_2. We hypothesized that this cleavage could be correlated with the mechanism of action of gastrin *in vivo* (Martinez *et al.*, 1985; Dubreuil *et al.*, 1987). More recently we have shown that a preparation of gastric-mucosal cells from various species, able to bind specifically gastrin, was able to hydrolyse tetragastrin and a series of gastrin analogues in the same manner as ACE (Dubreuil *et al.*, 1988, 1989). The enzymic system present in these preparations has been shown to closely to resemble ACE (Dubreuil *et al.*, 1989); however, its correlation with the gastrin receptor remains to be established.

ACE metabolizes gastrin analogues and CCK-8. It is a possibility, as has been suggested for endopeptidase 24.11, that ACE might be of some physiological significance in the metabolism of gastrin and CCK-8. The fact that gastric-mucosa-cell membranes (Dubreuil *et al.*, 1987) and gastric mucosal cells from different species can hydrolyse gastrin analogues such as ACE, and that captopril can inhibit this degradation (Dubreuil *et al.*, 1988, 1989) are consistent with this. However, the K_m values for CCK-8, [Leu[15]]gastrin-(11–17)-peptide and Boc-[Leu[15]]gastrin-(14–17)-peptide are higher than those of other substrates of ACE. Because we speculated that bound ACE in gastric-mucosal cells might be associated with the gastrin receptor and involved in the mechanism of action of gastrin, kinetic constants established with the soluble enzyme in the present work do not necessarily reflect those of a receptor-associated membrane-bound enzyme.

On the other hand, and taking into account our findings, it is possible that administration of ACE inhibitors, used clinically as antihypertensive drugs, could affect the metabolism of CCK-8 or gastrin fragments *in vivo*. Thus some of the actions of the ACE inhibitors *in vivo* could be due to inhibition of the hydrolysis of other peptides than antiotensin I or bradykinin.

REFERENCES

Bunnett, N. W., Orloff, M. S. & Turner, A. J. (1985) Life Sci. **37**, 599–606

Bunnett, N. W., Debas, H. T., Turner, A. J., Kobayashi, R. & Walsh, J. H. (1988) Am. J. Physiol. **255**, G676–G684

Bünning, P. & Riordan, J. F. (1983) Biochemistry **22**, 110–116

Cascieri, M. A., Bull, H. G., Mumford, R. A., Patchett, A. A., Thornberry, N. A. & Liang, T. (1983) Mol. Pharmacol. **25**, 287–293

Cushman, D. W. & Cheung, H. S. (1971) Biochim. Biophys. Acta **250**, 261–265

Das, M. & Soffer, R. L. (1975) J. Biol. Chem. **250**, 6762–6768

Deschodt-Lanckman, M., Pauwels, S., Najdovski, T., Dimaline, R. & Dockray, G. J. (1988) Gastroenterology **94**, 712–721

Dorer, F. E., Ryan, J. W. & Stewart, J. M. (1974) Biochem. J. **141**, 915–917

Dubreuil, Ph., Lignon, M. F., Magous, R., Rodriguez, M., Bali, J. P. & Martinez, J. (1987) Drug Design Delivery **2**, 49–54

Dubreuil, Ph., Galas, M. C., Lignon, M. F., Rodriguez, M., Bali, J. P. & Martinez, J. (1988) Forum Peptides, Nancy, France, abstr. P3

Dubreuil, Ph., Rodriguez, M., Fulcrand, P., Amblard, M., Laur, J. & Martinez, J. (1989) Proc. Am. Pept. Symp. 11th, in the press

Durieux, C., Pelaprat, D., Ruiz-Gayo, M., Gacel, G. & Roques, B. P. (1984) in Forum Peptides (Castro, B. & Martinez, J., eds.), pp. 121–124, Groupe Français des Peptides, Les Impressions DOHR, Nancy

Durieux, C., Charpentier, B., Pelaprat, D. & Roques, B. P. (1986) Neuropeptides **7**, 1–9

Erdös, E. G. (1976) Am. J. Med. **60**, 749–760

Erdös, E. G. (1979) Handb. Exp. Pharmacol. **25** (suppl), 427–487

Erdös, E. G. & Yang, H. Y. T. (1967) Life Sci. **6**, 569–574

Fulcrand, P., Rodriguez, M. & Martinez, J. (1988) J. Pept. Protein Res. **32**, 384–395

Hersh, L. B., Gafford, J. T., Powers, J. C., Tanaka, T., Erdös, E. G. (1983) Biochem. Biophys. Res. Commun. **110**, 654–659

Hooper, N. M. & Turner, A. J. (1987) Biochem. J. **241**, 625–633

Igic, R., Nakajima, T., Yek, H. S. J., Sorrells, K. & Erdös, E. G. (1973) in Pharmacology and the Future of Man (Acheson, G. H., ed.), vol. 5, pp. 307–319, Karger, Basel

Martinez, J., Bali, J. P., Rodriguez, M., Castro, B., Magous, R., Laur, J. & Lignon, M. F. (1985) J. Med. Chem. **28**, 1874–1879

Martinez, J., Rodriguez, M., Vali, J. P. & Laur, J. (1986*a*) Int. J. Pept. Protein Res. **28**, 529–535

Martinez, J., Rodriguez, M., Bali, J. P. & Laur, J. (1986*b*) J. Med. Chem. **29**, 2201–2206

McDermott, J. R., Dodd, P. R., Edwardson, J. A., Hardy, J. A. & Smith, A. I. (1983) Neurochem. Int. **5**, 641–647

Ondetti, M. A., Rubin, B. & Chusman, D. W. (1977) Science **196**, 441–444

Patchett, A. A. & Cordes, E. H. (1985) Adv. Enzymol. Relat. Areas Mol. Biol. **57**, 1–84

Peggion, E., Foffani, M. T., Wünsch, E., Moroder, L., Borin, G., Goodman, M. & Mammi, S. (1985) Biopolymers **24**, 647–666

Skeggs, L. T., Kahn, J. R. & Shumway, N. P. (1956) J. Exp. Med. **103**, 295–299

Skidgel, R. A. & Erdös, E. G. (1985) Proc. Natl. Acad. Sci., U.S.A. **82**, 1025–1029

Skidgel, R. A., Engelbrecht, S., Johnson, A. R. & Erdös, E. G. (1984) Peptides **5**, 769–776

Yang, H. Y. T. & Erdös, E. G. (1967) Nature (London) **215**, 1402–1403

Yokosawa, H., Endo, S., Ogura, Y. & Ishii, S. (1983) Biochem. Biophys. Res. Commun. **116**, 735–742

Received 29 March 1989/18 May 1989; accepted 24 May 1989