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# Hydrolysis of α-human atrial natriuretic peptide *in vitro* by human kidney membranes and purified endopeptidase-24.11

## Evidence for a novel cleavage site

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α-Human atrial natriuretic peptide (1–28) (hANP) is secreted by the heart and acts on the kidney to promote a strong diuresis and natriuresis. *In vivo* it has been shown to be catabolized partly by the kidney. Crude microvillar membranes of human kidney degrade $^{125}$I-ANP at several internal bonds generating metabolites among which the *C*-terminal fragments were identified. Formation of the *C*-terminal tripeptide was blocked by phosphoramidon, indicating the involvement of endopeptidase-24.11 in this cleavage. Subsequent cleavages by aminopeptidase(s) yielded the *C*-terminal dipeptide and free tyrosine. Using purified endopeptidase 24.11, we identified seven sites of hydrolysis in unlabelled α-hANP: the bonds Arg-4–Ser-5, Cys-7–Phe-8, Arg-11–Met-12, Arg-14–Ile-15, Gly-16–Ala-17, Gly-20–Leu-21 and Ser-25–Phe-26. However, the bonds Gly-16–Ala-17 and Arg-4–Ser-5 did not fulfil the known specificity requirements of the enzyme. Cleavage at the Gly-16–Ala-17 bond was previously observed by Stephenson & Kenny [(1987) Biochem. J. **243**, 183–187], but this is the first report of an Arg–Ser bond cleavage by this enzyme. Initial attack of α-hANP by endopeptidase-24.11 took place at a bond within the disulphide-linked loop and produced a peptide having the same amino acid composition as intact ANP. The bond cleaved in this metabolite was determined as the Cys-7–Phe-8 bond. Determination of all the bonds cleaved in α-hANP by endopeptidase-24.11 should prove useful for the design of more stable analogues, which could have therapeutic uses in hypertension.

## INTRODUCTION

The cardiac atria have been recently identified as an endocrine organ that contains multiple forms of natriuretic polypeptides. These hormones have potent biological activities on different systems involved in the regulation of intracorporal fluid volume. Their main actions are vasodilatation, a strong natriuretic and diuretic effect, and inhibition of aldosterone release [for reviews see Laragh (1985) and Ackermann (1986)]. Released by the atria following distension, the pro-hormone is cleaved either shortly after exocytosis or in the circulation where the two fragments, the inactive *N*-terminal moiety and the *C*-terminal peptide ANP-(Ser-1–Tyr-28), are found (Michener *et al.*, 1986; Bloch *et al.*, 1987).

The circulating form in humans is α-hANP-(1–28), a peptide consisting of a 17-amino-acid-residue ring linked by a disulphide bond. The ring structure and the *C*-terminal extension (Phe-Arg-Tyr) are required for full biological activity of the peptide (Misono *et al.*, 1984; Sugiyama *et al.*, 1984).

Atrial natriuretic peptides are short-lived molecules (Tang *et al.*, 1984; Murthy *et al.*, 1986*a*) that are catabolized partly by the kidney (Luft *et al.*, 1986). The cellular brush border of the proximal tubule contains a battery of peptidases, located on the external side of the epithelial plasma membrane, which are thought to be involved in the degradation of circulating peptides (for a review see Kenny & Maroux, 1982). Among these enzymes, endopeptidase-24.11 (EC 3.4.24.11; enkephalinase) has been shown to be involved in the degradation of α-hANP *in vitro* by pig microvillar kidney membranes (Stephenson & Kenny, 1987*b*).

In the present study we have extended these observations to human microvillar kidney membranes and have shown that enzymic attack of α-hANP was attributable to endopeptidase-24.11. Furthermore, using the purified human enzyme, we identified seven cleavage sites in α-hANP, among which is the Arg-4–Ser-5 bond, a position not predicted on the basis of the known specificity of the peptidase (Kerr & Kenny, 1974).

## MATERIALS AND METHODS

### Materials

$^{125}$I-labelled α-hANP (specific radioactivity approx. 2000 Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.); phosphoramidon

Abbreviation used: α-hANP, α-human atrial natriuretic peptide.

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was obtained from Peninsula (San Carlos, CA, U.S.A.), Arg-Tyr was from Bachem (Bubendorf, Switzerland), and bestatin and puromycin were from Boehringer (Mannheim, Germany).

Amastatin was generously given by Dr. Umezawa (Institute for Microbial Chemistry, Tokyo, Japan) and captopril by Dr. S. J. Lucania (Squibb Institute for Medical Research, Princeton, NJ, U.S.A.).

### Peptide synthesis

Merrifield's solid-phase method was applied to the syntheses of α-hANP and Phe-Arg-Tyr by using a Beckman model 990B peptide synthesizer. t-Butyloxycarbonyl-(*O*-2,6-dichlorobenzyl)tyrosine resin (0.34 mmol/g of resin) prepared from chloromethylated polystyrene resin (1% cross-linking) was submitted to repetitive cycles of deprotection [with 50% (v/v) trifluoroacetic acid in dichloromethane], neutralization [with 10% (v/v) triethylamine in dichloromethane] and coupling (with the use of a 2.5-fold molar excess of dicyclohexylcarbodi-imide). All amino acids were protected at the α-amino positions with t-butyloxycarbonyl groups and the following side-chain protectants were used: Asp, *O*-benzyl; Arg, *N*-ω-tosyl; Cys, *S*-*p*-methoxybenzyl; Ser, *O*-benzyl; Tyr, *O*-2,6-dichlorobenzyl (Steward & Young, 1984). The peptides were cleaved from the resin with anhydrous HF in the presence of dimethyl sulphide, *p*-cresol and *p*-thiocresol as scavengers (low-HF–high-HF procedure in accordance with Heath *et al.*, 1982). The crude peptides were extracted from the resin with 10% (v/v) acetic acid. Cyclization (disulphide-bridge formation) of α-hANP was performed by oxidation in aqueous solution with $K_3Fe(CN)_6$ at pH 6.8–6.9. The product was chromatographed on a Sephadex G-25 (fine grade) column in 0.1 M-acetic acid and purified by reverse-phase h.p.l.c. on a Vydac $C_{18}$ silica-gel column (30 nm porosity, 17 μm particle size, 25 mm × 250 mm) using the following conditions: solvent system A, 0.1 M-phosphoric acid/triethylamine, pH 3.5; solvent system B, 60% (v/v) acetonitrile in solvent A; gradient 25–35% (v/v) solvent B in 2 h; flow rate 9 ml/min. After extraction from the resin, the tripeptide Phe-Arg-Tyr was chromatographed on Sephadex G-10 in 0.1 M-acetic acid and purified by reverse-phase h.p.l.c. on a Macherey–Nagel $C_{18}$ silica-gel column (10 nm porosity, 7 μm particle size, 10 mm × 250 mm) using the following conditions: solvent system A, 0.1 M-formic acid/triethylamine, pH 4.0; solvent system B, 60% (v/v) acetonitrile in solvent A; gradient 5–45% (v/v) solvent B in 90 min; flow rate 3 ml/min. After being desalted on Sephadex G-10 in 10% (v/v) acetic acid, the peptides were freeze-dried. Homogeneity of the synthesized peptides was determined by h.p.l.c., t.l.c. and amino acid analysis.

### Crude microvillar membrane preparation

Human kidneys were obtained in the Department of Nephrology (Hôpital Erasme, Brussels, Belgium). These kidneys had been prepared initially for transplantation, but for various reasons were unsuitable for this purpose. The renal cortex was dissected and stored at −80 °C. All the following operations were carried out at 4 °C. The tissue was homogenized in 50 mM-Hepes/NaOH buffer, pH 7.5, containing 10 mM-$MgCl_2$. The homogenate was centrifuged for 15 min at 1500 *g* and the resulting supernatant centrifuged for 20 min at 15000 *g*. The pellet was washed, re-homogenized in the same buffer without $MgCl_2$ and stored in liquid $N_2$.

### Purification of kidney endopeptidase-24.11

The crude microvillar-membrane preparation from human kidneys was incubated for 45 min at 37 °C in 50 mM-Tris/HCl buffer, pH 7.6, containing 1% (w/v) Triton X-100. Endopeptidase-24.11 was purified essentially according to the method of Gafford *et al.* (1983). The solubilized extract was applied to a DEAE-Sepharose column (1.6 cm × 40 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.6, containing 0.1% Triton X-100 and eluted batchwise with the same buffer containing 25 mM-NaCl. The DEAE-Sepharose eluates containing endopeptidase-24.11 activity were concentrated and loaded on a chromatofocusing column (1.6 cm × 70 cm). The enzyme was eluted with a linear pH gradient from 6.4 to 4.0. A major peak of activity (pI 5.39) was collected, dialysed, concentrated and applied to a hydroxyapatite column (1.6 cm × 40 cm) equilibrated with 30 mM-sodium phosphate buffer, pH 8.3, containing 0.1% Triton X-100. The enzyme was eluted with a linear gradient increasing the molarity of the starting buffer from 30 to 250 mM. Endopeptidase-24.11 was eluted at 130 mM-sodium phosphate. A second chromatofocusing was performed as described above. On polyacrylamide-gel electrophoresis in the presence of SDS, the enzyme appeared to have been purified close to homogeneity; it emerged as a protein band of $M_r$ 95000, accounting for 90% of the starting material (Deschodt-Lanckman *et al.*, 1988*a*).

### Peptide incubation and h.p.l.c. analysis of the cleavage fragments

α-hANP (final concentration as indicated in the Figure legends) was incubated for various times at 37 °C with either kidney microvillar membranes or purified endopeptidase-24.11, usually in a volume of 200 μl. The reaction was stopped by adding 20 μl of pure trifluoroacetic acid. The samples were centrifuged and the supernatants injected on a μBondapak $C_{18}$ column. Elution was carried out at room temperature at a 1.0 ml/min flow rate with a linear acetonitrile gradient in 0.08% trifluoroacetic acid (see the Figure legends). In experiments with $^{125}$I-ANP 1.0 ml fractions were collected and their radioactivity was determined in a γ-radiation counter.

### Preparation of iodinated standards

Samples (1 μmol) of tyrosine, Arg-Tyr or Phe-Arg-Tyr were each treated for 1 min with 0.5 μmol of KI in the presence of 0.1 μmol, 1.0 μmol and 1.0 μmol of chloramine-T respectively. After the addition of 2.5 μmol of $Na_2S_2O_5$, the monoiodinated products were purified by isocratic h.p.l.c. runs with 10% (for $^{127}$I-Tyr) or 20% (for $^{127}$I-Arg-Tyr and $^{127}$I-Phe-Arg-Tyr) acetonitrile in 0.08% trifluoroacetic acid. These standards were detected by their absorbance at 214 nm.

### Amino acid analysis

Cleavage fragments of α-hANP were characterized by amino acid analysis performed by a modification of the method of Heinrikson & Meredith (1984). H.p.l.c. eluates for analysis were dried under vacuum in small glass tubes. These tubes were then placed inside a large hydrolysis tube that contained 0.5 ml of constant-boiling

HCl and 1% (v/v) phenol. The hydrolysis tube was flushed with $N_2$, evacuated, sealed and maintained at 108 °C for 20 h. Peptide hydrolysates were reconstituted in 0.1 ml of coupling buffer (methanol/water/triethylamine, 4:1:1, by vol.), freeze-dried and reconstituted in a further 0.1 ml of coupling buffer containing 5% (v/v) phenyl isothiocyanate. After 15 min reaction at room temperature, the mixture was dried and reconstituted in 0.1 ml of h.p.l.c. buffer A [0.16 M-sodium acetate buffer, pH 6.0, containing 0.05% (v/v) triethylamine]. Samples of the reconstituted reaction mixture (5–15 $\mu$l) were applied to a Waters Novapak $C_{18}$ column (3.9 mm × 150 mm) equilibrated with 90% buffer A and 10% buffer B (acetonitrile/water, 3:2, v/v), and eluted with a linear gradient to 50% buffer B over 15 min. The phenylthiocarbamoyl derivatives were detected at 254 nm and the peak areas integrated by using a Shimadzu C-R3A Chromatopak with reference to a standard mixture of amino acids.

### Protein assay

Proteins were measured by the Bradford (1976) method, with bovine serum albumin as a standard.

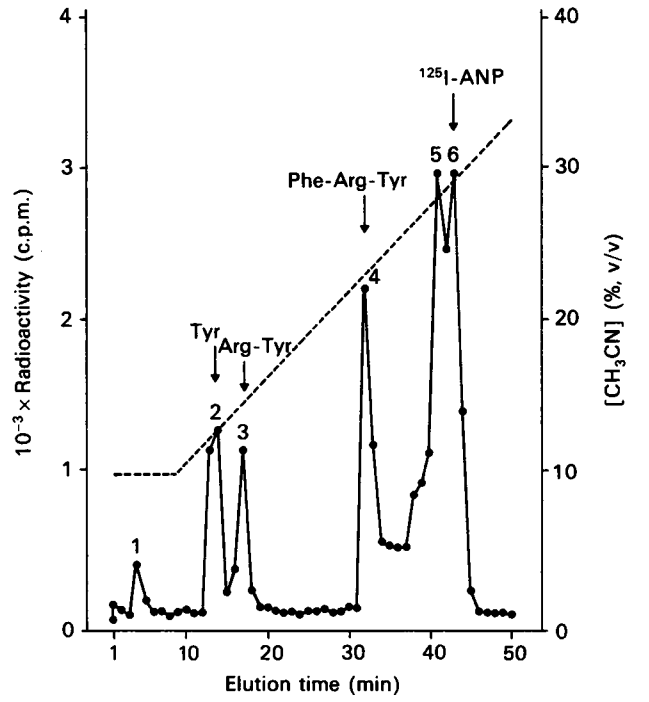


**Fig. 1. H.p.l.c. analysis of degradation fragments of $^{125}$I-ANP produced by crude microvillar kidney membranes**

Crude microvillar membranes from human kidneys (8 $\mu$g of protein/ml final concentration) were incubated for 30 min at 37 °C with $^{125}$I-ANP (approx. 15000 c.p.m./assay; 70 pM final concentration) in the presence of 100 $\mu$M-bestatin. Products were separated (●—●) on a $\mu$Bondapak $C_{18}$ column in a linear gradient of acetonitrile (10–35%, v/v) in 0.08% (v/v) trifluoroacetic acid over 45 min (------). Peak 1 corresponds to free $^{125}I_2$. Peaks 2, 3 and 4 were identified by co-elution with monoiodinated standards as indicated by the arrows; they correspond to $^{125}$I-Tyr, Arg-($^{125}$I-)Tyr and Phe-Arg-($^{125}$I-)Tyr respectively. See the Materials and methods section for further details.

## RESULTS

### Hydrolysis of radiolabelled α-hANP by human microvillar membranes

The use of $^{125}$I-labelled peptide allowed investigation of the degradation by kidney membranes at a substrate concentration approaching physiological values. Moreover, products of the *C*-terminal region of $^{125}$I-ANP could be readily detected since the only iodinated residue is the *C*-terminal Tyr-28. H.p.l.c. analyses of $^{125}$I-ANP incubated with crude microvillar membranes revealed six radioactive peaks: peak 1 corresponded to free iodine, peak 2 to iodinated Tyr, peak 3 to iodinated Arg-Tyr and peak 4 to iodinated Phe-Arg-Tyr. Peak 5 was a larger unidentified fragment and peak 6 was α-hANP (Fig. 1).

### Effect of inhibitors on the formation of radiolabelled ANP metabolites

The aminopeptidase inhibitors bestatin and amastatin decreased the formation of free tyrosine and increased the amounts of Arg-Tyr and Phe-Arg-Tyr, probably by protecting these fragments from subsequent cleavage by aminopeptidases. Puromycin was less effective in the protection of the *C*-terminal tripeptide. Captopril had no effect on the release of Arg-Tyr and Phe-Arg-Tyr, demonstrating that angiotensin-converting enzyme was not involved in these cleavages. The amounts of Arg-Tyr and Phe-Arg-Tyr were markedly decreased by phosphoramidon, indicating the involvement of endopeptidase-24.11 (Table 1). Using purified endopeptidase-24.11 we also observed cleavage of the Phe-Arg-Tyr peptide from $^{125}$I-ANP (results not shown). Dose–inhibition curves of phosphoramidon on the formation of Phe-Arg-Tyr by either the crude microvillar preparation or by purified endopeptidase-24.11 were nearly identical with an $IC_{50}$ of approx. 8–10 nM and complete inhibition at 0.3 $\mu$M-phosphoramidon (Fig. 2).

### Hydrolysis of unlabelled α-hANP by purified endopeptidase-24.11

**Identification of the products formed during long incubation periods.** Incubation of α-hANP with purified endopeptidase-24.11 for 10 h at 37 °C led to the accumulation of seven major peaks, as shown on the h.p.l.c. profile presented in Fig. 3(*a*). These peaks were collected and analysed for their amino acid content. As shown in Table 2, the fragments corresponded in their order of elution respectively to ANP-(1–4), ANP-(5–7)–(21–25), ANP-(8–11), ANP-(5–7)–(17–25), ANP-(1–7)–(21–25), ANP-(8–14) and ANP-(26–28). Amino acid analysis of peak 3 (Table 2) revealed a small amount of serine. As cysteine residues were not determined owing to their loss during acid hydrolysis, this fragment could correspond either to ANP-(6–11) or to ANP-(8–11). However, as we did not find other fragments derived from cleavage of the Ser-5–Ser-6 bond, it appears more probable that the serine found in the analysis represented a contaminant. The generation of all fragments was abolished in the presence of 1 $\mu$M-phosphoramidon (Fig. 3*b*).

The fragments identified were derived from hydrolysis at the Arg-4–Ser-5, Cys-7–Phe-8, Arg-11–Met-12, Arg-14–Ile-15, Gly-16–Ala-17, Gly-20–Leu-21 and Ser-25–Phe-26 bonds (Fig. 4).

**Table 1. Effect of inhibitors on the formation of $^{125}$I-ANP metabolites by crude microvillar membranes**

$^{125}$I-ANP was incubated as indicated in Fig. 1 legend in the presence and in the absence of proteinase inhibitors. Results are expressed as percentages of the control values obtained without inhibitors and are the means ± S.D. for three separate experiments.

| | Formation of metabolite (% of control) | | |
|---|---|---|---|
| Inhibitor | $^{125}$I-Tyr | Arg-($^{125}$I-)Tyr | Phe-Arg-($^{125}$I)-Tyr |
| Bestatin (100 μM) | 53.1 ± 6.6 | 738.3 ± 121.9 | 209.3 ± 23.8 |
| Amastatin (100 μM) | 54.4 ± 0.7 | 180.0 ± 12.0 | 988.5 ± 118.5 |
| Puromycin (100 μM) | 84.6 ± 5.7 | 248.6 ± 6.4 | 130.0 ± 9.0 |
| Phosphoramidon (100 μM) | 85.1 ± 4.9 | 36.0 ± 2.1 | 6.85 ± 1.4 |
| Captopril (100 μM) | 78.5 ± 6.3 | 102.0 ± 9.1 | 98.3 ± 7.9 |

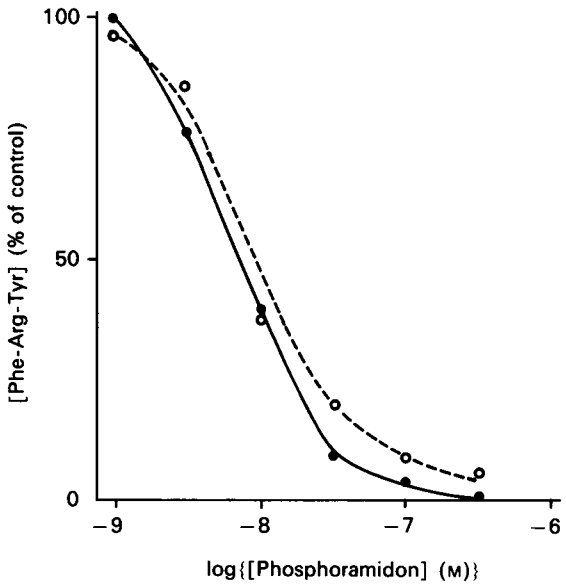


**Fig. 2. Dose–inhibition curves of phosphoramidon on the production of Phe-Arg-($^{125}$I-)Tyr by crude microvillar membranes and purified endopeptidase-24.11**

Crude microvillar membranes (●) (8 μg of protein/ml) or purified human endopeptidase-24.11 (○) (0.8 μg of protein/ml) were incubated as described in Fig. 1 legend. Results represent the means for three separate determinations.

**Identification of the first cleavage fragment produced in α-hANP hydrolysis.** During hydrolysis some peaks were formed and then disappeared, suggesting that they represent intermediary cleavage fragments. The first peak to appear (peak 8) after short incubation periods was eluted just before α-hANP in the h.p.l.c. profile (Fig. 5*a*). It had the same amino acid composition as intact α-hANP (Table 3). This finding indicates that the fragment might result from a cleavage occurring at a bond inside the disulphide-linked loop. To verify this assumption,

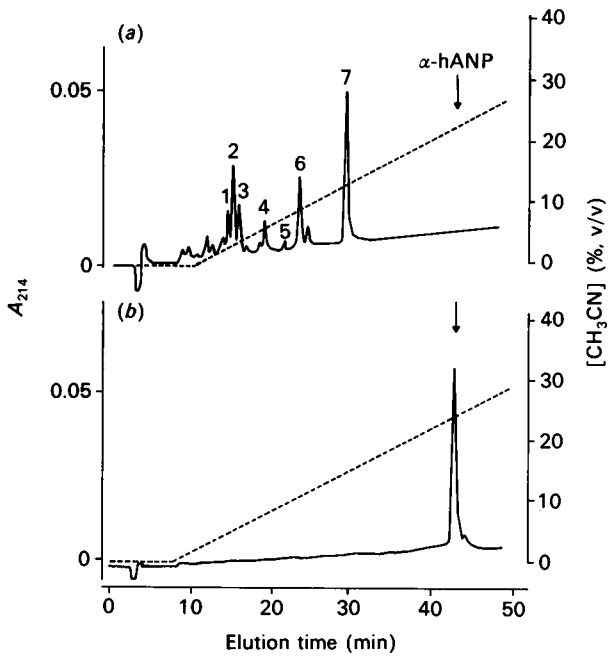


**Fig. 3. H.p.l.c. analysis of fragments of α-hANP produced by a long incubation period with endopeptidase-24.11**

α-hANP (25 μM) was incubated for 10 h at 37 °C with endopeptidase-24.11 (0.8 μg of protein/ml) and separated by h.p.l.c. in a 0–35 % (v/v) acetonitrile gradient in 0.08 % (v/v) trifluoroacetic acid over 50 min (------). (*a*) α-hANP incubated with enzyme alone. (*b*) α-hANP incubated with the enzyme in the presence of 1 μM-phosphoramidon.

the peak was collected and submitted to dithiothreitol reduction. The dithiothreitol-treated sample was then injected on the h.p.l.c. column, where it gave rise to two new peaks (Fig. 5*b*: peaks 9 and 10). Amino acid analyses of peaks 9 and 10 (Table 3) indicate that they corresponded to ANP-(1–7) and ANP-(8–28), demonstrating that the first bond cleaved was the Cys-7–Phe-8 bond.

## DISCUSSION

Atrial natriuretic peptides represent a new hormone system involved in extracellular fluid regulation. The short duration of activity of atrial peptides when injected *in vivo* has suggested a rapid metabolism. Reported half-lives varied from 17 s to 3.1 min (Tang *et al.*, 1984; Murthy *et al.*, 1986*a*; Katsube *et al.*, 1986), but all studies agreed on the rapid removal of the peptides from circulation. The sites of clearance of ANP include the kidneys (Weselcouch *et al.*, 1985; Luft *et al.*, 1986) and other vascular beds such as the mesenteric artery (Murthy *et al.*, 1986*b*). Clinical studies are also consistent with rapid metabolism of α-hANP in man (Yandle *et al.*, 1986). Crozier *et al.* (1986) have measured ANP concentrations in arterial and venous blood across the liver, kidney, lower limb and lung in patients undergoing cardiological investigation. Their results indicate little change during circulation through the lungs but a removal of the peptide by the liver, kidney and lower limb. The mechanisms of clearance are not known but

**Table 2. Identification of the end-products of α-hANP formed by incubation with endopeptidase-24.11**

A 270 nmol portion of α-hANP was incubated for 10 h at 37 °C with purified endopeptidase-24.11 (0.8 μg of protein/ml). Peaks (similar to those shown in Fig. 3) were separated by h.p.l.c. in a 0–25% (v/v) acetonitrile gradient in 0.08% (v/v) trifluoroacetic acid over 50 min. They were collected and repurified by isocratic runs for the amino acid analyses.

| | Composition (mol of residue/mol of peptide) | | | | | | |
|---|---|---|---|---|---|---|---|
| Residue | Peak 1 | Peak 2 | Peak 3 | Peak 4 | Peak 5 | Peak 6 | Peak 7 |
| Asp | 0.10 (0) | 1.04 (1) | 0.06 (0) | 0.91 (1) | 1.00 (1) | 1.07 (1) | 0.15 (0) |
| Glu | 0.10 (0) | – (–) | 0.05 (0) | 0.99 (1) | 0.27 (0) | 0.17 (0) | 0.22 (0) |
| Ser | 1.04 (1) | 2.89 (3) | 0.61 (0–1) | 3.81 (4) | 3.98 (4) | 0.11 (0) | 0.11 (0) |
| Gly | 0.14 (0) | 1.22 (1) | 2.08 (2) | 2.09 (2) | 1.43 (1) | 1.90 (2) | 0.11 (0) |
| His | – (–) | – (–) | – (–) | – (–) | – (–) | – (–) | – (–) |
| Arg | 2.16 (2) | – (–) | 0.93 (1) | – (–) | 2.04 (2) | 2.31 (2) | 1.10 (1) |
| Thr | – (–) | – (–) | – (–) | – (–) | – (–) | – (–) | 0.11 (0) |
| Ala | 0.10 (0) | – (–) | – (–) | 0.87 (1) | – (–) | 0.07 (0) | 0.11 (0) |
| Pro | – (–) | – (–) | – (–) | – (–) | – (–) | 0.01 (0) | 0.03 (0) |
| Tyr | – (–) | – (–) | – (–) | – (–) | – (–) | 0.01 (0) | 0.83 (1) |
| Val | – (–) | – (–) | – (–) | – (–) | – (–) | 0.01 (0) | 0.06 (0) |
| Met | – (–) | – (–) | – (–) | – (–) | – (–) | 0.75 (1) | – (–) |
| Cys | – (–) | – (–) | – (–) | – (–) | – (–) | – (–) | – (–) |
| Ile | – (–) | 0.08 (0) | – (–) | – (–) | – (–) | 0.01 (0) | 0.03 (0) |
| Leu | 0.80 (1) | 0.77 (1) | 0.21 (0) | 1.23 (1) | 1.86 (2) | 0.01 (0) | 0.11 (0) |
| Phe | 0.03 (0) | – (–) | 0.83 (1) | – (–) | – (–) | 0.97 (1) | 1.06 (1) |
| Lys | – (–) | – (–) | – (–) | – (–) | – (–) | 0.03 (0) | – (–) |
| Identification | (1–4) | (5–7)–(21–25) | (8–11) | (5–7)–(17–25) | (1–7)–(21–25) | (8–14) | (26–28) |

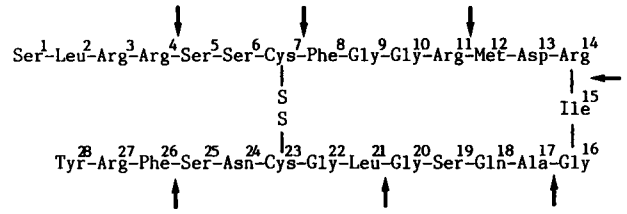


**Fig. 4. Sequence of α-hANP and identification of the bonds hydrolysed by endopeptidase-24.11**

The sites of cleavage are indicated by arrows.

could include breakdown by enzymes, binding to receptors and transfer to extravascular spaces.

The kidney, which is a target organ for atrial natriuretic peptides, is thought to play a major role in the degradation of many peptidic hormones since the brush border of the proximal-tubule epithelial cells contains ectoenzymes facing the extracellular medium (for a review see Kenny & Maroux, 1982). These enzymes include aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), dipeptidyl peptidase IV (EC 3.4.14.5), angiotensin-converting enzyme (peptidyl dipeptidase A, EC 3.4.15.1), endopeptidase-24.11 (enkephalinase, EC 3.4.24.11) and the recently discovered aminopeptidase W (Gee & Kenny, 1987). The human kidney has been shown to possess the same enzymic equipment (Abbs & Kenny, 1983).

Our results concerning the degradation of $^{125}$I-ANP by human kidney microvillar membranes showed cleavages in the *C*-terminal sequence producing Tyr, Arg-Tyr and Phe-Arg-Tyr (Fig. 1). It was clear from the inhibitory effect of phosphoramidon (Table 1 and Fig. 2) that the generation of Phe-Arg-Tyr was attributable solely to endopeptidase-24.11. This fragment was further processed by bestatin-sensitive aminopeptidase(s), possibly a combination of aminopeptidase N and aminopeptidase W, generating Arg-Tyr and Tyr. The removal of the *C*-terminal tyrosine residue did not appear to be due to a carboxypeptidase action since 3-guanidinoethylthio-2-mercaptomethylpropanoic acid was without effect (results not shown). The residual formation of $^{125}$I-tyrosine might be due to the action of bestatin-insensitive aminopeptidase(s) possibly contaminating the crude microvillar preparation. Removal of the *C*-terminal dipeptide Arg-Tyr was not produced by angiotensin-converting enzyme since captopril had no effect (Table 1). However, when the *C*-terminal pentapeptide fragment of α-hANP was incubated with the membrane preparation we observed cleavage at the Phe–Arg bond under the action of angiotensin-converting enzyme (results not shown), suggesting that the conformation of native α-hANP was probably responsible for the failure to hydrolyse the Phe–Arg bond. The lack of effect of angiotensin-converting enzyme (Stephenson & Kenny, 1987*b*; the present study) agree well with experiments carried out *in vivo* showing that angiotensin-converting enzyme did not inactivate the peptide during its passage through the lungs (Weselcouch *et al.*, 1985).

The observation made by Stephenson & Kenny (1987*b*) that endopeptidase-24.11 was the enzyme producing the initial breakdown of α-hANP in pig kidney membranes may be extended to the human kidney. Using endopeptidase-24.11 purified close to homogeneity from human kidneys (Deschodt-Lanckman *et al.*, 1988*a*) we observed hydrolysis of α-hANP at seven peptide bonds (Fig. 4). Endopeptidase-24.11 cleaves specifically to the amino side of hydrophobic amino acid residues (Kerr & Kenny, 1974). This specificity has been verified not only for the enkephalins but also for the cleavage of substance P (Matsas *et al.*, 1983), neurotensin (Skidgel *et al.*, 1984), cholecystokinin octapeptide (Matsas *et al.*, 1984; Naj-

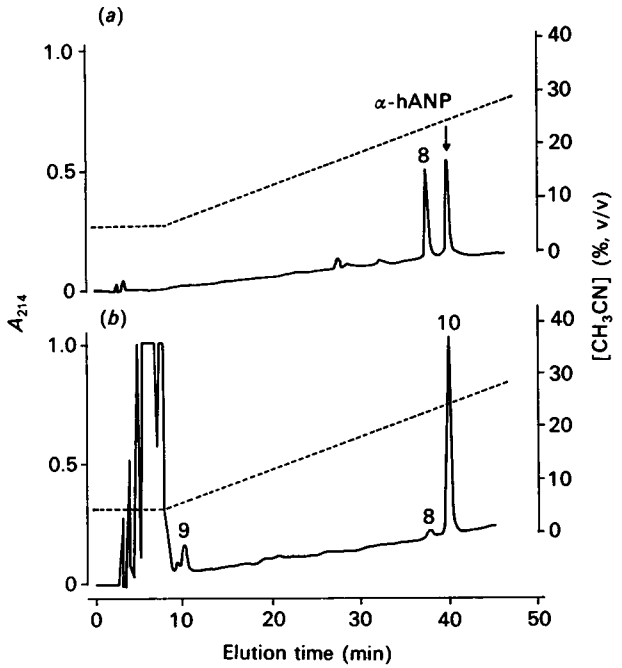


**Fig. 5. H.p.l.c. analysis of fragments of α-hANP produced by a short incubation period with endopeptidase-24.11**

α-hANP (300 μM) was incubated for 2 h at 37 °C with endopeptidase-24.11 (0.8 μg of protein/ml) and separated by h.p.l.c. in a 5–35 % (v/v) acetonitrile gradient in 0.08 % (v/v) trifluoroacetic acid over 50 min (------). (*a*) α-hANP incubated with the enzyme. (*b*) Fragments produced after reduction of peak 8 by 10 mM-dithiothreitol.

dovski *et al.*, 1985; Zuzel *et al.*, 1985) and gastrin heptadecapeptide (Deschodt-Lanckman *et al.*, 1988*a*). In the α-hANP sequence, cleavages at the Cys-7–Phe-8, Arg-11–Met-12, Arg-14–Ile-15, Gly-20–Leu-21 and Ser-25–Phe-26 bonds fulfil this requirement. Hydrolysis at the Gly-16–Ala-17 bond was unusual but had already been observed by Stephenson & Kenny (1987*b*) for the pig enzyme. However, cleavage at the Arg-4–Ser-5 bond was unexpected. The formation of the peptidic fragments ANP-(1–4) (peak 1), ANP-(5–7)–(21–25) (peak 2) and ANP-(5–7)–(17–25) (peak 4) derived from cleavage at the Arg-4–Ser-5 bond was completely abolished by 1 μM-phosphoramidon, suggesting that they were indeed produced by endopeptidase-24.11. It is possible that the nearby disulphide bridge has created a local hydrophobic conformation allowing the $S_1'$ subsite of the enzyme to interact with this structure near to Ser-5.

As previously described by Stephenson & Kenny (1987*a*,*b*), α-hANP was degraded by pig microvillar membranes at a rate comparable with that of substance P, bradykinin and angiotensins. Initial attack of α-hANP by endopeptidase-24.11 took place at a bond within the loop and produced a peptide, referred to as α-hANP′, having the same amino acid composition as intact ANP. We also found this metabolite (peak 8; Table 3). After reduction with dithiothreitol, ANP-(1–7) and ANP-(8–28) were generated (Table 3), indicating that the first bond cleaved was the Cys-7–Phe-8 bond. It may be noted that a similar fragment (ANP cleaved at the Cys-7–Phe-8 bond) was obtained as a predominant product when rat ANP was incubated with rat kidney-cortex membranes (Koehn *et al.*, 1987). During long incubation periods some minor peaks eluted before peak 1 (Fig. 3) appeared, but were not identified in our study and could correspond to small peptidic fragments such as Ile-Gly or Met-Asp-Arg.

Not surprisingly, the hydrolysis of seven peptide bonds in α-hANP (Fig. 4) by human endopeptidase-24.11 produced biologically inactive fragments, since the integrity of the disulphide-linked loop is required for full

**Table 3. Identification of the first bond hydrolysed in α-hANP by endopeptidase-24.11**

A 270 nmol portion of α-hANP was incubated for 2 h at 37 °C with endopeptidase-24.11 (0.8 μg of protein/ml). Peak 8 obtained after h.p.l.c. separation (see Fig. 5*a*) was further incubated with dithiothreitol, and the fragments generated (peaks 9 and 10, Fig. 5*b*) were collected and repurified for the amino acid analyses.

| | Composition (mol of residue/mol of peptide) | | | |
|---|---|---|---|---|
| Residue | ANP standard | Peak 8 | Peak 9 | Peak 10 |
| Asp | 1.78 (2) | 2.00 (2) | – (–) | 1.77 (2) |
| Glu | 1.11 (1) | 1.20 (1) | – (–) | 1.16 (1) |
| Ser | 4.56 (5) | 4.90 (5) | 2.82 (3) | 1.93 (2) |
| Gly | 5.10 (5) | 5.30 (5) | 0.20 (0) | 5.17 (5) |
| His | – (–) | – (–) | – (–) | – (–) |
| Arg | 5.28 (5) | 4.90 (5) | 2.08 (2) | 3.05 (3) |
| Thr | – (–) | – (–) | – (–) | – (–) |
| Ala | 1.09 (1) | 0.98 (1) | – (–) | 1.06 (1) |
| Pro | – (–) | – (–) | – (–) | 0.17 (0) |
| Tyr | 1.13 (1) | 0.78 (1) | – (–) | 1.01 (1) |
| Val | – (–) | – (–) | – (–) | 0.10 (0) |
| Met | 0.94 (1) | 0.62 (1) | 0.32 (0) | 0.93 (1) |
| Cys | – (–) | – (–) | – (–) | – (–) |
| Ile | 0.90 (1) | 0.72 (1) | – (–) | 1.01 (1) |
| Leu | 2.54 (2) | 1.90 (2) | 0.90 (1) | 1.24 (1) |
| Phe | 2.27 (2) | 2.00 (2) | – (–) | 2.05 (2) |
| Lys | – (–) | – (–) | – (–) | 0.10 (0) |
| Identification | (1–28) | (1–28) | (1–7) | (8–28) |

activity. The linear fragment ANP-(8–27) had a potency two to three orders of magnitude lower than that of ANP-(5–27), which contains the intact cyclic structure (Schiller *et al.*, 1985). Moreover, removal of the *C*-terminal tripeptide also diminished some of the biological activities of ANP (Sugiyama *et al.*, 1984).

The role of endopeptidase-24.11 may not be limited to the degradation of circulating α-hANP since the enzyme is also present in brain where it has a physiological role in the degradation of enkephalins (Schwartz *et al.*, 1981) and possibly substance P (Littlewood *et al.*, 1988). Moreover, the enzyme is at least in part responsible for the inactivation of $^{125}$I-ANP *in vitro* by hypothalamic and choroid-plexus membranes (Deschodt-Lanckman *et al.*, 1988*b*). Determination of all the bonds cleaved in α-hANP by this endopeptidase will prove useful for the design of more stable analogues that could have a therapeutic use in systemic or intracranial hypertension.

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