Volume 220, number 1, 214-216 FEB 05002 August 1987

In vitro processing of $rANF_{7-28}$-NH_2 by rat kidney homogenates

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Received 27 June 1987

The major rat kidney in vitro degradation products of the rat atrial natriuretic factor analog, H-Cys[7]-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr[28]-NH_2 (with a Cys^{27}-Cys^{23} disulfide bridge), have been identified. The degradation products are the C-terminal modified compounds $rANF_{7-27}$, $rANF_{7-26}$, and $rANF_{7-25}$.

Atrial natriuretic factor; Proteolytic degradation; (Rat kidney)

1. INTRODUCTION

The discovery of a potent family of vasodilatory, natriuretic and diuretic peptides (collectively referred to as atrial natriuretic factors) has led to considerable research activity (reviews [1-4]). However, from a therapeutic standpoint, one of the main drawbacks of ANF is the relatively short duration of its biological action (< 30 min). The short duration of action is most likely attributable to metabolism of the active peptide. Therefore, an attractive approach to overcome the stability problem is to design analogs that resist proteolysis but maintain full biological activity. This report details our initial attempts to identify the 'weak links' (proteolytic sites) present in the rANF analog $rANF_{7-28}$-NH_2, a shortened carboxypeptidase stabilized analog (residues 1-6 are deleted from the endogenous form) that shows full biological activity. We have examined kidney preparations because this tissue is both a potent source of proteolytic activity [5,6] and a target of ANF action [7].

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Abbreviation: rANF, rat atrial natriuretic factor

2. MATERIALS AND METHODS

2.1. *Peptide*

$rANF_{7-28}$-NH_2 was prepared by the solid-phase method [8] (details to be reported elsewhere).

2.2. *Enzymatic degradation*

For the degradation studies, rat kidneys were excised, minced and homogenized in ice cold 50 mM sodium phosphate-buffered saline (10 ml/kidney) at pH 7.4. The homogenate was centrifuged for 20 min at 48 000 × *g* at 4°C. The supernatant (100 μl) was added to 10 nmol $rANF_{7-28}$-NH_2 (freeze-dried powder), and incubated at 37°C for various times. Enzymatic activity was destroyed by dipping the sample tubes into boiling water for 2 min. The tubes were centrifuged at 4000 rpm for 2 min. The supernatants (50 μl) were then subjected to reverse phase HPLC [column: Vydac 218TP54 (25 cm × 4.6 mm ID), with a Macrosphere 300 Å C-4, 7 μm,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

guard column (1 cm × 4.6 mm ID)]. Mobile phase: A = H_2O/CH_3CN (85/15) containing 0.01 M ammonium acetate and B = H_2O/CH_3CN (50/50) containing 0.01 M ammonium acetate. A linear gradient was run from 0% B to 60% B over 30 min at a flow rate of 1.5 ml/min. For structure identification fractions from four separate 10 min incubations were pooled, freeze dried and analyzed.

2.3. *Amino acid sequence analysis*

Automated Edman degradations were performed on a model 470A protein-peptide sequencer (Applied Biosystems) with reagents, instructions, and standard programs supplied by the manufacturer. The phenylthiohydantoin (PTH)-derivatized amino acids were analyzed at each cycle on a model 120 PTH analyzer (Applied Biosystems) directly on-line with the sequencer.

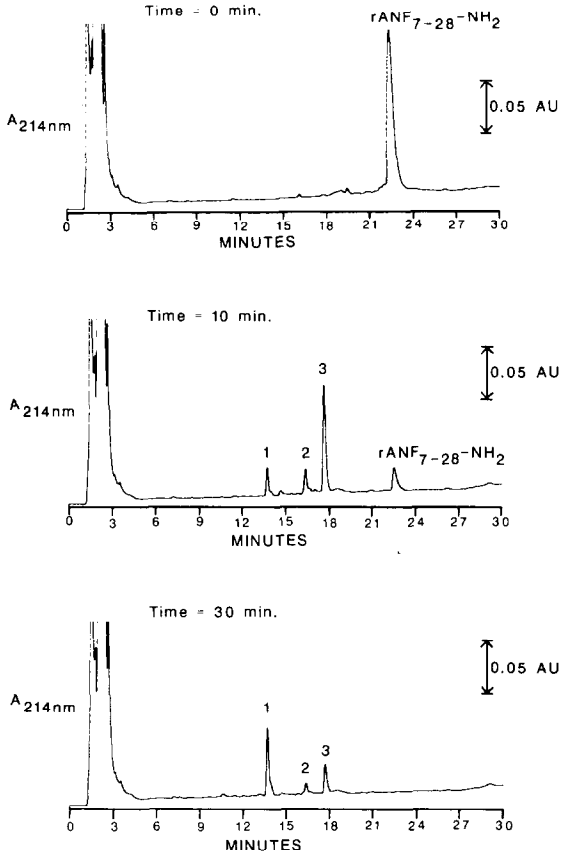

Fig.1. HPLC chromatogram of $rANF_{7-28}$-NH_2 after incubation with kidney homogenates.

Table 1

Peak	Peptide	[M + H] + calc[a]	[M + H] + obs[b]
1	$rANF_{7-25}$	1908	1908
2	$rANF_{7-26}$	2055	2055
3	$rANF_{7-27}$	2211	2211

[a] The calculated and observed [M + H] are reported using nominal masses

[b] VG analytical ZAB high field mass spectrometer operating at V_{acc} = 8 kV for 3000 mass range at full sensitivity

3. RESULTS AND DISCUSSION

The degradation of $rANF_{7-28}$-NH_2 by rat kidney homogenates proceeds quite rapidly ($t_{1/2}$ ~3.5 min). The HPLC elution profile of $rANF_{7-28}$-NH_2 after incubation with rat kidney homogenates shows several distinct metabolites (fig.1). These compounds, identified by amino acid analysis, sequencing and FAB-MS (table 1), are $rANF_{7-27}$, $rANF_{7-26}$, and $rANF_{7-25}$. These products most likely result from a trypsin-like cleavage at Arg^{27} (liberating Tyr-NH_2) and subsequent carboxypeptidase action. The involvement of a specific metallo dipeptidyl dipeptidase [9,10] for cleaving $rANF_{7-27}$ to $rANF_{7-25}$ appears plausible in this rat kidney preparation (note relatively reduced quantity of $rANF_{7-26}$). Previous structure-activity studies have demonstrated an order of magnitude loss in potency with the successive removal of Arg^{27} and Phe^{26} [11]. We conclude that the critical proteolysis of $rANF_{7-28}$-NH_2 that results in a relatively inactive rANF fragment occurs at the Phe^{26}-Arg^{27} linkage. We are currently examining the degradation profiles of $rANF_{7-28}$-NH_2 with various tissue preparations and peptidase inhibitors.

ADDENDUM

While our manuscript was in preparation reports describing ANF degradation by kidney brush border membrane enzymes were published [12,13]. Our results are qualitatively different and reflect degradation by soluble proteases present in rat kidney extracts.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the assistance of Drs Keith Robinson and Stephen Buck (kidney preparation), Dr Roger Sargent (amino acid analysis), Dr John Coutant (FAB-MS), and Charlene Mercer and Mary Dooley (manuscript preparation).

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