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Primary structure of a high M_r form of rat atrial natriuretic factor

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During the purification of rat atrial natriuretic factor (ANF), low, intermediate and high M_r forms were observed. In this report we describe the purification and amino acid sequence of a 73 residue peptide containing at its C-terminus the previously sequenced 33 amino acid ANF peptide. The cleavage necessary to produce the 33 amino acid ANF from the 73 amino acid precursor occurs at a Leu–Leu bond. We also report the amino acid composition of an even longer form of ANF containing about 103 residues, in which the extension is amino terminal to the 73 peptide. A computer data bank search showed that the determined sequence is a novel one and is not homologous to any known proteins or segment thereof. The natriuretic activity of the 73 amino acid form when compared to that of a synthetic ANF peptide, comprising the sequence of the last 26 amino acids of ANF, was found to be slightly lower.

Atrial natriuretic factor *Natriuresis* *Diuresis* *Peptide purification* *Propeptide*

1. INTRODUCTION

During the last year, a large effort was devoted to the purification and chemical characterization of the rat natriuretic factor from homogenates of heart atria. We recently reported the isolation and amino acid sequence of 4 homologous low M_r peptides containing the active natriuretic sequence and showed that the native and synthetic replicas exhibited comparable biological activity. These short forms of ANF contained 33, 32, 31, and 26 amino acids representing amino terminal truncated versions of the 33 amino acid form [1,2]. Two other groups of investigators have as yet only reported the amino acid composition of similar ANF peptides and no sequence data are yet available for comparison [3,4].

As previously reported [1,2], elongated forms of ANF were observed during the purification of the rat atrial natriuretic factor. These are now further subdivided into intermediate and high M_r forms of ANF based on amino acid composition and molecular sieving chromatography. We present the complete purification and sequence analysis of one of the intermediate forms and show that it is composed of 73 amino acids in which the amino terminal 40 amino acids precede the sequence of the previously reported 33 peptide. Furthermore, preliminary amino acid analysis and sequence suggest that one of the high M_r forms of ANF could contain up to 103 amino acids in which the 33 ANF peptide is again at the C-terminus.

2. MATERIALS AND METHODS

2.1. *Biological assay*

The natriuretic activity of the native and synthetic peptides was measured by a biological assay as in [5,6]. Briefly the native or synthetic ANFs were injected as a bolus of 1 ml in Krebs solution (pH 7.4) via a catheter inserted in the jugular vein of 200 g female Sprague-Dawley rats. Urine was collected for a 20 min period in preweighed vials. A constant infusion of 5% dextrose (2.1 ml/h) was given throughout the experiment. Results are expressed as μeq. Na excreted for a collection period

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of 20 min from which the sodium excretion of the control period has been subtracted.

2.2. *Purification of the high* M_r *forms of ANF*

The homogenization of 48 g of atria (650 rats), extraction of ANF by C_{19} Sep-Pak cartridges and gel filtration on Bio-Gel P-10 were done as in [1] except that the homogenisation buffer contained 5 mM instead of 1 mM EDTA. From the Bio-Gel P-10 column, 3 successive regions of different M_r containing active material were pooled. These regions were designated low (4000–6000), intermediate (6000–10000) and high (10000–15000) M_r forms. Each region was then further purified separately using a CM-Bio-Gel A column, a Mono S HR5/5 column and a CN μ-Bondapak column followed by a C_{18} μ-Bondapak column as in [1]. Final purification was achieved on a C_{18} μ-Bondapak column eluted with 0.13% (v/v) heptafluorobutyric acid and acetonitrile.

2.3. *Amino acid and sequence analysis*

Amino acid analyses of the native or reduced and carboxymethylated peptides were done in duplicate following hydrolysis in 5.7 N HCl in vacuo at 108°C for 24 h. The separation and quantitation of the amino acids were done as in [2].

The amino terminal Edman degradation on the reduced and carboxymethylated elongated form of ANF was performed using a 0.3 M Quadrol program and 3 mg Polybrene (Aldrich) [7] on a Beckman 890C sequenator equipped with a Sequemat P-6 autoconverter and a model SC-510 controller. Phenylthiohydantoins (PTHs) were identified and quantitated by HPLC [8], using a Varian 5500 liquid chromatograph equipped with a Vista 402 plotter/integrator.

3. RESULTS AND DISCUSSION

A large degree of peptide heterogeneity was observed during the various purification steps of the atrial natriuretic factor. At least 15 related peptides could be purified from 48 g of atria obtained from 650 rats. Initial molecular sieving on Bio-Gel P-10 allowed the crude subdivision of the various active molecular entities into low, intermediate and high M_r forms of ANF. The low M_r forms have been previously shown by sequence to be composed of at least 4 amino terminal truncated versions of a parent 33 amino acid ANF peptide [1,2]. All these homologous forms were shown to be highly active and of similar potency to a synthetic replica of ANF 8-33 [2].

From the intermediate and high M_r pools we were able to detect 7 elongated forms of ANF. Since the recovered amounts of most of these peptides were too low to allow further sequence characterization only 2 peptides obtained in yields between 50 and 100 μg were analysed extensively. These longer forms of ANF purified by reverse-phase HPLC on a C_{18} μ-Bondapak column using the trifluoroacetic acid/acetonitrile system [1] eluted around 35% acetonitrile, whereas the previously reported shorter forms [1,2] eluted between 22 and 26% acetonitrile. Final purification of the longer forms of ANF was achieved on a C_{18} μ-Bondapak column eluted with the heptafluorobutyric acid/acetonitrile system. The two peptides so purified are denoted ANF-H1 and ANF-H2. Fig.1 illustrates the elution profile of ANF-H1 in this final purification step. In this elution system, basic peptides are known to elute at a higher acetonitrile concentration as compared to the trifluoroacetic acid system. Indeed, ANF-H1 and ANF-H2 elute around 40% acetonitrile.

Table 1 gives the amino acid composition of

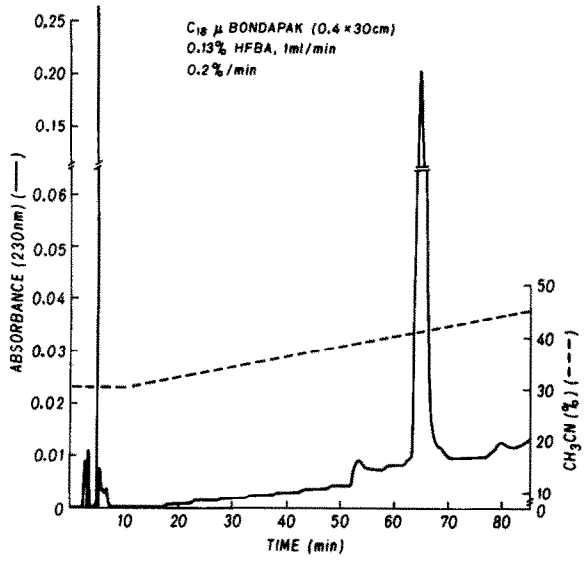

Fig.1. Chromatography of the peptide ANF-H1 on C_{18} μ-Bondapak column eluted with 30–50% acetonitrile in 0.13% heptafluorobutyric acid at a slope of 0.2%/min and a flow rate of 1 ml/min.

Table 1

Amino acid composition of ANF peptides

Amino acid	ANF-H1	ANF-H1 SEQ[a]	ANF-H2	ANF 1-33
Asx	6.42(6)	6	8.32(8)	2
Thr	1.05(1)	1	1.09(1)	0
Ser	8.42(9)	9	8.95(9)	5
Glx	4.42(4)	4	11.12(11)	1
Pro	6.69(6–7)	6	9.02(9)	1
Gly	10.69(11)	11	10.38(10)	6
Ala	5.49(5)	5	8.89(9)	2
Val	2.05(2)	2	3.98(4)	0
Met	0 (0)	0	1.55(2)	0
Ile	1.91(2)	2	2.00(2)	2
Leu	8.19(8)	8	11.99(12)	3
Tyr	0.91(1)	1	1.08(1)	1
Phe	2.13(2)	2	2.34(2)	2
His	0.17(0)	0	0.32(0)	0
Lys	1.53(1–2)	2	3.19(3)	0
Arg	9.45(9–10)	10	10.44(10)	6
Cys[b]	n.d.	2	2.2 (2)	2
Trp	n.d.	2	n.d.	0
Total	67–70[c]	73	95[c]	33

[a] ANF-H1 SEQ, the calculated ANF-H1 amino acid composition based on the determined sequence. This also applies to the values of ANF 1-33 obtained in [2]

[b] For ANF-H2 the Cys content was quantitated as carboxymethylcysteine. Otherwise for ANF-H1 the Cys was not determined (n.d.) since the composition was made on the native peptide

[c] This represents the calculated presumed total number of amino acids excluding Trp and Cys for ANF-H1 and excluding Trp for ANF-H2. These values have to be confirmed by sequence

Numbers in parentheses represent the nearest integer

both ANF-H1 and ANF-H2, together with that of ANF 1-33 deduced from its previously reported sequence [2]. First, it can be seen that ANF-H1 and ANF-H2 are composed of approx. 70 and 95 amino acids, respectively. Furthermore, the presence of the same proportions of Ile, Tyr and Phe as compared to ANF 1-33 suggests that these peptides could represent elongated forms of ANF. However, the composition does not allow precise localization of the extension nor does it provide irrevocable proof that these peptides are indeed elongated forms of ANF. For this purpose only amino acid sequencing can answer these questions unambiguously.

Fig.2 shows the identification and yield of the various PTH-amino acids obtained during the sequence of ANF-H1. The repetitive and initial yield for this sequence were computed from the linear regression line to be 94.2% and 4.97 nmol respectively with a correlation coefficient of 0.959. Clearly, this sequence proves that ANF-H1 represents an N-terminal extended form of ANF. This is evidenced by an 18 amino acid overlapping sequence starting at residue 41 of ANF-H1 which is identical to the first 18 amino acids of ANF 1-33 [2], as illustrated in fig.3. Based on the amino acid compositions of ANF-H1 and ANF 1-33 (table 1), together with the known presence of a single C-terminal Tyr in ANF 1-33, and the sequence shown in fig.2, it can be concluded that ANF-H1 is composed of 73 amino acids of which the last 32 amino acids (residues 41–73) represent the previously described sequence of ANF 1-33 (fig.3). Therefore, the obtainment of ANF 1-33 from ANF-H1 involves a cleavage between Leu_{40}–Leu_{41}. Such a Leu cleavage was also previously observed to occur within ANF 1-33, yielding the N-terminal truncated versions ANF 2-33 and ANF 8-33 [22].

Preliminary sequence data on ANF-H2 revealed that this peptide represents an N-terminally 30 amino acid extended form of ANF-H1, giving a total of 103 amino acids, thus providing evidence as to the relatedness of ANF-H2 to ANF 1-33. This result agrees reasonably well with the amino acid composition of ANF-H2 (table 1).

In order to verify the relatedness of the sequence of the 73 amino acid ANF-H1 to that of any known peptides or proteins and segments thereof, a computer data bank search was performed using the National Biomedical Foundation Mutation data Matrix program and Sequence Data Bank, Georgetown University, Washington, DC. When compared to the 2700 protein sequences in the data bank, no significant homology (>30%) was found. This confirms the novel nature of the sequence determined, similar to what was originally observed for the sequence of ANF 1-33 [2].

The natriuretic activity of the 73 amino acid ANF-H1 is compared to the synthetic ANF 8-33 [2] (amino acids 48–73 in fig.3) in fig.4. As expected ANF 8-33 gives a dose-response curve which levels off around 1000 pmol to produce a natriuretic

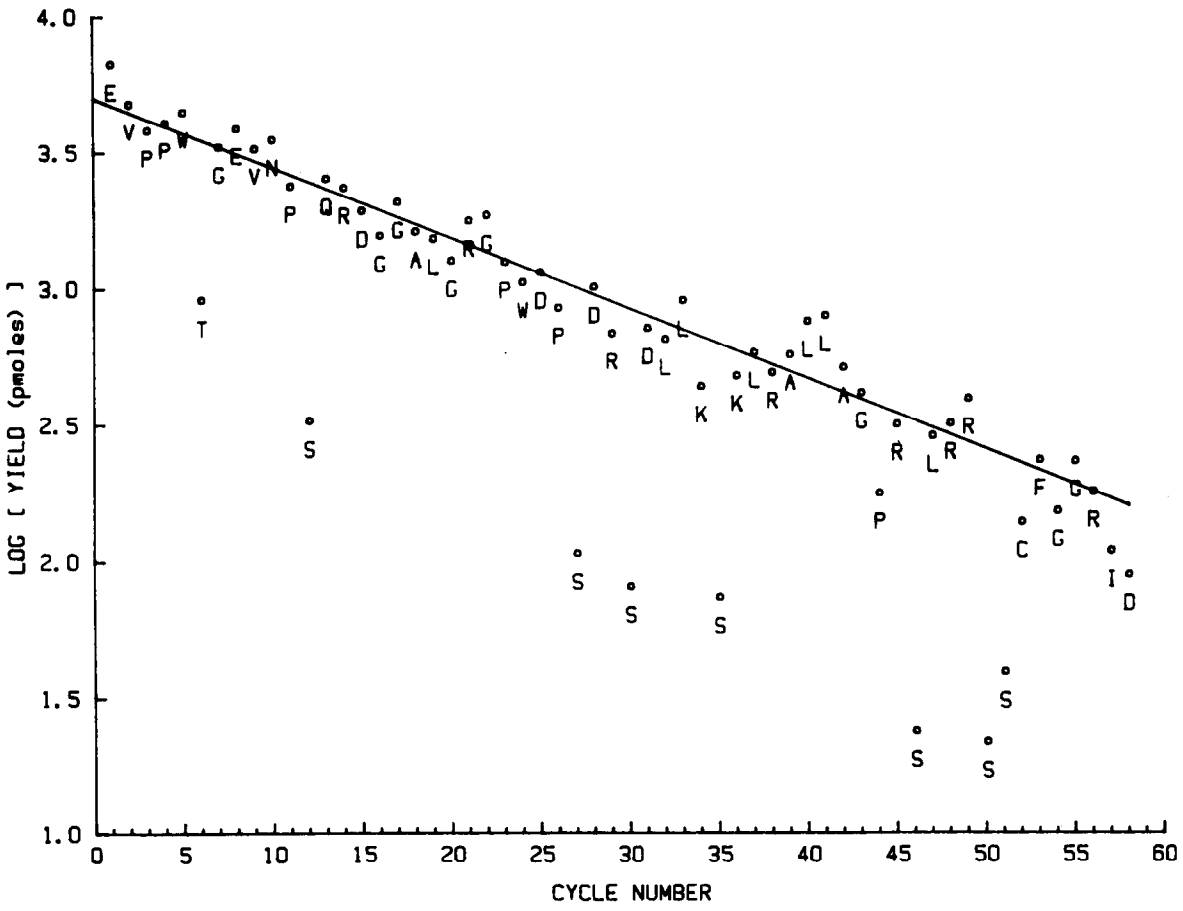

Fig.2. Automatic NH_2-terminal degradation of the ANF-H1 peptide. Quantitative yields of PTH-amino acids normalized to a PTH-norleucine internal standard are illustrated as a function of residue numbers. The slope and intercept were obtained by a linear regression analysis on selected stable PTH-amino acids giving the repetitive yield and initial yield respectively.

response of 350 μeq. Na/20 min. For comparison, only two doses of ANF-H1 could be used due to scarcity of the material. The observed natriuretic activity of ANF-H1 appears to be slightly lower than that of ANF 8-33 for the doses used (fig.4). It is rather surprising that this 73 amino acid form of ANF should cause a similar natriuretic effect to that of ANF 1-33. A possible explanation for this observed behavior could be the proteolysis of ANF-H1 in circulation once injected into the animal thereby releasing the active moiety. Alternatively, the 40 residue N-terminal extension may not change the conformation of the active sequence enough to cause the loss of biological activity. These hypotheses would, however, have to be verified experimentally.

In conclusion, it can now be stated that the atrium contains a potent natriuretic factor which is synthesized in the form of a precursor molecule containing at least 103 amino acids, of which the C-terminal 26 amino acids are enough to exhibit the potency of the observed natriuresis. Further work will be necessary to complete the amino acid sequence of the original mRNA translation form of ANF, which will be easier to achieve by molecular cloning. For this purpose the sequence of ANF-H1 provided should be useful to devise nucleotide probes for hybridization experiments

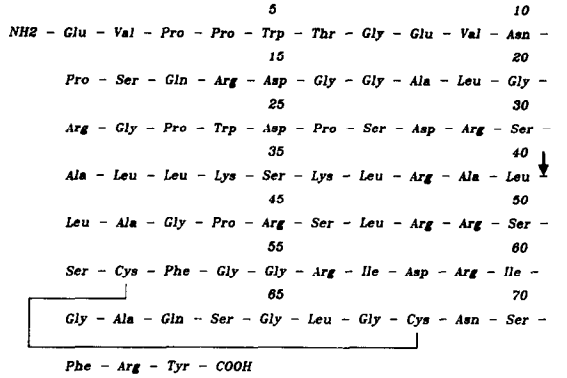

Fig.3. Complete amino acid sequence of ANF-H1 peptide. The arrow points to the junction between the first 40 amino acids and the beginning of the sequence of ANF 1-33 [2], emphasizing the Leu_{40}–Leu_{41} cleavage necessary in order to produce ANF 1-33. The disulfide bridge between cysteines occupying positions 52 and 68 is also indicated.

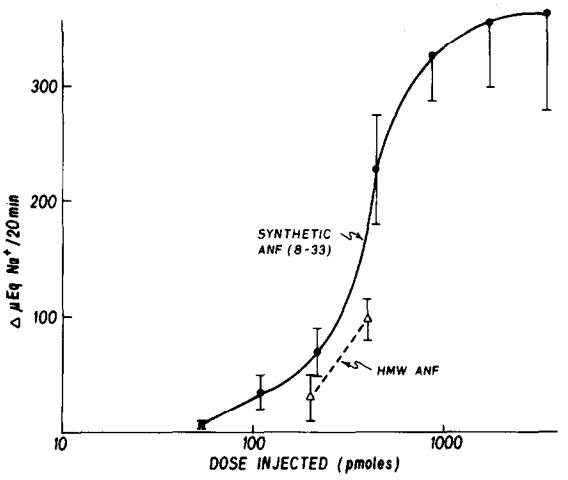

Fig.4. Dose-response curve of synthetic ANF (8-33) and of ANF-H1 (high M_r ANF) on natriuresis. Each dose is the mean (± SE) of at least 3 individual determinations.

aimed at the obtainment of a cDNA probe for ANF. This is especially evident from the presence of two strategically located tryptophan residues within the first 25 amino acid sequence of ANF-H1.

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