Atrial pronatriodilatin: a precursor for natriuretic factor and cardiodilatin

Amino acid sequence evidence

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Numerous peptides isolated from rat heart atria, including two containing 33 and 73 amino acids, were isolated and shown to exhibit natriuretic activities. Here, we describe the purification and partial amino acid sequence of a 106-residue peptide containing the previously sequenced 33- and 73-amino-acid ANF peptides. The determined sequence is a novel one and is not significantly homologous to any known protein or segment thereof. In fact, this sequence shows significant homology only to another novel partial sequence obtained from sequence analysis of a porcine peptide, called cardiodilatin, also found in heart atria. This relationship is taken as evidence that ANF and cardiodilatin are part of the same precursor molecule which would contain at the very least 126 amino acids.

Pronatriodilatin *Cardiodilatin* *Amino acid sequence* *Precursor*

1. INTRODUCTION

The presence in mammalian heart atria homogenates of numerous potent peptides possessing natriuretic and smooth muscle relaxant activities is now firmly established. Indeed, gel filtration separations have indicated that biological activities could be recovered in both high- and low-M_r material [1–5]. Chemical characterization including amino acid sequence determination has been successfully accomplished by many laboratories on the low-M_r peptides exhibiting natriuretic properties. Indeed, the atrial natriuretic factor (ANF) was shown by amino acid sequencing and by chemical synthesis to correspond to a peptide containing one disulfide bridge and comprising between 21 and 33 amino acids [6–9]. Moreover, all structures shown share the same common amino acid sequence except in the case of the human ANF (in preparation) [9] which contains a methionine residue instead of the isoleucine found in the rat ANF [6–8]. Also, in all cases, the isolated native peptide and its synthetic replica exhibited comparable biological activities. All these forms represent an amino-terminal truncated version of the 33-amino-acid peptide [6,10], except in the case of the peptides reported in [8] which are also truncated at the carboxy-terminal.

However, size heterogeneity of the biologically active molecule was observed in most studies and, in some of them, the presence of larger molecules of active material was reported [1–5]. Recently, the amino acid sequence of a 73-residue peptide, together with evidence of an even larger one, both containing at their C-termini the previously sequenced 33-amino-acid ANF peptide, were reported [3]. Moreover, demonstration of a heat-labile factor responsible for the conversion of high-M_r to low-M_r natriuretic activity was accomplished in rat atria [11] thus adding weight to the proposal that the low-M_r ANF is contained in a much larger protein or precursor.

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Interestingly, authors in [12] reported recently the presence in pig heart atria of a new low-M_r peptide which exhibits vasodilator effects but does not stimulate diuresis. Indeed this peptide, tentatively named 'cardiodilatin', has an M_r value of about 7500 and exhibits a strong smooth muscle relaxant activity when tested upon the aorta or renal artery. Here again, as in the case of ANF, numerous peptides present in the extract are able to exhibit a relaxing activity upon vascular smooth muscle. Here we present the purification and partial sequence analysis of the second high-M_r form identified (ANF-H2) in [3]. Furthermore, we show that it is composed of 106 amino acids in which the first 33 residues precede the 73 amino acids in ANF-H1. Moreover, we discuss the relationship of the presented sequence of ANF-H2 with the first 30-amino-acid sequence reported for cardiodilatin and propose that ANF and cardiodilatin [12] are part of the same precursor molecule. Both of these moieties, seemingly exhibiting two different biological activities, are thus contained in a protein or precursor having at the very least 126 amino acids.

2. MATERIALS AND METHODS

2.1. *Biological assay*

The natriuretic activity of the purified peptide was determined in 200 g anesthetized female Sprague-Dawley rats as in [2,13]. Results were calculated as μeq. Na^+ excreted/20 min.

A chick rectum assay [5] was used to determine the vasorelaxant activity. Results were expressed in comparison with synthetic ANF.

2.2. *Purification of the ANF-H2 form*

Purification of the ANF-H2 form from 48 g atria (650 rats) was accomplished as in [3]. 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 reduced and carboxymethylated ANF-H2 were done in duplicate following hydrolysis in 5.7 N HCl in vacuo at 108°C for 24 h. Separation and quantitation of the amino acids were done as in [3].

Automatic amino-terminal Edman degradation on the reduced and alkylated ANF-H2 was performed using a 0.33 M Quadrol program on a Beckman 890C sequenator equipped with a Sequemat P6 autoconvertor and a model SC-510 controller. Prior to sequencing, two precycles were run to clean the Polybrene (3 mg, Aldrich). Modifications to the running order of cycles included a double coupling procedure on cycle 1 to increase the initial yield and a double cleavage procedure following two previously localized proline residues at cycles 4 and 12. Furthermore, in an effort to decrease the background amount of phenylthiohydantoin (PTH) amino acid derivatives and to eliminate minor contaminating protein sequences, blockage of all the primary amino groups was accomplished prior to cycle 4. As suggested originally in [14], this can be done at a cycle corresponding to a proline residue by using a reagent, such as fluorescamine, reacting irreversibly with free primary amino groups. In our case, it was accomplished by using a solution containing 0.25% (w/v) *o*-phthalaldehyde (OPA; Fluoropa, Pierce) in methanol (HPLC grade, Burdick and Jackson); this reagent is used during the blocking cycle instead of the normal coupling reagent. After addition of the OPA solution, normal programming is resumed to the end, including the cleavage and extraction step, followed by a normal sequencing cycle to remove the unmodified proline residue. PTH-amino acid derivatives were identified and quantitated using PTH-norleucine as internal standard by HPLC [15] on a Varian 5500 liquid chromatograph equipped with a Vista 402 plotter/integrator and with a WISP (Waters) automatic injector.

3. RESULTS AND DISCUSSION

As mentioned, molecular sieving on Bio-Gel P10 of the crude extract of rat atria resulted in the subdivision of the various active molecular entities. From the low-M_r pool it was possible to purify and analyze, including amino acid sequencing, at least 4 amino-terminal truncated forms of the longest 33-residue ANF peptide [6,10]. All these homologous forms were shown to be biologically active and comparable in potency to a synthetic replica containing the last 26 amino acids [6]. From the high-M_r pool, numerous elongated forms of ANF were identified but the amounts recovered

in most cases did not permit further characterizations. Thus only two peptides, denoted ANF-H1 and ANF-H2, recovered in yields between 50 and 100 μg, could be further analyzed. ANF-H1 was recently shown to be a 73-residue peptide containing at its C-terminus the 33-amino-acid ANF while ANF-H2 was also reported to be an amino-terminal extended form [3]. Furthermore, a subfragment of rat ANF-H1 has been isolated and characterized [16]; the amino acid sequence presented is absolutely identical with the last 48 residues of ANF-H1. Fig.1 illustrates the elution profile of ANF-H2 purified on a C_{18} μ-Bondapak column eluted with the trifluoroacetic acid–acetonitrile system: material corresponding to the bar under the peak was pooled together and repurified using the heptafluorobutyric acid–acetonitrile system where it elutes around 40% acetonitrile.

The amino acid composition of purified ANF-H2 did not reveal any marked difference with the preliminary reported composition [3], except that

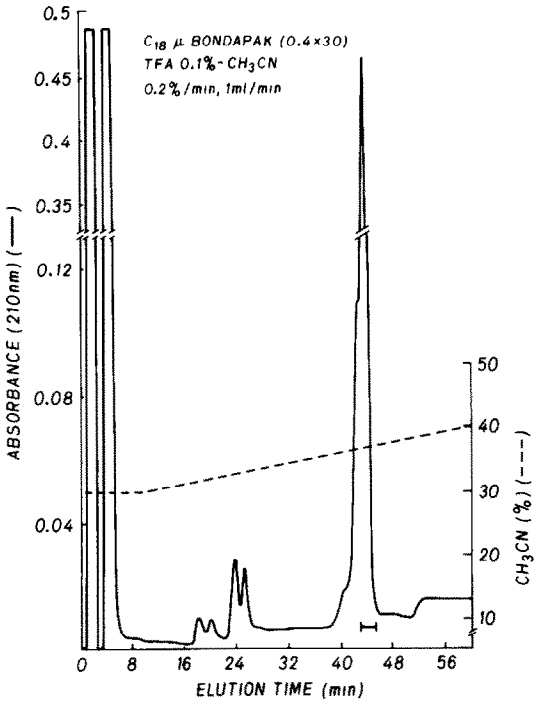

Fig.1. Chromatography of peptide ANF-H2 on a C_{18} μ-Bondapak column eluted with acetonitrile (30–40%) in 0.1% trifluoroacetic acid at a slope of 0.2%/min and a flow rate of 1 ml/min.

one more Thr was found together with a higher number of Ser (11 instead of 9) and Gly (12 instead of 10). All other features, namely the high number of Ser, Gly, Glu, Pro, Leu and Arg combined with the same proportion of Ile, Tyr and Phe as compared to ANF 1-33, were found to be unchanged.

The natriuretic and relaxant activities as determined by the natriuretic assay [2,13] and the chick rectum assay [5] of both high-M_r forms of ANF, ANF-H1 and ANF-H2 are compared in table 1 to the synthetic ANF comprising the last 26 residues located at the C-terminus. In both cases, the observed biological activities of ANF-H1 and ANF-H2 are 5- and 10-times lower, respectively, than for the synthetic ANF.

Preliminary amino acid sequence data obtained while analyzing the products isolated during HPLC purification revealed the presence of at least 3 chains, namely a major one corresponding to Glu-Lys-Met-Pro... (50.1% based on recovery at cycle 1) and two others corresponding to His-Leu-Glu-Glu... and Lys-Met-Pro... (21.1 and 28.8%, respectively). Upon further purification we were able to remove the His-Leu-Glu-Glu so that the amino acid sequencing was carried out on the major chain, the contribution of the other contaminating sequence, Lys-Met-Pro... (representing the N-1 sequence), being eliminated by treatment with OPA at cycle 4.

Fig.2 shows the identification and yield of the various PTH-amino acids obtained during sequen-

Table 1

Biological activity of high-M_r forms of ANF compared to synthetic ANF (8–33)

	Chick rectum assay[a]	Natriuretic assay[b]
Synthetic ANF	100	270 ± 47.1
ANF-H1	41.7 ± 21.9 (n = 4)	32.3 ± 21.6 (n = 4)
ANF-H2	17.8 ± 8.3 (n = 5)	16.6 ± 13.3 (n = 3)

[a] The relaxant activity was compared to an equimolar dose of synthetic ANF

[b] The natriuretic activity was measured in change of μeq. Na^+ excreted in 20 min for a dose of 500 pmol for each peptide

Values are expressed as %

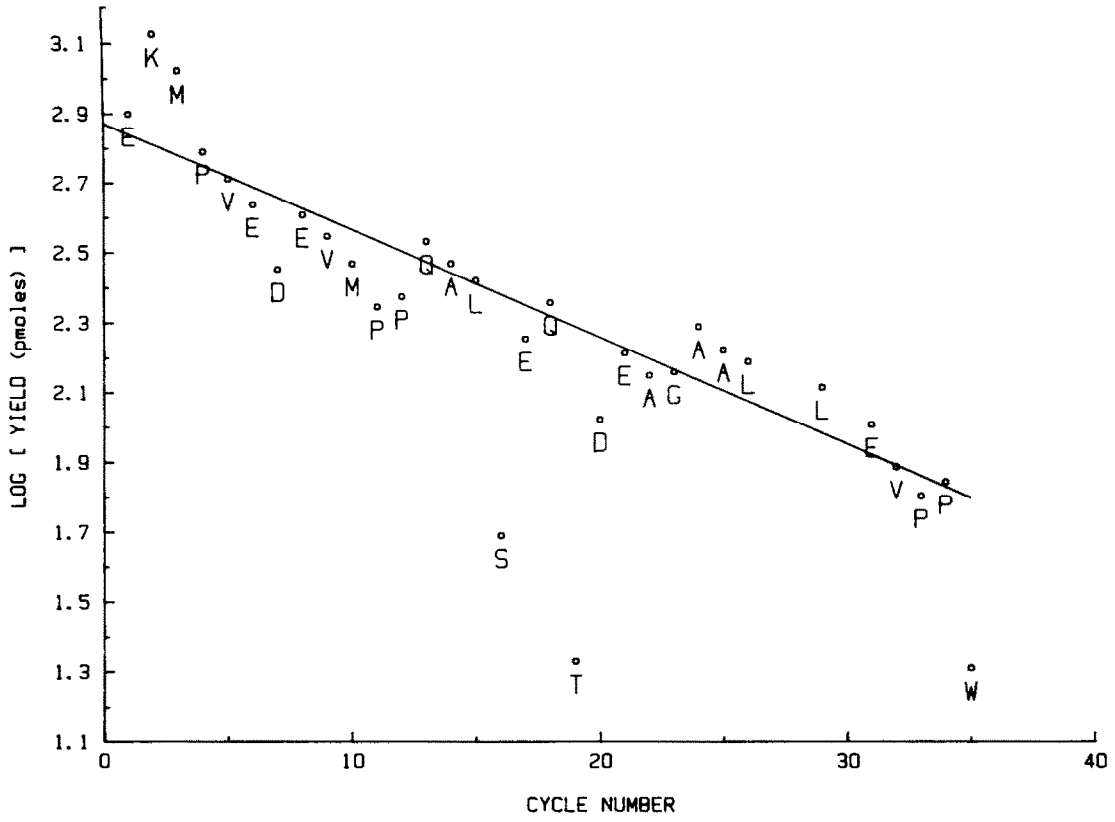

Fig.2. Automatic NH_2-terminal degradation of the ANF-H2 peptide. Quantitative yields of PTH-amino acids normalized to a PTH-norleucine internal standard are illustrated as a function of residue numbers. The slope and intercept were obtained by a linear regression analysis on selected stable PTH-amino acids giving the repetitive yield and initial yield, respectively.

cing of ANF-H2. The repetitive and initial yield were computed from the linear regression line to be 93.17% and 742 pmol, respectively, with a correlation coefficient of 0.866. As can be seen in fig.2, amino acids occupying positions 27, 28 and 30 could not be quantitated nor could they be identified positively. This can probably be best explained by the presence in those cycles of labile amino acids that are destroyed during conversion (like Ser and Thr), since even at this low level (100 pmol) the amino acid sequence was clearly seen up to cycle 35. Moreover, addition of OPA at cycle 4, as described in section 2.3, was certainly not deleterious to the repetitive yield and, in contrast, it helped to remove the contribution of the Lys-Met-Pro... sequence representing 25% of the sequenceable material and of another unidentified sequence present in very low amounts. After treating the sequenced chain at the Pro residue with OPA solution, we experienced some loss of amino-terminal function as evidenced by the recovery of 1 nmol Met at cycle 3 as compared to 600 pmol Pro at cycle 4 even though this could also be due to the known resistance of bonds involving Pro residues during the cleavage reaction. Nevertheless, from that cycle onwards, only one sequence was observed with minimal background thus allowing the sequence to continue up to 35 cycles. Clearly, as shown in fig.3, based upon the presented data of the sequence of ANF-H2 and the presence of the His-Leu-Glu-Glu sequence, the identity proposed solely from the amino acid composition of ANF-H2 is established as being an amino-terminal extended form of ANF-H1 and of ANF 1-33. Indeed, one can find a 5-amino-acid stretch, namely Glu-Val-Pro-Pro-Trp, at the end

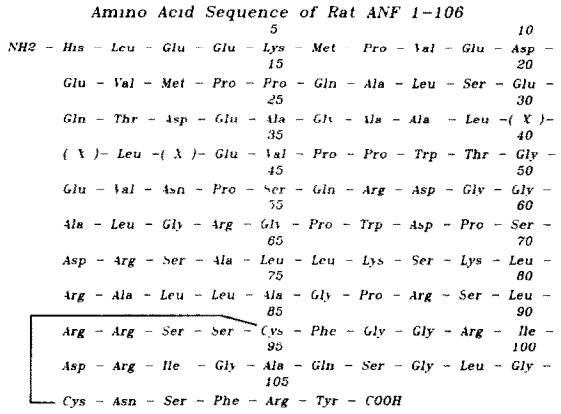

Fig.3. Complete amino acid sequence of the 106-residue ANF peptide also comprising the sequence of ANF-H1 and of the 33-residue ANF starting at positions 34 and 74, respectively.

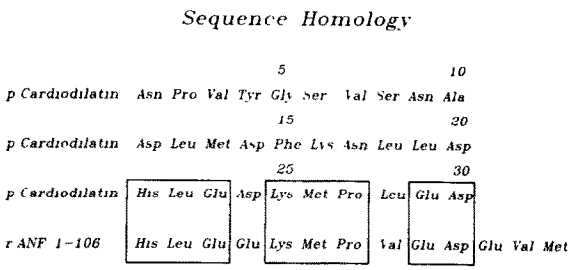

Fig.4. Amino acid sequence homology between cardiodilatin [12] and the first 13 residues of the 106-residue ANF. Identical amino acids are enclosed in boxes for comparison.

of the sequence of ANF-H2 which overlaps the sequence starting at position 34 of ANF-H1. Thus, it can be concluded, based upon those sequence data and on amino acid composition, that the low-M_r ANF peptide is located at the C-terminal portion of ANF-H1 which itself is contained in the larger ANF-H2.

As found in the case of ANF-H1 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 confirming the novel character of that sequence. On the other hand, authors in [12] recently reported preliminary data including a partial amino acid sequence concerning a new peptide, cardiodilatin, able to exert a relaxing activity upon vascular smooth muscle. This amino acid sequence, corresponding to the first 30 amino acids of cardiodilatin, was also shown to be novel since no significant homology could be found using the same procedure as for ANF-H2. Interestingly, when one compares these two novel sequences, a strongly homologous region, as indicated in fig.4, can be identified between the last 10 residues of cardiodilatin and the first 10 of the 106-residue ANF-H2. Indeed, in that sequence 8 amino acids, namely His, Leu, Glu, Lys, Met, Pro, Glu and Asp, are identical in both sequences while the two others could result from a single base change in the codon. Moreover, those two substitutions are of a conservative nature since Asp and Leu are replaced by Glu and Val, respectively; they could result from species differences because cardiodilatin was isolated from porcine atrial tissue while ANF-H2 was isolated from rat atrial tissue. Considering the novel character of both sequences and the almost perfect match of 10 amino acids as far as physicochemical characteristics are concerned (80% homology in true identity), it can thus be concluded that both molecules, cardiodilatin and ANF, are part of the same precursor molecule. The ANF moiety is located at the C-terminus while cardiodilatin is much closer to the as yet unidentified N-terminus. Moreover, the complete cDNA sequence coding for both cardiodilatin and ANF confirms entirely those results and, furthermore, shows that only 6 amino acid differences exist between the sequence of rat and the first 30 amino acids of porcine cardiodilatin (in preparation). Thus, this molecule of at least 126 residues could be responsible for the distribution of both biological activities, the natriuresis and vascular smooth muscle relaxation, in high-M_r fractions when atrial extracts are analyzed by molecular sieving. It can also be surmised, based upon the relationship between the two activities, that a way must exist in which both activities, present in the same molecule, are separated. Indeed, authors in [12] reported that highly purified cardiodilatin, when tested upon renal function, results in loss of diuretic activity while crude fractions are able to stimulate it; this could thus be explained by the presence of the ANF moiety in the crude preparation.

Conceivably, that molecule of 126 amino acids must be processed so as to release both activities; this could be accomplished by the heat-labile factor identified in [11]. Based upon the presented sequence, that molecule, which might be called 'pronatriodilatin', is almost devoid of pairs of basic amino acids which are known to be recognized and cleaved during maturation of prohormone precursors [17]. The only pair, Arg-Arg, present is located at position 81–82 in the 106-residue sequence and seems to be slightly cleaved since small amounts of fragment 83–106 have been isolated [6–10].

Furthermore, the different ANF molecules could possibly arise by non-specific chemical or proteolytic degradation during the extraction procedure as exemplified by the 48-amino-acid fragment characterized in [16] which could result from cleavage at an acid-labile Asp–Pro bond. Alternatively, they could be obtained from cleavage arising at various Leu residues located at positions 2, 32, 73, 74 and 80 yielding ANF4-106, ANF34-106, ANF74-106, ANF75-106 and ANF81-106, respectively. In some cases, further degradation by an aminopeptidase, made plausible by the presence of an N-1 sequence found during sequencing of ANF-H2, would produce the observed fragments. Interestingly, the fragmentation pattern seems to follow the distribution of Leu pairs of which two can be located at position 18–19 of cardiodilatin thus preceding the 103- and 106-residue fragments and at position 73–74 of ANF 1-106 preceding the smaller (<33 residues) ANF peptides. The presence of consecutive Leu residues that could act as recognition sites for processing was also observed by authors in [18]; they isolated 4 different naturally occurring fragments arising from cleavages at the carboxyl side of consecutive Leu residues in the glycopeptide representing the C-terminal of propressophysin [19]. Other Leu cleavages are known to occur during maturation of prorelaxin to relaxin at the junction of the B and C chain [20], in the production of LPH 61-76 [21] and in the renin–angiotensin system [22]. Finally, it is worth noting that production of kallidin (Lys-bradykinin) by glandular kallikrein is accomplished through cleavage on kininogen of a neutral amino acid, Met, following a Leu residue [23]; this cleavage is quite similar to that observed in pronatriodilatin where bonds located one residue away from Leu are cleaved. On the other hand, the ANF peptides starting at position 79 described in [7] and [9] could result from cleavage at a single Arg residue; however, this cleavage cannot explain the occurrence of the 73-, 103- and 106-residue forms. Nevertheless, cleavages at a single Arg were observed in the case of prohormones, notably during conversion of chicken proalbumin [24], propressophysin [19], procholecystokinin [25], prosomatocrinin [26] and prosomatostatin [27].

Finally, it can be concluded that cardiodilatin and ANF are part of the same precursor molecule, pronatriodilatin, which contains a minimum of 126 residues as derived from protein sequencing. However, the heterogeneity of the various peptides responsible for natriuresis and smooth muscle relaxant activity still remains to be resolved. Furthermore, the maturation pathway, if any, for that precursor is at present unclear. Indeed, in view of its similarity to prohormone and proneuropeptide processing relying on single or paired basic residues, and to the angiotensin or bradykinin system relying more on the substrate specificity of the relevant enzymes, the maturation pathway can now be related to both. Knowing the 126-residue sequence and the nucleotide sequence should help greatly in following the steps during post-translational processing by allowing the use of region-specific antibodies and microsequencing, thus leading to a better knowledge of the biological activities of both ANF and cardiodilatin. Finally, it is interesting that a molecule like atrial pronatriodilatin might contain at least two bioactive fragments having complementary hypotensive effects.

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