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IDENTIFICATION OF THE RELEASED FORM OF ATRIAL NATRIURETIC FACTOR BY THE PERFUSED RAT HEART

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Abstract. Atrial natriuretic factor (ANF), released by the isolated perfused rat heart, was extracted from the perfusates by C_{18} Sep-Pak cartridges and then isolated by immunoaffinity chromatography and by reverse phase HPLC. About 500 ng of immunoreactive material were so obtained and submitted to amino acid sequencing. The C-terminal Tyr was detected by radiolabelling. Identification of these residues indicated that the primary structure corresponds to ANF (Ser 99 - Tyr 126) which is identical to the circulating form in the rat. These results indicate that the ANF released by the atria corresponds to a short peptide. Therefore, its maturation process may therefore take place either intracellularly or during secretion and implicates a tryptic-like cleavage after a single Arg residue in position 98.

Introduction. Atrial natriuretic factor (ANF) is now known to be a circulatory peptide. Radioimmunoassay techniques using ANF as a standard have demonstrated an immunoreactive product in both human and rat plasma (1-5). By the use of HPLC these immunoreactive circulating forms have been identified as small peptides of 20 to 40 residues (1, 3) which probably represent the active C-terminal of ANF propeptide.

The circulating peptide in the rat has already been identified as ANF (Ser 99 - Tyr 126) (6, 7). However, there is still a possibility that this peptide represents only a major degradation product of a longer secreted form of ANF. To investigate this possibility, ANF has been directly isolated from the effluent of the perfused rat heart.

Materials and Methods. Male Sprague-Dawley rats (300-350 g) were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), the thorax was opened and the heart rapidly removed and washed in a cold Krebs solution. The aorta was cannulated and the coronary circulation was washed with heparinized Krebs. The spontaneously beating heart was perfused according to the method of Langendorff with Krebs solution at a constant flow of 10 to 15 ml/min enough to keep a perfusion pressure between 80 to 100 mmHg. The solution was maintained at 37°C and oxygenated with a mixture of 95% O_2 and 5% CO_2. The composition of the Krebs solution used in this study was (mol/l): NaCl, 119; KCl, 4.7; KH_2PO_4, 1.8; $MgSO_4 \cdot 7H_2O$, 1.17; $CaCl_2\ 6H_2O$, 2.5; $NaHCO_3$, 25.0; and dextrose, 5.5. After washing the heart for 15 min the effluent was passed through C_{18} Sep - Pak cartridges (Water Associates, Milford, Mass) and collected during 60 min. In order to verify the possible degradation of ANF in the effluent, iodinated ANF (Ser 99 - Tyr 126) or ANF (Gly 54 - Tyr 126), labelled by the lactoperoxidase method (8), were injected into the perfusion liquid. Radioactive products of the effluent were collected on C_{18} Sep-Pak and analyzed on a C_{18} μ Bondapak column as described.

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The cartridges were washed with 0.1% trifluoracetic acid and the material eluted with 100% acetonitrile containing 0.1% trifluoracetic acid. The organic solvent was evaporated on a Savant Speed Vac (Hicksville, N.Y.). The pellet was reconstitued in 0.05 M Tris-HCl, pH 7.4, plus 0.5 M NaCl in order to be processed by immunoaffinity chromatography.

Monoclonal antibodies against ANF (Arg 101 - Tyr 126), kindly provided by R.S. Milnes, were obtained from mouse ascites fluid by precipitation with ammonium sulfate at a final saturation of 35%. The proteins, dissolved in 0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl, were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the instructions of the manufacturer with a ratio of 7.5 mg of proteins per ml of gel. The immunoaffinity matrix was poured into a 0.5 X 10 cm column and alternatively washed with 0.05 M Tris-HCl, pH 7.4, 0.5 M NaCl, 0.25% Triton X-100 and with 1.0 M propionic acid, 10% p-dioxane. The extracted material from the Sep-Pak cartridges was applied to the column, washed with 40 ml of Tris buffer containing Triton X-100, 40 ml of Tris buffer without Triton X-100 and then eluted with 30 ml of 1.0 M propionic acid, 10% p-dioxane. The propionic acid fractions containing the immunoreactive ANF were lyophylized, reconstituted in 0.1% trifluoracetic acid, 10% CH_3CN, and processed by reverse phase HPLC on a C_{18} µ Bondapak column (0.78 X 30 cm) using an acetonitrile gradient (15 to 55% CH_3CN in 0.1% trifluoracetic acid, 0.5%/min, 2 ml/min). The immunoreactive peak was then purified on a C_{18} Vydac column (0.3 X 25 cm) (15 to 30% CH_3CN in 0.1% trifluoracetic acid, 0.2%/min, 1 ml/min).

The immunoreactivity of the material in the various fractions was measured by radioimmunoassay as previously described (1) using ANF (Arg 101 - Tyr 126) as a standard, ^{125}I-ANF as a tracer and rabbit antiserum against ANF (Arg 101 - Tyr 126). Amino acid sequence of the HPLC-purified ANF was performed on a Beckman 890 M sequenator using a 0.3 M Quadrol program and 3 mg of Polybrene. The phenylthiohydantoin-amino acids were analyzed by HPLC using norleucine as an internal standard (9). In order to identify the Tyr in position 126, about 200 ng of immunoreactive ANF of the first HPLC column were radiolabelled by the lactoperoxidase method (8) and rechromatographed by reverse phase HPLC. The immunoreactivity of the radioactive peak eluting at about 30% of CH_3CN was verified using rabbit antiserum against ANF (Arg 101 - Tyr 126). Protein concentration was measured by the Bradford assay as modified by Spector (10). Synthetic ANFs were purchased either from Peninsula Laboratories (Belmont, CA) or from Institut Armand Frappier (Laval, Quebec). ANF (Glu 54 - Tyr 126) was obtained by purification (11).

Results. The concentration of immunoreactive ANF in the perfusate of the isolated rat heart preparation ranges between 200 to 1,000 pg/ml. About 60 hearts, each one perfused for 60 min, were used. A total of 15 to 20 µg of immunoreactive material were collected. Analysis by HPLC of the material retained by the cartridges indicates, as shown in figure 1A, that it is mainly composed of ANF eluting around 30% of acetonitrile and thus representing short forms of ANF. Longer forms of ANF eluted between 35 and 45% represent minute amounts and may be due to partial necrosis of the tissue. Injection of either iodinated ANF (Ser 99 - Tyr 126) or ANF (Glu 54 - Tyr 126) in the perfusion liquid demonstrated, as shown in Fig. 1B and C, that they were not modified by their passage through the heart since they elute at the same position as the original material.

The material eluted from the Sep-Pak cartridges was processed by immunoaffinity chromatography. All the immunoreactive ANF was retained by the immunoaffinity matrix. Since glycine buffer or acetic acid were ineffective, propionic acid plus dioxane were used to elute ANF, probably indicating a high affinity of the monoclonal antibody. In spite of these drastic conditions, the affinity matrix could be used repetively.

The two final purification steps on reverse phase HPLC columns yielded a single immunoreactive peak associated with a sharp absorbance peak (Fig. 2). The RIA indicated that it contained about 500 ng of immunoreactive ANF.

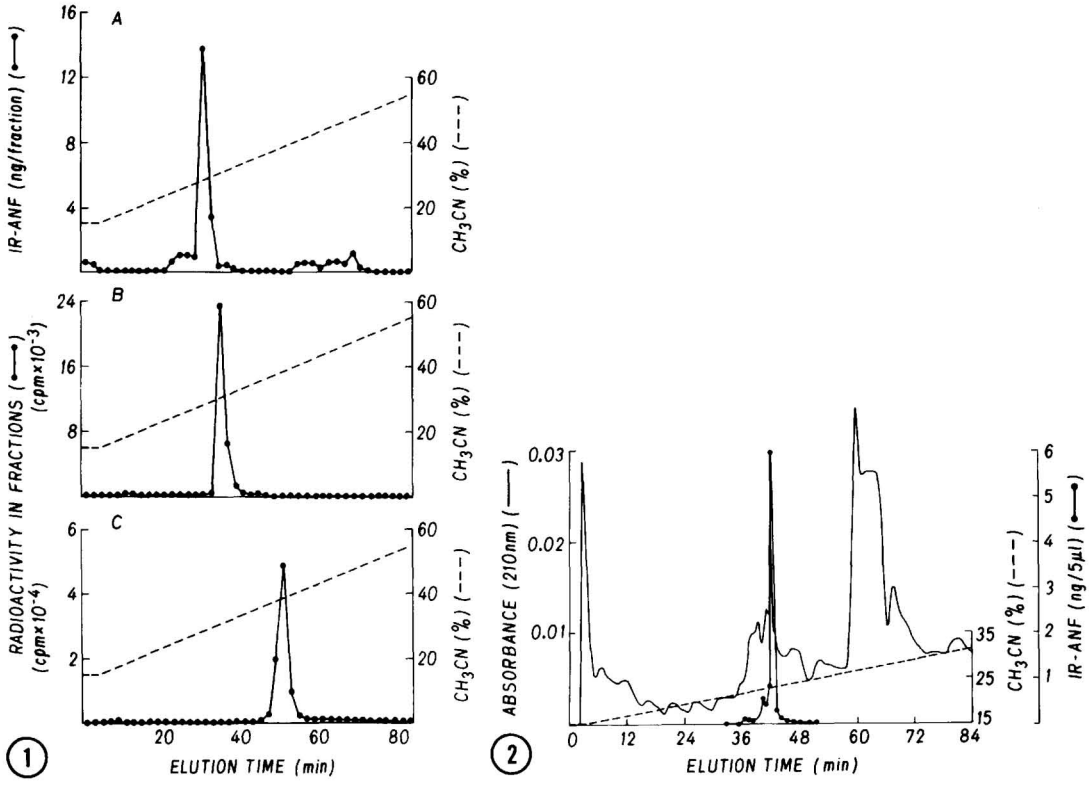

Figure 1 HPLC patterns on a C_{18} µ Bondapak column. Linear gradient of acetonitrile with a slope of 0.5%/min at 1 ml/min.
Panel A. Extracted material from C_{18} Sep-Pak.
Panel B. Injection of ^{125}I-ANF (Ser 99 - Tyr 126) in the perfusion liquid and analysis of the recovered material.
Panel C. Injection of ^{125}I-ANF (Glu 54 - Tyr 126) in the perfusion liquid and analysis of the recovered material.

Figure 2 Chromatography on a C_{18} Vydac column of immunoreactive ANF (IR-ANF). Gradient slope: 0.2% CH_3CN/min at 1 ml/min.

This isolated peptide was submitted to amino acid sequencing for 10 cycles. The Table 1 gives the yield of the residues. Eight positions out of 10 were positively identified. By correspondance with the known primary structure of the propeptide of ANF these residues represent the amino acids Leu, Arg, Arg, Ser, Ser, Phe, Gly and Gly in positions 100, 101, 102, 103, 104, 106, 107 and 108 respectively. In order to determine if Tyr was present at the C-terminal, 200 ng of IR-ANF from the first HPLC purification step were radiolabelled and then purified by reverse phase HPLC to remove free iodine. The radioactive material which elutes between 25 to 40% was tested using antiserum against ANF (Arg 101 - Tyr 126). Two immunoreactive fractions, eluting respectively at 28.5 and 31% of CH_3CN which corresponds to the mono and diiodinated forms (8), were detected. The isolated peptide was therefore identified as ANF (Ser 99 - Tyr 126).

Discussion. We have already isolated the circulating form of ANF which corresponds to ANF (Ser 99 - Tyr 126) (6). Similar results were obtained by Schwartz et al. from plasma of rat previously injected with vasopressin (7). In the present work we demon-

TABLE 1

AMINO ACID SEQUENCE OF ANF ISOLATED FROM EFFLUENT OF RAT HEART

CYCLE NO.	AMINO ACID	YIELD (pmoles)
1	Ser	-
2	Leu	150
3	Arg	65
4	Arg	62
5	Ser	15
6	Ser	16
7	Cys	-
8	Phe	98
9	Gly	66
10	Gly	62

strate that the perfusates of rat heart contain a short form of ANF which has been identified as ANF (Ser 99 - Tyr 126). Lang et al. (3) have also shown that effluent of isolated heart contains mainly a small form of ANF.

These data indicate that ANF (Ser 99 - Tyr 126) is most probably the peptide which is released by the atrial cardiocytes and it does not appear to be further cleaved in the blood. This finding implicates that the intracellular maturation of the propeptide which probably requires a tryptic-like cleavage between the Arg 98 - Ser 99 bond takes place intracellularly or during secretion. Bloch et al. (12) and Glembotski et al. (13) have reported that atrial cardiocytes in culture released in the medium the precursor of ANF. The discrepancies between these results and ours are not clear. The precursor may be secreted by the cardiocytes and then immediately processed by an extracellular protease which may be membrane bound.

The propeptide of ANF contains a pair of basic amino acids in positions 101 and 102 which does not appear to be involved in the maturation processing of ANF since ANF (Ser 103 - Tyr 126) was only isolated from boiled acid extracts of atria (14) or as a minor product in the rat plasma (7). Pair of Arg or Lys are often involved in the maturation of hormonal peptide (15) although some peptides can also be generated after a single Arg residue (16-19).

In summary, we have shown that ANF (Ser 99 - Tyr 126) is not only the major circulating form of ANF but also the released form of ANF by the perfused heart. Whether or not the released form of ANF corresponds to the secreted form by the cardiocytes still remains to be elucidated.

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References

1. Gutkowska, J., K. Horky, G. Thibault, P. Januszewicz, M. Cantin, J. Genest. Atrial natriuretic factor is a circulating hormone. Biochem. Biophys. Res. Comm. **125**: 315-323, 1984.
2. Larose, P., S. Meloche, P. du Souich, A. De Léan, H. Ong. Radioimmunoassay of atrial natriuretic factor: human plasma levels. Biochem. Biophys. Res. Comm. **130**: 553-558, 1985.
3. Lang, R.E., H. Tholken, D. Ganten, F.C. Luft, H. Ruskoaho, T. Unger. Atrial natriuretic factor - a circulating hormone stimulated by volume loading. Nature **314**: 264-266, 1985.

4. Manning, P.T., D. Schwartz, N.C. Katsube, S.W. Holmberg, P. Needleman. Vasopressin-stimulated release of atriopeptin: endocrine antagonists in fluid homeostatis. Science **229**: 395-397, 1985.
5. Takayanagi, R., I. Tanaka, M. Maki, T. Inagami. Effects of changes in water-sodium balance on levels of atrial natriuretic factor messenger ANF and peptides in rats. Life Sciences **36**: 1843-1848, 1985.
6. Thibault, G., C. Lazure, E.L. Schiffrin, J. Gutkowska, L. Chartier, R. Garcia, N.G. Seidah, Chrétien, J. Genest, M. Cantin. Identification of a biologically active circulating form of rat atrial natriuretic factor. Biochem. Biophys. Res. Comm. **130**: 981-986, 1985.
7. Schwartz D., D.M. Geller, P.T. Manning, N.R. Siegel, K.F. Fok, G.E. Smith, P. Needleman. Ser-Leu-Arg-Arg-Atriopeptin III: The major circulating form of atrial peptide. Science **229**: 397-400, 1985.
8. Murthy, K.K., G. Thibault, E.L. Schiffrin, R. Garcia, L. Chartier, J. Gutkowska, J. Genest, M. Cantin. Disapperance of atrial natriuretic factor form circulation in the rat. Peptides **7**: In press, 1986.
9. Lazure, C., N.G. Seidah, M. Chrétien, R. Lallier, S. St-Pierre. Primary structure determination of *Escherichia coli* heat-stable enterotoxin of porcine origin. Can. J. of Biochem. Cell Biol.**61**: 287-292, 1983.
10. Spector, T. Refinement of the Coomasie Blue method of protein quantitation. Anal. Biochem. **86**: 142-146, 1978.
11. Thibault, G., R. Garcia, M. Cantin, J. Genest, C. Lazure, N.G. Seidah, M. Chrétien. Primary structure of a high Mr form of rat atrial natriuretic factor. FEBS Letter **167**:352-356, 1984.
12. Bloch, K.D., J.A. Scott, J.B. Zisfein, J.T. Fallon, M.N. Margolies, C.E. Seidman, G.R. Matsueda, C.J. Honrey, R.M. Graham, J.G. Seidman. Biosynthesis and secretion of proatrial natriuretic factor by cultured rat cardiocytes. Science. **230**:1168-1171, 1985.
13. Glembotski, C.C., T.R. Gibson. Molecular forms of immunoactive atrial natriuretic peptide released from cultured rat atrial myocytes. Biochem. Biophys. Res. Comm. **132**:1008-1017, 1985.
14. Geller, D.M., M.G. Currie, K. Waketani, B.R. Cole, S.P. Admas, K.F. Fok, N.R. Siegel, S.R. Eubanks, G.R. Galluppi, P. Needleman. Atriopeptins: a family of potent biologically active peptides derived from mammalian atria. Biochem. Biophys. Res. Comm. **120**: 333-338, 1984.
15. Lazure, C., N.G. Seidah, D. Pelaprat, M. Chrétien. Proteases and post translational processing of prohormones: a review. Can. J. of Biochem. Cell Biol. **61**: 501-515, 1983.
16. Rosen, A.M., D.M. Geller. Chicken microsomal albumin: amino terminal sequence of chicken proalbumin. Biochem. Biophys. Res. Comm. **78**: 1060-1066, 1977.
17. Land, H., G. Schutz, H. Schmale, D. Richter. Nucleotide sequence of cloned cDNA encoding bovine arginine vasopressin - neurophysin II precursor. Nature. **255**: 299-303, 1982.
18. Kurjan, J., I. Herskowitz. Structure of a yeast pheromone gene (MFα): a putative α-factor precursor contains four tandems copies of mature α-factor. Cell. **30**: 933-943, 1982.
19. Schiller, R.H., J.F. Jackson, L.B. McAllister, B.S. Rothman, E. Mayeri, R. Axel. A single gene encodes multiple neuropeptides mediating a stereotyped behavior. Cell. **32**: 7-22, 1983.

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