Aatrioactivase, a Specific Peptidase in Bovine Atria for the Processing of Pro-atrial Natriuretic Factor

PURIFICATION AND CHARACTERIZATION*

T eruaki Imada, Ryoichi Takayanagi, and Tadashi Inagami
From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

(Received for publication, February 19, 1988)

A seryl protease which catalyzes conversion of pro-atrial natriuretic factor (ANF) to the active circulating form, ANF(99-126), was purified from a particulate fraction of bovine atria. The enzyme was solubilized with 1.6 M KCl. The molecular mass of the purified enzyme was 580 kDa on gel filtration, whereas by sodium dodecyl sulffate-polyacrylamide gel electrophoresis a cluster of six bands with molecular masses around 30 kDa was observed. The purified enzyme produced ANF(99-126) from partially purified bovine pro-ANF by the selective cleavage of the arginyl peptide bond in the -Pro$_{97}$-Arg$_{98}$-Ser$_{99}$-sequence in pro-ANF. The enzyme was localized mainly in the microsomal fraction rather than the granule fraction. It is likely that the enzyme selectively cleaves the Arg$_{98}$-Ser$_{99}$ peptide bond in pro-ANF during the process of secretion.

Atrial natriuretic factor (ANF) is a peptide hormone synthesized mainly in atrial myocytes and stored in specific granules as a 126-residue precursor, pro-ANF (1). The circulating form of ANF in rat plasma is a shorter peptide consisting of the 28-residue segment ANF(99-126) in the carboxy-terminal part of pro-ANF (2, 3). These findings indicated that pro-ANF is converted to the active form during or just after secretion from the atrium. This view receives further support because blood collected in the coronary sinus, where the ANF concentration is highest and hence is considered the site from which freshly secreted ANF is drained, contains exclusively the small form of ANF (4). Moreover, isolated rat heart perfused with Krebs-Ringer buffer secretes ANF(99-126), but not pro-ANF (5).

Knowledge of the enzymes involved in the conversion of the prohormone to the active hormone is scanty. The activation of rat pro-ANF seems to provide an ideal system for the study of prohormone conversion. The ANF in the storage granules of the atrium is in the prohormone form (126 residues) and in the process of secretion is almost completely converted to the active hormone (28 residues) by cleavage at Arg$_{98}$-Ser$_{99}$ in the sequence Ala$_{95}$-Gly$_{96}$-Pro$_{97}$-Arg$_{98}$-Ser$_{99}$. By avoiding the problems that arise when prohormone and active hormone are mixed in storage granules and in blood, the distinct localization of pro-ANF and active ANF in different spaces facilitates identification of the activating enzyme.

Proteases (or a protease) in serum were found to produce ANF$_{99-126}$ from pro-ANF (6, 7). However, further work indicated that it is unlikely that serum enzymes are involved because plasma has no processing activity (8). It was further shown that conversion does not take place in blood after secretion because addition of protease inhibitors to the perfusing buffer had no effect on the appearance of ANF$_{99-126}$ from a rat heart Langendorf perfusion (9).

Recently we obtained evidence for the presence of a pro-ANF processing enzyme in rat atria (10). To facilitate detection of the enzyme we used Boc-Ala-Gly-Pro-Arg-MCA (AGPR-MCA) which contains the sequence on the amino-terminal side of the peptide bond cleaved by the processing enzyme. The enzyme was found to be bound to the microsomal membrane fraction of rat atrial extract and was solubilized by 1.6 M KCl solution. The enzyme possesses general properties compatible with a seryl protease and produces 28-residue ANF by selectively cleaving the Arg$_{98}$-Ser$_{99}$ bond rather than other arginyl peptide bonds such as those in Arg$_{101}$-Arg$_{102}$-Ser$_{103}$. Although the complete amino acid sequence of bovine pro-ANF is not yet known, we found that the rat atrial enzyme substrate could be used for the determination of bovine enzyme activity. In the present study we purified the enzyme from bovine atria and report its properties.

EXPERIMENTAL PROCEDURES

Materials

DFP, p-aminobenzamidine, leupeptin, soybean trypsin inhibitor, lima bean trypsin inhibitor, aprotinin, HEPES, heparin (from porcine intestinal mucosa), heparin-agarose, arginine-agarose, aprotinin-agarose, N-ethylmaleimide, and iodoacetate were obtained from Sigma. Boc-Ala-Gly-Pro-Arg-MCA was from the Peptide Institute (Osaka, Japan). Human ANF$_{99-126}$, ANF$_{99-126}$, ANF$_{99-126}$, ANF$_{99-126}$, ANF$_{99-126}$, and Z-Phe-Arg-MCA were from Peninsula Laboratories. [3H]DFP was from Amersham Corp. N-Glycosidase F (N-Glycanase$^{	ext{TM}}$) was from Genzyme Corp.

Measurement of Enzymatic Activity

Activity of the enzyme was measured fluorometrically using AGPR-MCA as substrate (10). To a cuvette containing 2 ml of 50 mM Tris-HCl, pH 8.0, and 0.2 ml of 1 mM substrate which had been dissolved in 1 mM HCl was added 50 ml of an enzyme fraction. The reaction was followed at room temperature by determining the increase in fluorescence emitted at 460 nm and excited at 380 nm in a Perkin-Elmer model 650-15 spectrofluorometer. One unit of the enzymatic activity was defined as the amount of enzyme which releases 1 pmol of 7-amino-4-methylcoumarin from the substrate/min.
### Purification of the Enzyme

**Enzyme Extraction**—Ten bovine atria (360 g) freshly obtained from a local slaughterhouse were minced and homogenized in a Polytron homogenizer for 30 s in 2 liters of a high ionic concentration buffer, 10 mM HEPES, pH 7.0, containing 0.25 M sucrose and 1 mM EDTA, and 1.6 M KCl to release the enzyme from the membrane fraction. The homogenate was centrifuged for 1 h at 10,000 × g, and the supernatant was dialyzed against 10 mM HEPES, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA (buffer A).

**Chromatography on Heparin-Agarose**—The dialysate was applied to a heparin-agarose column (2.7 cm × 10 cm) equilibrated with buffer A and subsequently washed with the buffer. The enzyme was eluted with a linear gradient from 0.1 to 1.5 M NaCl in buffer A. Fractions containing activity were collected and dialyzed against buffer A containing heparin at 10 µg/ml (buffer B).

**Chromatography on Arginine-Agarose**—The eluate from heparin-agarose was reapplied to an arginine-agarose column (1.3 × 4 cm) equilibrated with buffer B, and the column was eluted by a linear gradient of NaCl from 0.1 to 1.0 M. Fractions containing the enzyme (total 10 ml) were directly concentrated in a Centricon-30 cartridge (Amicon) to 1 ml. The recovery was nearly 90%.

**Gel Filtration on Sephacryl S-300**—The concentrate was applied to a Sephacryl S-300 column (1.8 cm × 90 cm) pre-equilibrated and eluted in a solution of 50 mM Tris for pH 7.0-9.0, and carbonate for above pH 9.0.

### Amino Acid Sequence Analysis

Amino acid sequence analysis was performed on an automated gas-phase instrument (model 470A, Applied Biosystems) by the method described by the manufacturer.

### Preparation of Bovine Pro-ANF

Partially purified bovine pro-ANF was prepared by the method of Trippodo et al. (19). Bovine atrium was boiled in 1 N acetic acid for 15 min and homogenized by sonication for 10 s using a Polytron homogenizer. After centrifugation to obtain a clear supernatant, the crude extract was applied to a Sephadex G-100 column (1.0 × 90 cm). The pro-ANF peak fractions identified by radioimmunoassay and elution position were pooled. The concentration of immunoreactive pro-ANF was estimated by radioimmunoassay.
the solubilization step and heparin-agarose chromatography may be due to instability of the enzyme. We found that heparin stabilizes the activity in a low ionic concentration buffer. Therefore, we added heparin to all the buffer systems.

Enzyme activity was labeled with [3H]DFP, electrophoresed in the mass of these pairs were estimated as 31.5 and 31.0 kDa, labeled by [3H]DFP. Relative intensities of the radioactivity and silver staining were correlated by visual inspection.

ACRYLAMIDE GEL ELECTROPHORESIS PATTERN OF THE PURIFIED ENZYME. The 60-kDa band in egg albumin after the heparin-agarose step. Without heparin activity was lost during purification.

Estimation of Molecular Mass—Fig. 1 shows the SDS-polyacrylamide gel electrophoresis pattern of the purified enzyme. As shown in lane A the final product consisted of three double bands with approximate masses of 30 kDa. The apparent masses of these pairs were estimated as 31.5 and 31.0 kDa, 29.0 and 28.5 kDa, and 26.5 and 26.0 kDa. The purified enzyme was labeled with [3H]DFP, electrophoresed in the same SDS gel, and the [3H]DFP-labeled proteins identified (Fig. 1, lane B). All the bands observed by silver staining were labeled by [3H]DFP. Relative intensities of the radioactivity and silver staining were correlated by visual inspection.

By contrast, gel filtration of the purified enzyme on a TSK-G3000 SW column showed a mass of 580 kDa (Fig. 2). The cluster of bands (Fig. 3, lane A) collapsed to two distinct bands (a major band at 28.0 kDa and a minor band at 30.0 kDa) as shown in Fig. 3 (lane B).

Effect on Pro-ANF—Partially purified pro-ANF was treated with the enzyme, and the resultant product was fractionated by gel filtration. Fig. 4a shows the elution profile. Partially purified bovine pro-ANF was eluted in fraction 35 in this system. After enzyme treatment the elution position of immunoreactive ANF was shifted to the elution position of the circulating form, ANF(99-126). The recovery was 76%.

Further examination of the product from the enzyme treatment by reverse-phase HPLC revealed that the elution position was shifted from that of pro-ANF to that of ANF(99-126). This position was distinguishable from those of ANF(105-121), ANF(102-126), ANF(101-126), or ANF(96-126), as shown in Fig. 4b.

In order to obtain unequivocal identification of the ANF produced by the enzyme, the peak fractions (shown by a bar in Fig. 4b) were collected, purified by HPLC, and subjected to sequence analysis. The following amino-terminal sequence was determined: Ser-Leu-Arg-Arg-Ser-Ser-. In addition the amino acid composition of the peptide was compatible with that of ANF(99-126).

Subcellular Distribution of the Enzyme—The subcellular distribution of the enzyme was determined with fractions obtained from a homogenate of bovine atria (15). In addition to the enzyme activity detected by AGPR-MCA, we also measured nonspecific protease activity with Z-Phe-Arg-MCA (ZFR-MCA), immunoreactive ANF, and alkaline phosphatase.

### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6 M KCl extract</td>
<td>6000</td>
<td>20.8</td>
<td>0.0035</td>
<td>1.0</td>
</tr>
<tr>
<td>Heparin-agarose</td>
<td>40</td>
<td>11.7</td>
<td>0.293</td>
<td>83.7</td>
</tr>
<tr>
<td>Arginine-agarose</td>
<td>2.31</td>
<td>4.58</td>
<td>1.98</td>
<td>565.7</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>2.0</td>
<td>5.26</td>
<td>2.63</td>
<td>751.4</td>
</tr>
<tr>
<td>Aprotinin-agarose</td>
<td>0.258</td>
<td>4.08</td>
<td>15.8</td>
<td>4514.3</td>
</tr>
<tr>
<td>Arginine-agarose</td>
<td>0.097</td>
<td>3.06</td>
<td>31.9</td>
<td>914.3</td>
</tr>
</tbody>
</table>

*a One unit is defined as the enzyme activity which hydrolyzes 1 pmol of Boc-Ala-Gly-Pro-Arg-MCA/min.

### Table II

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>100 mg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Lima bean trypsin inhibitor</td>
<td>100 mg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>100 mg/ml</td>
<td>58</td>
</tr>
<tr>
<td>p-Aminobenzamidine</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>DFP</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>100 mg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>100 mg/ml</td>
<td>100</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>5 mg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>5 mg/ml</td>
<td>7</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>200 mg/ml</td>
<td>7</td>
</tr>
<tr>
<td>EDTA</td>
<td>6 mM</td>
<td>0</td>
</tr>
</tbody>
</table>
ANF Processing Enzyme

FIG. 3. Effect of N-glycosidase F (N-Glycanase™) treatment on the purified enzyme. The enzyme (100 ng) was treated for 24 h with N-glycosidase F by the method given in the manufacturer's instruction. Lanes A and B show control (incubation without N-glycosidase F) and sample incubation with N-glycosidase F, respectively. Standards used for calibration are described in the legend to Fig. 1.

The enzyme (100 ng) was treated for 24 h with N-glycosidase F by the method given in the manufacturer's instruction. Lanes A and B show control (incubation without N-glycosidase F) and sample incubation with N-glycosidase F, respectively. Standards used for calibration are described in the legend to Fig. 1.

FIG. 4. a, detection of molecular weight change in immunoreactive pro-ANF on treatment with atrial enzyme. Fifty μl of the reaction mixtures (pro-ANF incubated with or without the enzyme) were applied to the column and the amount of immunoreactive ANF in the fractions determined by the method described under "Experimental Procedures." Elution positions of standard proteins indicated by arrows are: BSA, bovine serum albumin; Lys, lysozyme; Glu, glucagon. Elution positions of pro-ANF and ANF99-126 are indicated by arrows A and B, respectively. b, characterization of the product from atrial enzyme-treated pro-ANF on an octadecylsilane column. Fifty μl of the reaction mixture (pro-ANF incubated with the enzyme) were applied to the column, and the amount of immunoreactive ANF in the fractions was determined by the method described under "Experimental Procedures." Synthetic ANFs used here are: I, ANF103-123; II, ANF105-123; III, ANF106-123; IV, ANF96-123; V, ANF99-126; VI, ANF103-126. The fractions shown by the bar were collected and subjected to sequence analysis after purification by chromatography on ODS and CN columns.

activity (Table III). The AGPR-MCA activity appeared mainly in the microsomal fraction, whereas ZFR-MCA hydrolyzing activity was predominantly localized in the cytosolic fraction (high speed supernatant). Immunoreactive ANF was found mainly in the granular fraction, as expected from previous studies (15).

TABLE III

<table>
<thead>
<tr>
<th>Subcellular distribution of the enzyme</th>
<th>ir-ANP&lt;sup&gt;a&lt;/sup&gt; alkaline</th>
<th>Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AGPR-MCA</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Mitochondrial fraction</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Granular fraction</td>
<td>10</td>
<td>1.2</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>36</td>
<td>0.3</td>
</tr>
<tr>
<td>High speed supernatant</td>
<td>20</td>
<td>97</td>
</tr>
</tbody>
</table>

<sup>a</sup> Immunoreactive ANF.

DISCUSSION

In the proteolytic conversion of rat pro-ANF to the circulating form ANF(99-126), specific cleavage of the Arg<sup>98</sup>-Ser<sup>99</sup> peptide bond occurs in the following sequence (2, 3): -Ala<sup>95</sup>-Gly<sup>96</sup>-Pro<sup>97</sup>-Arg<sup>98</sup>-Ser<sup>99</sup>-Leu<sup>100</sup>-Arg<sup>101</sup>-Arg<sup>102</sup>-Ser<sup>103</sup>. In the previous paper (10) we identified the presence of such a pro-ANF processing activity in rat atria using the synthetic fluorogenic substrate AGPR-MCA.

The present study represents the identification and purification of a protease in bovine atrial membrane fractions capable of catalyzing the specific processing of pro-ANF to the circulating form of ANF. Although the amino acid sequence of active bovine ANF was shown to be the same as that of human ANF (14), the entire sequence of bovine pro-ANF has not yet been determined. However, we found the substrate used in the rat study served as a substrate for the bovine enzyme.

The purified enzyme has the following characteristics: 1) it has a high affinity to heparin-agarose and is stabilized by heparin; 2) the mass is 580 kDa by gel filtration, whereas on SDS-polyacrylamide gel electrophoresis a cluster of bands is seen around 30 kDa. We could not exclude the possibility of nonspecific aggregation of the enzyme, but a sharp single peak of the activity in Fig. 2 suggests that the enzyme exists in a 580-kDa form.

Three doublets observed on SDS-polyacrylamide gel electrophoresis around 30 kDa indicate that the enzyme is an aggregate of smaller subunits. To examine the possibility that these multiple bands arise from proteolytic cleavage of the native subunits or are due to heterogeneous glycosylation, three experiments were performed. First, the enzyme was purified in the presence of N-ethylmaleimide. This cysteiny1 protease inhibitor, which has been shown not to inhibit the present enzyme (Table II), was added to all the buffers at 1 mM during purification to prevent proteolytic cleavage by cathepsin-like cysteiny1 proteases which are often present in the cytosol. The resultant product gave an identical pattern on SDS-gel electrophoresis.

Second, we labeled the crude extract with [³H]DFP and subjected it to SDS-gel electrophoresis. The pattern of radioactive bands obtained from the crude extract was identical to that (Fig. 1) obtained from the purified and [³H]DFP-treated enzyme (data not shown). In the third experiment, we treated the enzyme with an N-glycosidase. This treatment reduced the mass and the number of the bands, indicating that the multiplicity of the bands is mainly due to heterogeneity in glycosylation, similar to the pattern observed with trypsinase isolated from the pituitary gland (22).

Recently several high molecular weight seryl proteases have been obtained from various tissues (22-26). The enzyme purified by us has characteristics similar to these, but does not seem identical. Tryptases isolated from human lung (23, 24) and human pituitary (22) were shown to have high affinity to
heparin and to be stabilized by heparin. However, their masses are 120–130 kDa, which are much smaller than that of the atrial enzyme. While high ionic concentrations stabilize tryptases, the atrial enzyme rapidly lost activity under the same conditions.

An enzyme isolated from rat liver has been shown to have a mass of 600 kDa (25), which is close to that of the atrial enzyme. However, the liver enzyme exists only in the cytosol, and the substrate specificity is completely different from the atrial enzyme.

The enzyme we identified seems to be different from that reported by Baxter et al. (27). The present enzyme is a serin protease whereas their enzyme is a cysteine protease.

The observation that the present enzyme exists mainly in the microsomal fraction distinguishes it from the protease activity detected by ZFR-MCA which is localized in the cytosol. Twenty percent of the AGPR-MCA hydrolyzing activity is only one bond in -ProGlyProArgSer instead of bonds involving a double basic residues. While high ionic concentrations stabilize tryptases, the atrial enzyme rapidly lost activity under the same conditions.

The microsomal fraction obtained by the present method also contains plasma membranes, as indicated by the presence of alkaline phosphatase activity, a plasma membrane marker (as shown in Table III). Further investigation of the distribution of the enzyme may disclose the path of processing ANF.

HPLC and sequence analysis of the peptide produced from pro-ANF by this enzyme demonstrates that ANF (99-126) is the major or exclusive product. Since the amino acid sequence of bovine pro-ANF is not known and since antibodies to the amino-terminal segment of bovine pro-ANF are not available, it is not feasible to investigate whether this enzyme hydrolyzes other arginyl peptide bonds in the amino-terminal segment of pro-ANF. However, recently Michener et al. (8) made an interesting approach to this problem by utilizing an antibody to the NH2-terminal fragment of rat pro-ANF. They detected only one (14 kDa) molecular form, which corresponds to the 98-residue NH2-terminal fragment in rat blood, and concluded that processing of pro-ANF is performed at only one site, -Gly-ProArgSer.

In view of the specific functional features of the enzyme we propose to call it "atrioactivase." One of the unique features of this enzyme is the specific cleavage of the arginyl peptide bond in -ProArgSer instead of bonds involving a double basic residue sequence such as -Arg-ProArgSer. Cleavage of the latter peptide bonds given rise to physiologically active peptides with 24 or 25 residues. Although such ANFs have not been found in plasma, they have been reported in rat brain (29, 30). In the brain the processing of pro-ANF may be catalyzed by proteases specific for double basic residues, which are commonly found in the processing of precursors of many peptide hormones such as preopiomelanocortin.

REFERENCES