Brain Endo-Oligopeptidase B: A Post-Proline Cleaving Enzyme that Inactivates Angiotensin I and II

LEWIS J. GREENE, PH.D., AUGUSTO CESAR C. SPADARO, PH.D., ANTONIO R. MARTINS, M.D., PH.D., WANDA DRAGHETTA PERUSSI DE JESUS, B.S., AND ANTONIO C. M. CAMARGO M.D., PH.D.

SUMMARY Rabbit brain endo-oligopeptidase B inactivates angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) by hydrolysis of the Pro'-Phe* peptide bond. The site of hydrolysis was determined in preparative and analytical experiments in which both products were recovered in a molar ratio of 1:1, and the sum of the products plus unhydrolyzed substrate accounted for the starting material. The enzyme has a $K_m$ of $6.3 \times 10^{-6}$ M for angiotensin II at pH 8.3 and is activated 30-fold with 4.8 mM dithiothreitol. BPP$_{aa}$ (<Gln-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, SQ 20,881) inhibits the inactivation of angiotensin II with an $IC_50$ of $5 \times 10^{-9}$ M. BPP$_{aa}$ (<Glu-Lys-Trp-Ala-Pro, SQ 20,475) is less active and D-3-mercapto-2-methylpropanoyl-L-proline (captopril, SQ 14,225) has essentially no activity. These compounds are significantly more potent as inhibitors of angiotensin-converting enzyme. The role of brain endo-oligopeptidase B in angiotensin I and II metabolism remains to be established.

KEY WORDS • brain endo-oligopeptidase B • angiotensinase • captopril • inhibition of angiotensinase by BPP$_{aa}$

The release of renin (EC 3.4.99.19) into the circulation triggers the formation of angiotensin II (AII) via conversion of angiotensin I (AI) by angiotensin-converting enzyme (EC 3.4.15.1). AII produces vasoconstriction and secretion of mineralocorticoid hormones, which lead to salt and water retention and increased arterial blood pressure by acting directly on arterial and adrenal receptors. Several lines of evidence have suggested the presence of the renin-angiotensin system and AII in the central nervous system. The effects elicited by central application of AII are synergistic with those obtained by systemic injection of this hormone.

Although several hydrolytic enzymes are capable of terminating the effect of AI and AII, little detailed information is available about those that are responsible for the physiological inactivation of these hormones in brain and peripheral tissues. Previous reports from this laboratory described the isolation of endopeptidases A and B from rabbit brain using the peptide hormone bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) for the detection of peptidase activity. Endopeptidase A and B hydrolyze bradykinin at the Phe'-Ser* and Pro'-Phe* peptide bond, respectively. These enzymes were designated endo-oligopeptidases because they hydrolyze internal peptide bonds of oligopeptides but not of oligopeptides attached to soluble polymers. Endo-oligopeptidase B also inactivates luliberin and is inhibited by the nonapeptide BPP$_{aa}$ (SQ 20,881, <Gln-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, SQ 20,475) is less active and D-3-mercapto-2-methylpropanoyl-L-proline (captopril, SQ 14,225) has essentially no activity. These compounds are significantly more potent as inhibitors of angiotensin-converting enzyme. The role of brain endo-oligopeptidase B in angiotensin I and II metabolism remains to be established.
Methods

The enzyme was prepared from the supernatant fraction of rabbit brain by step elution chromatography on DEAE-cellulose, gel filtration on Sephadex G-100, and isoelectric focusing as described by Oliveira et al. with the modifications in DEAE-cellulose chromatography described by Martins et al. The specific activity of the enzyme was 0.78 units/mg protein for the hydrolysis of 8 X 10^-8 M bradykinin in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl, 5 X 10^-4 M DTT at 37°C.7 The enzyme hydrolyzed only the Pro-Phe bond of bradykinin2 and gave only one band after polyacrylamide gel electrophoresis at pH 8.9.7

Determination of the Site of Hydrolysis of Angiotensin I and II

The conditions for hydrolysis are given in the legend to figure 1. Products and unhydrolyzed substrate were isolated by chromatography on Aminex A-5 resin (0.9 X 30 cm), equilibrated and developed at 22 ml/hour, 60°C. The resin was equilibrated with pyridine-acetic acid buffer, 0.2 M pyridine, pH 3.5 (16.1 ml pyridine, 235 ml acetic acid and H2O to 1 liter). After sample application, the column was developed with equilibrating buffer, pH 3.5 for 1 hour, and then eluted with a linear gradient prepared from 250 ml each of 0.9 M pyridine-acetic acid buffer pH 4.25 (72.0 ml pyridine, 120 ml acetic acid and H2O to 1 liter) and 4.0 M pyridine-acetic acid buffer pH 5.0 (320 ml pyridine, 280 ml acetic acid, and H2O to 1 liter). The initial period of equilibrium chromatography was used to separate some of the constituent amino acids of AI. The elution volumes were: Asp, 49 ml; Val, Ile, Leu, 57 ml; Tyr, 63 ml; Phe, 70 ml; His, 127 ml; Arg, 231 ml. The column effluent was divided into two streams: 45% was collected with a fraction collector (1.6 ml/tube) and the remainder was submitted to alkaline hydrolysis and reaction with TNBS in a flow system utilizing a Technicon autoanalyzer, as described by Delaney. Sodium sulfite was used to increase the sensitivity of the TNBS reaction. A complete description of the apparatus and analytical system is given by Spadaro.11

Effluent corresponding to TNBS-positive material was pooled and submitted to amino acid analysis after acid hydrolysis and to high voltage electrophoresis on Whatman 3 mm paper at pH 3.5 at 40 V/cm for 3.5 hours in a Savant apparatus. Ninhydrin (0.5% in acetonitrile) and fluorescamine were used for detection. Then 25 nmoles or more of each peptide or amino acid was applied to permit the detection of contaminating material at levels of a minimum of 5% to 10%, if they had been present.

Measurement of Endo-Oligopeptidase B Activity

AI was the substrate for studies of the effect of DTT on enzyme activity and for Km determination. The initial rates of phenylalanine release were determined using the short column (0.9 X 15 cm) of the amino acid analyzer eluted with 0.2 N sodium citrate buffer, pH 3.25, at 58°C. Both AI and AII were substrates used for studies of the time course of hydrolysis and inhibition by BPP and related compounds. All the products of the reactions, Phe, [Des-Phe]-AII, and Phe-His-Leu, as well as unhydrolyzed substrates, were determined using an automatic amino acid analyzer. This chromatographic system, capable of detecting 5 to 50 nmoles of amino acids and peptides employs Aminex A-5 resin, sodium citrate buffers, and ninhydrin for detection. Recoveries were calculated by averaging the amount of each product and adding the amount of unhydrolyzed substrate. The incubation conditions are given in the legends to the figures and tables.

Results

Site of Hydrolysis

Figure 1 shows the elution diagrams obtained for hydrolysates of A1 (top) and AII (bottom) by rabbit brain endo-oligopeptidase B. The middle panel is the elution diagram of a mixture of AII, His-Leu, and AI. Endo-oligopeptidase B produced only two products from each substrate. The TNBS-positive peak eluted near 115 ml did not contain detectable amino acids either before or after acid hydrolysis. The effluent corresponding to each peak was homogeneous by electrophoresis and amino acid analysis, and the peptides were identified on the basis of amino acid composi-
Chromatography on Aminex A-5 resin of brain endo-oligopeptidase B hydrolysate of angiotensin I and II (AI and AII) The column, 0.9 x 50 cm, was developed with pyridine-acetic acid buffers. Fifty-five percent of the effluent was used for peptide detection by reaction with TNBS after alkaline hydrolysis, as described in Methods. AI (120 nmoles) and II (140 nmoles) were each incubated with 2.5 µg endo-oligopeptidase B in 0.6 ml 0.1 M sodium phosphate buffer, pH 8.5, containing 0.1 M NaCl and 2.0 x 10^{-4} M DTT for 80 minutes at 37°C. The reaction was stopped by acidification to pH 2.5. Top Panel: Hydrolysate of AI. Middle panel: Standard Peptides. AI (40 nmoles), AH (40 nmoles), and His-Leu (65 nmoles). Bottom Panel: Hydrolysate of AII. Table 1 gives the amino acid composition, electrophoretic mobility, and recovery for each peptide.

Identification was confirmed by comparing the chromatographic behavior and electrophoretic mobility of the products with those of peptides of known structure. [Des-Phe⁹]-AII and Phe-His-Leu, the complementary peptides of AI, were recovered in 41% and 43% yields, respectively. The products plus unhydrolyzed substrate, 36%, accounted for 76% to 79% of the initial substrate. Similarly, [Des-Phe⁹]-AII and Phe plus unhydrolyzed octapeptide were recovered in 64% to 69% yield (table 1). In separate experiments to determine recoveries of the peptides from the column, AI, AII, and [Des-Phe⁹]-AII (50 to 100 nmoles) were recovered in yields of 70% to 80%. Thus, quantitative overall recovery was achieved in the preparative experiments when chromatographic losses are considered.

Hydrolysates of AI and AII by endo-oligopeptidase B were also characterized analytically using a chromatographic system based on an amino acid analyzer. The data presented in table 2 show that the products were liberated in equimolar amounts during the reaction and that within the experimental error of the method (± 10%) all of the material was accounted for as product or unhydrolyzed substrate. No free amino acids were detected when separate aliquots of the hydrolysates were analyzed by standard amino acid analysis where 1% of free amino acids would have been readily observed. These data, taken with the information that [Des-Phe⁹]-AII is biologically inactive, show that brain endopeptidase B acts as an angiotensinase by hydrolyzing the Pro⁴-Phe⁷ peptide bond of both AI and AII.

Dithiothreitol Activation of Endo-Oligopeptidase B

The data in figure 2 show that endo-oligopeptidase B was strongly thiol-dependent. In the absence of DTT, the enzyme activity was only 3% that obtainable with 4.8 mM DTT. With 2 mM DTT, the amount routinely used in assays, 88% of the maximum activity is obtained. The activation by high concentrations of DTT may be due to the reduction of disulfide bridges or to the chelation of metals which inactivate the enzyme.

Km Determination

A Km of 6.3 x 10^{-8} M for the hydrolysis of AII at pH 8.5, 2.0 mM DTT, 37°C, by endo-oligopeptidase B was calculated from the data presented in figure 3.
TABLE 1. Characterization of Peptides Released from Angiotensin I and II by Brain Endo-oligopeptidase B

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Angiotensin I (fig. 1, top) (moles amino acid/moles peptide)</th>
<th>Angiotensin II (fig. 1, bottom) (moles amino acid/moles peptide)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Des-Phe&lt;sup&gt;8&lt;/sup&gt;] Ang I Ang II Phe-His-Leu</td>
<td>[Des-Phe&lt;sup&gt;8&lt;/sup&gt;] Ang II Phe-His-Leu Ang I Ang II Phe</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.60 (2) 0.99 (1)</td>
<td>0.86 (1) 0.94 (1)</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.84 (1) 1.03 (1)</td>
<td>1.00 (1) 0.99 (1)</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.80 (1) 0.81 (1)</td>
<td>0.70 (1) 0.70 (1)</td>
</tr>
<tr>
<td>Proline</td>
<td>1.00 (1) 0.99 (1)</td>
<td>0.96 (1) 0.96 (1)</td>
</tr>
<tr>
<td>Valine</td>
<td>0.94 (1) 1.05 (1)</td>
<td>0.98 (1) 0.98 (1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.86 (1) 0.95 (1)</td>
<td>0.90 (1) 0.90 (1)</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.05 (1)</td>
<td>1.04 (1)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.83 (1) 0.30 (1)</td>
<td>0.32 (1) 0.32 (1)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.92 (1) 0.72 (1)</td>
<td>1.00 (1) 1.00 (1)</td>
</tr>
<tr>
<td>Hydrolysate R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>0.62 0.52 0.70</td>
<td>0.50 0.52 0.22</td>
</tr>
<tr>
<td>Standards R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>0.61 0.54 0.70</td>
<td>0.50 0.53 0.22</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>36 41 43</td>
<td>49 20 15</td>
</tr>
</tbody>
</table>

Amino acid analyses were performed on 22-hour hydrolysates. Theoretical values based on the amino acid sequence of angiotensin I and II are given in parentheses. Amino acids present in concentrations of 0.05 residues/mole or less are not reported (see figure 1).

Inhibition of Endo-Oligopeptidase B by Angiotensin Converting Enzyme Inhibitors

Table 3 shows that 1.8 × 10<sup>-4</sup> M BPP<sub>sa</sub> (SQ 20,881), at a concentration near that of the substrate, inhibited 76% of the angiotensinase activity of the enzyme on AI and 90% of the activity for AII. At concentrations of 4.2 × 10<sup>-4</sup> M, BPP<sub>sa</sub> completely inhibited endo-oligopeptidase B. The I<sub>50</sub> value for the inhibition of endo-oligopeptidase B by BPP<sub>sa</sub> was approximately 5 × 10<sup>-4</sup> M. BPP<sub>sa</sub> was a weaker inhibitor of endo-oligopeptidase B but at high concentra-

TABLE 2. Hydrolysis of Angiotensin I and II by Brain Endo-oligopeptidase B

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Incubation time (min) 0</th>
<th>30</th>
<th>90</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td>96</td>
<td>78</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Phe-His-Leu</td>
<td>30</td>
<td>88</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>[Des-Phe&lt;sup&gt;8&lt;/sup&gt;]AII</td>
<td>29 91 96</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td>110%</td>
<td>109%</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>AII</td>
<td>100</td>
<td>82</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>Phe</td>
<td>22</td>
<td>61</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>[Des-Phe&lt;sup&gt;8&lt;/sup&gt;]AII</td>
<td>21 55 68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td>104%</td>
<td>90%</td>
<td>88%</td>
<td></td>
</tr>
</tbody>
</table>

Angiotensin I (1.9 × 10<sup>-4</sup> M) and angiotensin II (2.0 × 10<sup>-4</sup> M) were each incubated with endo-oligopeptidase B (2.2 μg) in 0.5 ml 0.1 M sodium phosphate buffer, pH 8.5, containing 0.06 M NaCl, 2.0 × 10<sup>-3</sup> M DTT at 37°C. The reaction was stopped by the addition of 0.5 ml of 0.2 M sodium citrate buffer, pH 2.2, containing 15% polyethylene glycol. Products and unhydrolyzed substrate, reported as nanomole/0.5 ml reaction mixture, were measured simultaneously with an amino acid analyzer. The specific activity of the enzyme was 1.00 μmole/min/mg protein<sup>1</sup> and 0.76 μmole/min/mg protein<sup>1</sup> for AI and II, respectively.

![Figure 2. Dithiothreitol activation of endo-oligopeptidase B. The enzyme was preincubated with DTT for 2 minutes at concentration two times that shown on the ordinate in assay buffer in the absence of substrate. Assay conditions: angiotensin II, 2 × 10<sup>-4</sup> M, 1.3 μg endo-oligopeptidase B in 0.5 ml 0.1 M sodium phosphate buffer, pH 8.5, 0.06 M NaCl, for 10 minutes at 37°C. The reaction was stopped by the addition of 0.5 ml of 0.2 M sodium citrate buffer pH 2.2 containing 15% polyethylene glycol. Phenylalanine was determined with an amino acid analyzer.](http://hyper.ahajournals.org/Downloadedfrom)
The reaction was stopped by acidification at 4, 7 and 10 minutes and phenylalanine was measured with an amino acid analyzer. The Km of 6.3 \times 10^{-6} M was calculated from the initial rates of reaction.

**Table 3. Inhibition of Brain-Endo-oligopeptidase B - Angiotensinase Activity**

<table>
<thead>
<tr>
<th>Inhibitor concentration</th>
<th>Activity change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BPP&lt;sub&gt;sa&lt;/sub&gt; (SQ 20,881)</td>
</tr>
<tr>
<td></td>
<td>AI</td>
</tr>
<tr>
<td>7.0 \times 10^{-4} M</td>
<td>-8</td>
</tr>
<tr>
<td>2.8 \times 10^{-4} M</td>
<td>-36</td>
</tr>
<tr>
<td>1.8 \times 10^{-4} M</td>
<td>-76</td>
</tr>
<tr>
<td>4.2 \times 10^{-5} M</td>
<td>-100</td>
</tr>
</tbody>
</table>

The enzyme was incubated with each compound in 0.1 M sodium phosphate buffer, pH 8.5 containing 0.06 M NaCl and 2.0 mM DTT for 10 minutes at 37°C and then with substrate for 30 minutes. Assay conditions: enzyme, 2.2 \mu g/ml; substrate, 2 \times 10^{-4} M; inhibitor concentrations are given in the table. The reaction was stopped by acidification. Unhydrolyzed substrate and products were measured simultaneously with an amino acid analyzer. ND = not determined.

**Discussion**

Rabbit brain endo-oligopeptidase B hydrolyzes the Pro-Phe peptide bond in bradykinin and in a series of analogs and fragments. In this paper, we show that the enzyme also acts as an angiotensinase for the precursor肽 AI as well as for AII, is thiol-activated, and hydrolyzes AII with a Km of 6 \times 10^{-6} M. In preparative and analytical experiments, all of the products of hydrolysis of AI and AII plus unhydrolyzed substrate were accounted for quantitatively, indicating that hydrolysis occurred only at the Pro-Phe peptide bond. Endo-oligopeptidase B hydrolyzes AIII as well as analogs of AI and II when Tyr, Leu, Ile, or Val occupy the position of Phe in the scissile bond.

The peptides <Gln-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro-BPP<sub>sa</sub>, SQ 20,881> and <Gln-Lys-Trp-Ala-Pro-BPP<sub>sa</sub>, SQ 20,475>, isolated from Bothrops jararaca venom, potentiates the action of bradykinin and inhibit the conversion of AI to II in vivo and in vitro and have been used to demonstrate the participation of the renin-angiotensin system in some forms of hypertension. These substances are potent inhibitors of angiotensin-converting enzyme and have little activity against other enzymes tested. The results presented here indicate that BPP<sub>sa</sub> inhibits angiotensin-converting enzyme by its acidic pH optima, 6.0 \times 10^{-4} M (mixed inhibitor), and 1.7 \times 10^{-4} M (competitive inhibitor), respectively. These substances are potent inhibitors of angiotensin-converting enzyme and have little activity against other enzymes tested. The results presented here indicate that BPP<sub>sa</sub> inhibits angiotensin-converting enzyme by its acidic pH optima, 6.0 \times 10^{-4} M (mixed inhibitor), and 1.7 \times 10^{-4} M (competitive inhibitor), respectively. In contrast, captopril does not appreciably inhibit endo-oligopeptidase B. Although the inhibition of the enzyme by BPP<sub>sa</sub> is 1 to 2 orders of magnitude weaker than that of ACE, these differences may not be significant in in vivo experiments where large doses of inhibitors are routinely used and local concentrations are unknown. Thus, the interpretation of the pharmacological properties of BPP<sub>sa</sub> at least when injected intracerebroventricularly, must be made not only in terms of the inhibition of angiotensin-converting enzyme but also in terms of the inhibition of brain endo-oligopeptidase B which inactivates several hormones. Comparison of the effects of BPP<sub>sa</sub> and captopril in physiological experiments may provide a way to distinguish between the activity of angiotensin-converting enzyme and of endo-oligopeptidase B. Several types of angiotensinases have been described. The cathepsin-like enzymes prolylcarboxypeptidase and cathepsin A also liberate Phe from AII and can be easily distinguished from endo-oligopeptidase B by their acidic pH optima, molecular weight, and because they act as carboxypeptidases.
Cathepsin C, dipeptidyl aminopeptidase,\textsuperscript{a} angiotensinase B\textsuperscript{a} hydrolyze All at sites other than Pro-Phe.

Recently, several enzyme preparations capable of hydrolyzing the Pro-X peptide bond have been isolated from nervous tissue of several species: "thyroliberin-deamidating enzyme",\textsuperscript{4,5} "prolyl endopeptidase",\textsuperscript{4} "proline endopeptidase",\textsuperscript{4} and "post-proline cleaving enzyme."\textsuperscript{4,6} Each of these enzymes has been shown to hydrolyze the Pro-X peptide bond in one or more of the following peptide hormones: bradykinin, AI, All, AIII, Iuliberin, thyroliberin, and Substance P. They may be closely related to or identical with rabbit brain endo-oligopeptidase B described by us in 1976.\textsuperscript{4,6}

Extracts of peripheral tissue release the same set of peptides from bradykinin as nervous tissue\textsuperscript{a} and hydrolyze fluorogenic substrates having the general structure carbobenzyoxy-X-L-prolyl-4-methoxy-\beta-naphthylamide,\textsuperscript{8} suggesting that endo-oligopeptidase B or similar enzymes are present both in peripheral as well as in nervous tissues. The term "post-proline cleaving enzyme" was first used by Koida and Walter for a lamb kidney enzyme of molecular weight 115,000 which hydrolyzes All and bradykinin.\textsuperscript{4,8} However, Ser-Pro-Phe-Arg is hydrolyzed faster than bradykinin by endo-oligopeptidase B but not hydrolyzed by the lamb kidney enzyme.\textsuperscript{11}

Although the role of brain endo-oligopeptidase B remains to be established, inactivation by peptide bond hydrolysis provides a mechanism to regulate the biological activity of peptides, which act as hormones or neurotransmitters in nervous and peripheral tissue. In view of the fact that most hormones are small peptides that contain proline and that most tissue peptidases and proteases do not hydrolyze prolyl-peptide bonds, endo-oligopeptidase B may be considered a likely candidate for this regulatory role by virtue of its specificity for proline in the scissile bond and selectivity for "small" peptides.\textsuperscript{11}

Acknowledgments

We acknowledge the expert technical assistance of Jane D. Berti Terra, and the skillful preparation and typing of the manuscript by Eletra Greene.

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Hypertension. 1982;4:178-184
doi: 10.1161/01.HYP.4.2.178

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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