Substrate Specificity of Tonin from Rat Submaxillary Gland

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SUMMARY The substrate specificity of tonin from rat submaxillary gland was examined with a series of synthetic peptides encompassing the C-terminus of the decapeptide substrate angiotensin I. In contrast to angiotensin I-converting enzyme from plasma or lung, only angiotensin I, (des-Asp')-angiotensin I, and (des-Asp')-angiotensin I, are substrates of tonin with Km values of 34.5 μM, 39.3 μM, and 54.4 μM, respectively, while the shorter C-terminal peptides are not hydrolyzed. Thus, the N-terminal sequence extending from position 1 to 3 is the enzymatic binding site for tonin. Turnover numbers of 33.4 sec⁻¹, 42.8 sec⁻¹, and 6.5 sec⁻¹ are observed for the hydrolysis of angiotensin I, (des-Asp')-angiotensin I, and (des-Asp')-angiotensin I, respectively. The relative percentage rates of hydrolysis (proportional to V/Km) at low substrate concentrations ([S] < Km) are almost identical for (des-Asp')-angiotensin I, angiotensin I, and the tetradecapeptide substrate, indicating that these three peptides are equally good substrates at low physiological concentrations. The observed high specificity of the enzyme lends support to the possible important role of tonin for local conversion in tissue. The conversion of (des-Asp')-angiotensin I to (des-Asp')-angiotensin II (angiotensin III) is of particular interest in relation to the recently suggested, potential role of angiotensin II in aldosterone release.

IN RECENT reports the identification and partial purification of an angiotensin I-converting enzyme from rat submaxillary gland was described. The enzyme, for which the name tonin has been suggested, has recently been purified to homogeneity. The subsequent enzymatic characterization revealed major differences between tonin and the classic angiotensin I-converting enzyme found in plasma, lung, and other tissues. Compared to angiotensin I-converting enzyme, tonin has a much lower molecular weight of only 28,700, a different pH optimum (pH 6.5–7.2), and no chloride requirement for enzymatic activity. The inhibitors of angiotensin I-converting enzyme, ethylenediaminetetraacetic acid (EDTA), dipyridyl, and the pentapeptide Pyr-Lys-Trp-Ala-Pro have no effect on tonin which, on the other hand, is strongly inhibited by a plasma component. In contrast to the classic converting enzyme, tonin is devoid of kininase II activity. Preliminary studies with the frequently used tripeptide substrates of angiotensin I-converting enzyme showed that these compounds are very poor substrates of tonin. However, it was found that tonin can form angiotensin II directly from renin tetradecapeptide substrate by cleavage of the Phe-His bond. On the basis of the suspected high specificity of the enzyme and the presence of a strong plasma protein inhibitor, it has been suggested that tonin may play an important role in the local generation of angiotensin II in tissue.

The purpose of this study was to further elucidate the substrate specificity of tonin by use of a series of synthetic polypeptides related to the C-terminus of angiotensin I. One of these peptides, (des-Asp')-angiotensin I, is of particular interest as a substrate for possible conversion to (des-Asp')-angiotensin II (angiotensin III) which recently has been proposed as a potentially important agonist in its own right for aldosterone release.

Methods

SYNTHESIS AND PURIFICATION OF PEPTIDES

Precoated plates (Silicagel G, 250 μm, Analtech) were used for ascending thin layer chromatography in the following solvents: (1) n-butanol-acetic acid-H2O (BAW) (4:1:5); (2) n-butanol-pyridine-acetic acid-H2O (BPAW) (15:10:3:12); (3) ethyl acetate-pyridine-acetic acid-H2O (EPAW) (5:5:1:3). Compounds on the chromatograms were detected with the chlorine peptide spray or ninhydrin or with both. The deprotected peptides were hydrolyzed in 6 N HCl for 24 hours and amino acid analyses were performed with a Beckman model 120C amino acid analyzer. Melting points were determined with a Fisher electrothermal melting point apparatus and are uncorrected.

The Merrifield method was used for the synthesis of peptides, with the aid of a Beckman model 990 peptide synthesizer. t-Boc-amino acids were either purchased from Beckman or synthesized according to the method of Schwyzer et al. The following side chain-protected t-Boc-amino acids were used: t-Boc-im-tosyl-L-histidine, t-Boc-O-benzyl-L-tyrosine, t-Boc-nitroarginine, and t-Boc-L-aspartic acid-β-benzyl ester. t-Boc-L-leucine was reacted with chloromethyl resin (Bio-Beads S-X1, Bio-Rad Laboratories, 200–400 mesh, 1.34 mEq/g), whereby a yield of 0.400 mmol of t-Boc-amino acid per g of resin was obtained. The coupling of each subsequent t-Boc-amino acid was performed according to the following protocol: The esterified resin was (1) washed with CHCl3 and (2) prewashed with 25% (vol/vol) CF3COOH in CHCl3 for 5 minutes, (3) the Boc group was removed by treatment with 25% (vol/vol) CF3COOH in CHCl3 for 30 minutes, (4) washed with CHCl3, (5) washed with CH2Cl2, (6) neutralized with 10% Et3N (vol/vol) in CH2Cl2 for 30 minutes, and (7) washed with CH2Cl2 six times for 1.5 minutes; (8) t-Boc-amino acid in CH2Cl2

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(2.5-fold excess) was added; in the case of t-Boc-im-tosyl-L-histidine and t-Boc-L-nitroarginine the t-Boc-amino acid was first dissolved in a minimal amount of N,N-dimethylformamide (DMF) and the resulting solution was subsequently brought to the desired volume by addition of CH₂Cl₂; (9) dicyclohexylcarbodiimide (2.5-fold excess in CH₂Cl₂; (9) dicyclohexylcarbodiimide (2.5-fold excess in CH₂Cl₂, (10) washed with CHCl₃, and (11) washed with CHCl₃.

Unless otherwise mentioned all washings were carried out three times for 1.5 minutes. After each cycle completeness of the reaction was checked with the color test. After completion of the last cycle the peptide was cleaved from the resin and completely deprotected by treatment with HF. The reaction was carried out for 1 hour at 0°C with 20 ml of HF and in the presence of 2 ml of anisole as scavenger per g of resin. After removal of the HF the resin was extracted with 90% acetic acid and the resulting solution was lyophilized.

The crude reaction products were purified by partition chromatography on a Sephadex G-25 column (2.5 × 100 cm) with the solvent system n-butanol-acetic acid-H₂O (4:1:5). In each case 200 mg of crude product were applied to the column and fractions of 7 ml were collected at a flow rate of 70 ml/hr. All peptides could be completely separated from contaminating side-products by this one chromatographic step. Fractions containing the pure peptide were pooled, evaporated in vacuo, and lyophilized from 90% AcOH. The overall yield of the purified peptides, based on initial amino acid attached to the polymer, was 19–35%.

(Ile*)-angiotensin I: Melting point (mp) = 204°C with decomposition (dec); thin layer chromatography (TLC) (silica gel) retention factor (Rᵢ) = 0.20 (BAW), 0.55 (BPAW), and 0.49 (EPAW). Amino acid analysis: Asp, 1.07; Arg, 1.09; Val, 1.00; Tyr, 0.82; Ile, 0.93; His, 1.99; Pro, 1.16; Phe, 1.02; Leu, 1.09.

(des-Asp¹,Ile*)-angiotensin I: mp = 191°C; TLC (silica gel) Rᵢ = 0.16 (BAW), 0.49 (BPAW), and 0.58 (EPAW). Amino acid analysis: Arg, 1.03; Val, 1.05; Tyr, 0.88; Ile, 0.86; His, 1.91; Pro, 1.14; Phe, 1.03; Leu, 1.00. Lyophilization of this peptide yielded an oil; the product was obtained in solid form through precipitation from DMF/ether.

(des-Asp¹,des-Arg³,Ile*)-angiotensin I: mp = 153°C dec; TLC (silica gel) Rᵢ = 0.28 (BAW), 0.62 (BPAW), and 0.66 (EPAW). Amino acid analysis: Val, 0.99; Tyr, 0.96; Ile, 0.92; His, 2.11; Pro, 1.15; Phe, 1.00; Leu, 1.06.

The synthesis of Z-Phe-His-Leu-ÖH was reported in an earlier paper and the peptides H-Pro-Phe-His-Leu-ÖH, H-Ile-His-Pro-His-Leu-ÖH and H-Tyr-Ile-His-Pro-Phe-His-Leu-ÖH were obtained from the Papanicolaou Cancer Research Institute, Miami).

PREPARATION AND ASSAY OF TONIN

Tonin was purified from rat submaxillary glands by a procedure involving differential centrifugation, ammonium sulfate precipitation, gel filtration on Sephadex G-150 and ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, phosphocellulose, SP-Sephadex C-25, and SP-Sephadex C-50. Homogeneity of the enzyme was demonstrated by disc gel electrophoresis and by analytical ultracentrifugation analysis.

The enzyme assay was performed in 0.1 m potassium phosphate buffer, pH 6.8, containing bovine serum albumin (20 mg/ml), disopropyl fluorophosphate (DFP) (0.70 mM), dipipryridyl (8 mM), and EDTA (1.3 mM); 2.0 ml of this incubation medium containing a known amount of enzyme was mixed with 150 µl of a solution of angiotensin I in 25% methanol (MeOH) and the mixture was incubated for 30 minutes at 37°C. The enzymatic reaction was followed through determination of histidylleucine release by means of the fluorometric assay based on the reaction of dipeptide with o-phthalaldehydë. A Technicon Auto-Analyzer was employed for automation of the entire enzymatic assay. The same assay conditions were used for all the other peptide substrates described in this paper.

One unit of tonin activity is defined as the amount of tonin that hydrolyzes 1 nm of angiotensin I per minute at 37°C under the conditions described above. Purified tonin has a specific activity of 1.80 × 10⁵ U/mg of protein.

RESULTS

HYDROLYSIS OF SUBSTRATES BY TONIN

The extent of the tonin-catalyzed hydrolysis of the various substrates relative to angiotensin I is presented in Table 1. The respective peptide substrate (0.4 µmol) was incubated with 450 ng of purified tonin under standard assay conditions and the histidylleucine release was determined after 30 minutes. No hydrolysis of H-Tyr-Ile-His-Pro-Phe-His-Leu-ÖH and the shorter C-terminal fragments of angiotensin I could be detected; (des-Asp¹,des-Arg³)-angiotensin I was hydrolyzed only to an extent of 20% as compared to angiotensin I and (des-Asp¹)-angiotensin I under the conditions used.

The hydrolysis of angiotensin I, (des-Asp¹)-angiotensin I, and (des-Asp¹,des-Arg³)-angiotensin I was studied in greater detail by determination of the Michaelis-Menten constants and turnover numbers. Figure 1 shows the determination of the kinetic parameters by means of the double-reciprocal plot (Lineweaver-Burk). The obtained parameters are tabulated in Table 2, which also contains the calculated turnover numbers and relative percentage rates of hydrolysis. The latter constitute the most relevant parameters for comparison of substrates at low, physiological substrate concentration.

Discussion

The obtained results show that aside from the differences in localization, physical parameters, ion requirements, and...
inhibition between angiotensin I-converting enzyme and tonin, the substrate requirements of the two enzymes are far from identical. Angiotensin I-converting enzyme is relatively nonspecific because it is able to hydrolyze a large number of peptide substrates ranging from N-protected tripeptides to the B chain of insulin.4 However, it is strictly a peptidyl dipeptidase, as demonstrated by the fact that the nonapeptide, (des-Leu')-angiotensin I, 14> lB  and the undecapeptide, (Leu')-angiotensin I, 14> lB  are not hydrolyzed to form angiotensin II. Within the series of peptides related to the C-terminal sequence of angiotensin I, angiotensin I-converting enzyme does not have an important recognition site for the N-terminal amino acid residues of angiotensin I, Asp-Arg-Val. In the case of partially purified angiotensin I-converting enzyme from hog plasma, peptides encompassing the C-terminal sequence of angiotensin I maintain approximately the same \( V_{\text{max}} \) with increasing chain length while \( K_m \) is steadily lowered.13 Thus the longer peptides are somewhat better substrates than the shorter ones. In a more recent study with partially purified angiotensin I-converting enzyme from pig lung, the shorter C-terminal sequences of angiotensin I were hydrolyzed with a high \( V_{\text{max}} \) which gradually decreased in the course of chain extension until a minimal value was reached with (des-Asp')-angiotensin I.14 Concomitantly a decrease in \( K_m \) was observed and the ratio \( V_{\text{max}}/K_m \) was fairly constant for the series of peptides examined in this investigation. Studies with D-amino acid-substituted analogues of angiotensin I revealed the C-terminal sequence, Phe-His-Leu, as the enzymatic binding site for angiotensin I-converting enzyme in vivo and in vitro.15 In contrast to the situation with angiotensin I-converting enzyme, tonin does not hydrolyze any of the shorter peptides related to the C-terminal sequence of angiotensin I until a chain length of eight amino acid residues is reached. Incubation of equimolar amounts of angiotensin I, (des-Asp')-angiotensin I and (des-Asp',des-Arg')-angiotensin I with purified tonin for 30 minutes showed that the octapeptide is hydrolyzed to an extent of 20% as compared to angiotensin I, whereas the nonapeptide is hydrolyzed as rapidly as angiotensin I. It thus appears that the N-terminal sequence of angiotensin I, Asp-Arg-Val, is the important recognition and binding site for tonin. In particular, the presence of the positively charged arginine residue is essential for fast hydrolysis; possibly this residue anchors the substrate at the active site through ionic interaction with a negatively charged moiety of the enzyme or through formation of a hydrogen bond. Alternatively, it is also conceivable that this residue stabilizes a particular conformation of the peptide, thereby rendering it a better substrate for fast hydrolysis. In this context it is interesting to note that no product inhibition of tonin with angiotensin II is observed (unpublished results). As reported earlier,4 tonin also hydrolyzes the tetradecapeptide substrate to form angiotensin II directly and there is some preliminary evidence that the enzyme also can generate angiotensin II from a natural substrate in plasma.5 On the other hand, the nonapeptide, (des-Leu')-angiotensin I is not a substrate for tonin.5 It thus appears that tonin can specifically cleave the Phe-His-bond of N-terminal peptide sequences of the natural substrate, provided that the peptide chain at the C-terminus is at least extended to the length of

### TABLE 2  Michaelis-Menten Parameters for the Hydrolysis of Peptides by TONIN

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ) (µM)</th>
<th>( V_{\text{max}} ) (nmol x min(^{-1}))</th>
<th>Turnover number (sec(^{-1}))</th>
<th>Relative percentage rate of hydrolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin I</td>
<td>34.5</td>
<td>20.6</td>
<td>33.4</td>
<td>91.3</td>
</tr>
<tr>
<td>(des-Asp')-Angiotensin I</td>
<td>39.5</td>
<td>25.7</td>
<td>42.8</td>
<td>100</td>
</tr>
<tr>
<td>(des-Asp',des-Arg')-</td>
<td>54.4</td>
<td>3.9</td>
<td>6.5</td>
<td>11.0</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The relative percentage rate of hydrolysis is proportional to \( V_{\text{max}}/K_m \), which is the first-order rate constant at low substrate concentrations ([S] ≪ \( K_m \)): \( v = (V_{\text{max}}/K_m)[S] \).
angiotensin I. On the basis of these findings tonin has to be
classified as a highly specific endopeptidase.

The $K_m$ values of tonin for (des-Asp$^{1}$)-angiotensin I and
angiotensin I are almost identical, whereas that of (des-
Asp$^{1}$,des-Arg$^{2}$)-angiotensin I is approximately doubled
compared to the values observed with the latter two peptides. The $K_m$
values determined for angiotensin I and
angiotensin I-converting enzyme from various sources are of
the same order of magnitude ($20-70 \mu M$)$^{1, 17, 18, 20}$ as that
observed with tonin. The turnover numbers obtained for
angiotensin I and (des-Asp$^{1}$)-angiotensin I are
33.4 sec$^{-1}$ and 42.8 sec$^{-2}$, respectively, whereas that deter-
mined for (des-Asp$^{1}$,des-Arg$^{2}$)-angiotensin I is significantly
lower (6.5 sec$^{-1}$). The turnover number for angiotensin I is
about 7 times smaller than that observed with pulmonary
angiotensin I-converting enzyme (257 sec$^{-1}$).$^{21}$ For the
hydrolysis of hog renin substrate by purified hog renin a
turnover number of 4.7 sec$^{-1}$ has recently been published as a
preliminary estimate.$^{21}$ The relative percentage rate of
hydrolysis is the most relevant parameter for comparison of
different substrates at low, physiological concentrations.$^{13}$

The almost identical values obtained with tonin for angi-
tensin I and (des-Asp$^{1}$)-angiotensin I demonstrate that these
two peptides are equally good substrates, whereas the 10
times lower hydrolysis rate observed with (des-Asp$^{1}$,des-
Arg$^{2}$)-angiotensin I reveals that the octapeptide is a poor
substrate. Earlier data with partially purified tonin demon-
strated that relative to angiotensin I an 8 times lower $K_m$
value is observed with the tetradecapeptide substrate.\textsuperscript{3}
However, this difference in the $K_m$ values is almost compen-
sated for by the 6 times lower $V_{max}$ determined for the
tetradecapeptide substrate as compared to angiotensin I. Similar relative percentage rates of hydrolysis are therefore
obtained for these two peptides and they are almost equally
good substrates for tonin. The difference in the Michaelis-
Menten parameters may be due to a conformational change
produced by the extension of the chain at the C-terminus in
the tetradecapeptide substrate.

All these data corroborate the high specificity of tonin
which is consistent with the concept of tonin playing an
important role in the intracellular generation of angiotensin
II in tissue.$^{3}$

The heptapeptide (des-Asp$^{1}$)-angiotensin II (angiotensin
II) has recently been described as a possible important
agonist in its own right for stimulation of aldosterone
release.$^{6}$ Angiotensin III either could be formed from angi-
tensin II through action of angiotensinase A or from
(des-Asp$^{1}$)-angiotensin I through cleavage of the Phe-His
bond by converting enzymes. Our results show that tonin
can generate angiotensin III from (des-Asp$^{1}$)-angiotensin I
equally well as it forms angiotensin II from angiotensin I. In
this respect tonin is quite similar to pig lung angiotensin
I-converting enzyme which in partially purified form was
shown to cleave the Phe-His bond of both angiotensin I and
(des-Asp$^{1}$)-angiotensin I with again only small differences in the
kinetic parameters.$^{17}$ The physiological significance of these
in vitro findings of angiotensin III formation is at the
moment unclear, since nothing is known about the circulat-
ing levels of intracellular concentrations of angiotensin III
and (des-Asp$^{1}$)-angiotensin I. The possible role of these two
peptides as third and fourth agonists in the renin-angiotensin
system has yet to be demonstrated by means of specific
radioimmunoassays or other methods.

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