# Angiotensin-Converting Enzyme: I. New Strategies for Assay

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The disposition of converting enzyme (kininase II) on the luminal surface of pulmonary endothelial cells is well established. Further, it is known that there is a net conversion of angiotensin I into angiotensin II as blood passes through the lungs. However, little is known about modulations of converting enzyme activity that may arise through, e.g., changes in the quality of inhalants, blood flow, or blood oxygenation. There are few data on the effects of lung disease. A major barrier to studies to examine for pathophysiologic modulations of converting enzyme is that of assay. The enzyme can be measured in terms of the rate of formation of angiotensin II from a known quantity of angiotensin I. However, both peptides are biologically active, and lungs contain other enzymes capable of degrading them. We have developed a series of radiolabeled, acylated tripeptides to improve our ability to examine for changes in the net converting enzyme of intact lungs. The enzyme, a dipeptidyl carboxypeptidase, is capable of removing C-terminal dipeptides from a variety of oligopeptides. We have prepared benzoyl-Gly-Gly-Gly (I), benzoyl-Pro-Phe-Arg (II), benzoyl-Gly-His-Leu (III), benzoyl-Phe-Ala-Pro (IV), and benzoyl-Phe-His-Leu (V), each containing a $^3$H-atom in the *para* position of the benzoyl moiety. Substrates I and III have been used previously in photometric assays of low sensitivity. II is the acylated C-terminal tripeptide of bradykinin, IV is an acylated tripeptide analog of BPP$_{5a}$ (<Glu-Lys-Trp-Ala-Pro) and V is the acylated C-terminal tripeptide of angiotensin I. These substrates can be used *in vitro* or *in vivo* to measure converting enzyme. The $^3$H-labeled product is separable by partitioning between an organic solvent and acidified aqueous solution. The product is quantified by scintillation counting of the organic phase. The choice of substrate depends on the goals of the experiment: substrate I or III when wide variations in substrate concentrations are needed but high sensitivity is not; substrate IV when high sensitivity is needed.

## Introduction

Angiotensin-converting enzyme appears to occur in association with endothelial cells of virtually all vascular beds (*1*). The enzyme also occurs in association with brush border of renal proximal tubule (*2, 3*) and small intestine (*4*). However, bulk conversion of angiotensin I into angiotensin II occurs, apparently unaided by blood enzymes, within the pulmonary vascular space. Angiotensin-converting enzyme is situated on the luminal surface of pulmonary endothelial cells (*5-8*) and is strategically placed for bulk processing, not only of angiotensin I but also bradykinin and perhaps other oligopeptides (neurotensin, enkephalins) having adequate affinity for the enzyme. The lungs have an enormous vascular bed through which flows the entire cardiac output. Further, because of the peculiar situation of the

lungs within the general circulation, their venous effluent empties directly into the arterial circulation. Hence, the activity or inactivity of lung angiotensin converting enzyme may matter greatly to the quality of arterial blood and to the function of distant organs. Whether angiotensin-converting enzyme is important to intrinsic lung function remains to be determined.

A major barrier to improved understanding of the pathophysiologic role of lung angiotensin-converting enzyme is that of adequate techniques for assay. In principle, one should be able to measure modulations of enzyme activity by examining for variations in the pulmonary arteriovenous gradients of angiotensin I, angiotensin II, and bradykinin. However, these substances occur in blood at concentrations of less than $10^{-10}M$. Radioimmunoassays can be used, but the precision of such assays is not great and their specificities, especially for bradykinin, are not proved. The assay problem is not substantially improved by following the changes of activity of

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intravenously administered bradykinin or angiotensin I, either by radioimmunoassay or the dynamic, on-line bioassay system of Vane (9). Furthermore, the bioassay is not readily adaptable for use with human subjects.

In an effort to develop simpler and more precise means of measuring angiotensin-converting enzyme, we have synthesized a series of acylated tripeptides, each bearing a radioisotope in the moiety used for acylation. Most have been prepared to contain a $^3$H-atom, but $\gamma$-emitting isotopes, e.g., $^{125}$I, can be used as well. Angiotensin-converting enzyme functions as a dipeptidyl carboxypeptidase and acts on acylated tripeptides to yield a dipeptide and an acyl-amino acid (10, 11). By careful selection of amino acid residues one can vary the affinity of substrate for enzyme. As has been shown for benzoyl-Gly-His-Leu (hippuryl-His-Leu) (12, 13), the acyl-amino acid product can be separated from the acyl-tripeptide substrate by partitioning between acid-aqueous solvent and an organic solvent such as ethyl acetate or toluene. The efficiency of the extraction technique is a function of amino acid residues and their sequence. Thus an aromatic or aliphatic amino acid is advantageous for the $^3$H-labeled product and a basic amino acid is useful when placed in the dipeptide leaving group.

Six radiolabeled acylated tripeptides have been prepared, and four have been characterized in detail as substrates for angiotensin converting enzyme. Three of the latter compounds can be used to measure converting enzymes of lungs perfused with artificial salt solutions. We believe that these three substrates can be used to measure lung converting enzyme in vivo.

## Materials and Methods

Synthetic bradykinin and its higher homologs, angiotensin I and its lower homologs, and BPP$_{9a}$ and its lower homologs were prepared by the solid-phase peptide synthesis technique (14). The completed peptides were purified by two or more of the following techniques: counter-current distribution, molecular sieve chromatography (Bio-Gel P-2, Sephadex G-25), and partition chromatography (LH-20 within 6% butanol in H$_2$O; Sephadex G-25 with butanol, acetic acid, H$_2$O; 4:1:5). The peptides were synthesized by J. M. Stewart, University of Colorado Medical Center and G. H. Fisher, University of Miami (15).

The compounds, $p$-I-benzoyl-Gly-Gly-Gly, $p$-I-benzoyl-Pro-Phe-Arg, $p$-I-benzoyl-Gly-His-Leu, $p$-I-benzoyl-Phe-Ala-Pro, $p$-I-benzoyl-Phe-His-Leu, and $p$-I-benzoyl-Phe-Ser-Pro, were synthesized by stepwise solution methods (16, 17). Purifications

were accomplished with one or more of the following techniques: molecular sieve chromatography (Sephadex G-10), ion-exchange chromatography (CM-Sephadex or DEAE-Sephadex with ammonium acetate buffers). The $p$-$^3$H-benzoyl derivatives were prepared by New England Nuclear Corp. by dehalogenation in $^3$H$_2$ gas at 39 psi for 1 hr over 5% rhodium on calcium carbonate in dimethylformamide and H$_2$O, 1:1 ($v/v$). Each of the $^3$H-labeled acyl-tripeptides was obtained at specific radioactivities of >20 Ci/mmole. The $^3$H-labeled compounds were purified on Sephadex G-10, and each behaved as a pure substance in two or more thin-layer chromatography systems (16) and on paper electrophoresis at pH 2.0 and 5.0 (18). The $^3$H-labeled compounds, stored at 1 mCi/ml of ethanol at −28°C, did not undergo detectable radiolysis over a period of 12 months.

Our studies used angiotensin-converting enzyme partially-purified from guinea pig lung or urine and human urine (17). Pure rabbit lung angiotensin converting enzyme was provided by R. L. Soffer, Cornell University Medical College (11).

The experiments using anesthetized rats or perfused isolated rat lung were performed as described by Roblero et al. (19) and Ryan et al. (20). For in vitro assays, we used the protocol described by Ryan et al. (16). In brief, reactions were stopped by adding 0.1N HCl to a pH <2. The H-labeled acyl-amino acid product was extracted into an equal volume of organic solvent. Ethyl acetate was used for all substrates except $^3$H-benzoyl-Phe-Ala-Pro. Toluene was used for extraction of reaction mixtures containing the latter substrate (17).

## Results and Discussion

### Recognition Sites of Angiotensin-Converting Enzyme

In the early 1970's, it was recognized that angiotensin-converting enzyme is misnamed. The enzyme acts as a dipeptidyl carboxypeptidase capable of removing the C-terminal dipeptide of a variety of oligopeptides (10, 21, 22). In fact, bradykinin is a better substrate (lower $K_m$) than is angiotensin I, and the enzyme is sometimes known as kininase II. In terms of $K_m$, the venom peptide, BPP$_{5a}$ (23) is the best of the known substrates ($K_m < 10^{-6}M$).

Clearly, the enzyme is not specific, but there is reason to believe that the enzyme is highly selective. Further, it is evident that the enzyme possesses a number of recognition sites; knowledge of which is useful for design of synthetic substrates.

In our early studies on the metabolism of kinins by intact lungs, we noted that bradykinin is inactivated

more rapidly than any of its $N$-extended higher homologs (19, 24). When pure lung angiotensin converting enzyme became available (25), it was found that much of the selectivity of processing of kinins by intact lungs could be explained in terms of one enzyme (see Table 1). Clearly, the enzyme is sensitive to amino acid residues at a distance from the cleavage site. Chain length and charge of the substrates appear to be important factors.

Angiotensin I and its lower homologs are also treated differently by converting enzyme. As shown in Table 2, des-Asp[1]-angiotensin I is a somewhat better substrate than is angiotensin I itself (lower $K_m$, higher $V_{max}/K_m$). In terms of $V_{max}/K_m$, the C-terminal pentapeptide is a better substrate than angiotensin I. In comparison with some of the acylated tripeptide substrates to be described below, the C-terminal hexapeptide is a surprisingly poor substrate. Nonetheless, Table 2 emphasizes the points that converting enzyme is sensitive to amino acid residues distant from the cleavage site and that chain length is an important factor.

Table 1. Processing of bradykinin and its higher homologs by isolated, perfused rat lungs and by angiotensin-converting enzyme purified from pig lung.[a]

| | Relative reaction rates, %[b] | |
| | Intact lungs | Converting enzyme |
| --- | --- | --- |
| Bradykinin (BK) | 100 | 100 |
| Lys-BK | 96 | 65 |
| Met-Lys-BK | 80 | 55 |
| Lys-Lys-BK | 0 | 12 |
| Gly-Arg-Met-Lys-BK | <10 | 5 |
| Polistes kinin | 0 | 2 |

[a]Further experimental details as described by Dorer et al. (22)
[b]Results are expressed as relative reaction rates (the rate for bradykinin = 100%). Rates of hydrolysis by pure enzyme were measured in terms of the formation of ninhydrin-reactive products (21). Rates of degradation of kinins by isolated lungs were measured by bioassay (19, 24).

Greater insight into the importance of enzyme recognition sites has come from studies of two inhibitors of converting enzyme, BPP$_{9a}$ (<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and BPP$_{5a}$ (<Glu-Lys-Trp-Ala-Pro); peptides purified from the venom of *Bothrops jararaca* (23, 26). Cushman et al. (27) have examined for the inhibitory potencies of a series of analogs of the two inhibitors, and we (15) have examined for the inhibitory potencies of BPP$_{9a}$ and its lower homologs. Both studies have shown that Trp of BPP$_{9a}$ interacts with an important recognition site, apparently distant from the catalytic site of the enzyme. However, the distant binding site may not be fixed or stationary, as the N-terminal homolog of BPP$_{9a}$, <Glu-Trp-Pro-Arg-Pro-Gln-Ile, and its lower homolog, Trp-Pro-Arg-Pro-Gln-Ile, are virtually as potent as is BPP$_{9a}$ itself (respective $I_{50}$ values: 54, 34, and 28 nM). Results obtained with some of the homologs are shown in Table 3.

At present, it appears that binding of a substrate or inhibitor to angiotensin converting enzyme is initiated by interaction of the free $\alpha$-carboxy group of the substrate with an ionic recognition site. Our results and those of others indicate that the ring of proline or the side-chain of isoleucine aids in binding (29, 30). Clearly, the side chains of C-terminal arginine, as in bradykinin, and leucine, as in angiotensin I, are not unfavorable for binding. Similarly, phenylalanine or histidine are not unfavorable penultimate residues, but alanine (as in BPP$_{5a}$) may be the most favorable. Less attention has been paid so far to the importance of the side-chain of the third amino acid residue; i.e., that on the $N$-terminal side of the cleavage site. Such data as are available indicate that an aromatic amino acid (e.g., Trp of BPP$_{5a}$ or Phe of angiotensin I) is favorable for binding. Judging from the low $I_{50}$ of the C-terminal tripeptide of BPP$_{9a}$, Ile-Pro-Pro (Table 3), the side chain of isoleucine fits well. Through such considerations, we have prepared six acylated tripeptide substrates for converting enzyme.

Table 2. Reaction of pig lung angiotensin-converting enzyme with angiotensin I and its C-terminal lower homologs.[a]

| | $K_m$, $\mu M$ | $V_{max}$ $\mu$mole/min-mg | $V_{max}/K_m$ |
| --- | --- | --- | --- |
| Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu | 33 | 1.98 | 0.060 |
| Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu | 11 | 0.99 | 0.090 |
| Val-Tyr-Ile-His-Pro-Phe-His-Leu | 170 | 7.94 | 0.047 |
| Tyr-Ile-His-Pro-Phe-His-Leu | 100 | 8.83 | 0.088 |
| Ile-His-Pro-Phe-His-Leu | 1000 | 37.71 | 0.038 |

[a]Experimental details as given previously (28). Reaction rates were measured in terms of the appearance of the fluorophor formed by reacting His-Leu with $o$-phthaldialdehyde. $V_{max}$ is expressed in terms of $\mu$mole/min per mg of enzyme protein.

**Table 3. Inhibition of angiotensin-converting enzyme by BPP[9a] and some of its lower homologs[a]**

| Structure | $I_{50}$, nM[b] |
|---|---|
| <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 28 |
| Trp-Pro-Arg-Pro-Gln-Ile | 34 |
| Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 50 |
| <Glu-Trp-Pro-Arg-Pro-Gln-Ile | 54 |
| Pro-Arg-Pro-Gln-Ile-Pro | 170 |
| Ile-Pro-Pro | 330 |
| Gln-Ile-Pro-Pro | 760 |
| Arg-Pro-Gln-Ile-Pro-Pro | 1,100 |
| Pro-Gln-Ile-Pro-Pro | 1,500 |
| <Glu-Trp-Pro | 42,000 |
| <Glu-Trp-Pro-Arg | 450,000 |

[a]Data at Fisher et al. (15).

[b]Compounds were tested for their abilities to inhibit the hydrolysis of ^{3}H-hippuryl-His-Leu by human urinary angiotensin-converting enzyme (17).

## Design of Synthetic Substrates

We have synthesized the following compounds: $p$-I-benzoyl-Gly-Gly-Gly (I), $p$-I-benzoyl-Pro-Phe-Arg (II), $p$-I-benzoyl-Gly-His-Leu (III), $p$-I-benzoyl-Phe-Ala-Pro (IV), $p$-I-benzoyl-Phe-His-Leu (V), and $p$-I-benzoyl-Phe-Ser-Pro (VI). Each of the compounds can be labelled by dehalogenation in [3]H₂ gas (16, 17). Compounds I-V have been so labeled and each was obtained at a specific radioactivity of >20 Ci/mmole. Further, each compound was labeled specifically in position 4 of the benzoyl ring.

Compound I, after tritation, is ^{3}H-hippuryl-Gly-Gly. Hip-Gly-Gly is commonly used as a substrate for converting enzyme (31) and appears to be remarkably resistant to hydrolysis by other enzymes. However (Table 4), Hip-Gly-Gly has relatively little affinity for converting enzyme, and its ^{3}H-derivative cannot be used to obtain a sensitive assay.

Compound II, the acylated C-terminal tripeptide of bradykinin, yields a much more sensitive assay, although its $V_{max}/K_m$ suggests that one or both of the products is a poor leaving group. Under some conditions, II is hydrolyzed by a carboxypeptidase B-like enzyme and is not specific. The encouraging results obtained with III, Hip-His-Leu, deserves comment, as the magnitude of its $K_m$ is heavily dependent on selection of buffer and salts. Cushman and Cheung (12, 13) were the first to use Hip-His-Leu as a substrate for converting enzyme, and under their conditions of assay (0.1$M$ phosphate buffer, pH 8.3, plus 0.3$M$ NaCl), Hip-His-Leu has a relatively low affinity for the enzyme ($K_m$>2000 $\mu M$). Quite by accident, we found that its affinity is greatly increased by Na₂SO₄ at 0.75$M$ (17). Phosphate is inhibitory, thus the most sensitive assay uses ^{3}H-Hip-His-Leu in

0.05$M$ Hepes buffer, pH 8.0, plus 0.1$M$ NaCl and 0.75$M$ Na₂SO₄. As yet, we have no explanation for the dramatic effects of SO₄. Nonetheless, our work and that of Dorer et al. (31) indicate that it will be important to check for effects of organ perfusion solutions (e.g., Krebs-Henseleit solution) on the interactions of a given substrate with angiotensin converting enzyme. Plasma and blood are likely to present their own problems.

**Table 4. Characteristics of the reactions of acylated tripeptides with angiotensin-converting enzyme.**

| | Substrate | $V_{max}$, $\mu$mole/min | $K_m$, $\mu$M | $V_{max}/K_m$ |
|---|---|---|---|---|
| I | benzoyl-Gly-Gly-Gly | 75.6 | 3490 | 0.022 |
| II | benzoyl-Pro-Phe-Arg | 4.7 | 120 | 0.039 |
| III | benzoyl-Gly-His-Leu | 18.7 | 103 | 0.182 |
| IV | benzoyl-Phe-Ala-Pro | 3.6 | 5.4 | 0.667 |
| V | benzoyl-Phe-His-Leu | 2.1 | 5.6 | 0.375 |

[a]Each of the ^{3}H-labeled substrates, with varying concentrations of unlabeled carrier, was reacted with guinea pig urinary angiotensin-converting enzyme such that no more than 10% of initial substrate was used (17). Similar results were obtained when rabbit lung converting enzyme was used.

Compound IV, an acylated analog of the C-terminal tripeptide of BPP₅a, can be used in assays 40 times more sensitive than those using Hip-Gly-Gly (I). Neither II nor IV requires Cl⁻, and each is apparently insensitive to added Na₂SO₄. The $K_m$ for IV is intermediate between that for angiotensin I (33 $\mu M$) and that for bradykinin (0.85 $\mu M$) (11, 21, 28).

## Concluding Remarks

Depending on the goals of the experiment, compound I, III, or IV, can be used to measure angiotensin converting enzyme of plasma, tissue homogenates, cells in culture, or isolated, perfused lungs. Compound IV may well be suitable for use with lungs of intact animals. Because of the wide range of affinities of the substrates for converting enzyme and their high specific radioactivities, it should be possible to examine for modulations of converting enzyme activity using a wide range of substrate concentrations. Each of the ^{3}H-labeled substrates, without carrier, is labeled such that 1$nM$ solutions contain easily detectable radioactivity. Thus the range of concentrations readily tested extends from 1 $nM$ to >2$K_m$. So far as is known, none of the substrates or products is biologically active or toxic. The acyl-tripeptides are relatively inexpensive and can be made in quantity.

Again, according to the goals of a given experiment, an acyl-tripeptide substrate can be selected to give <10% to >90% hydrolysis during one transit

through the lungs. Thus, it should be possible to define conditions to assess easily effects of variables such as perfusion rate, pressure, toxic inhalants and oxygenation.

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