Membrane-bound Angiotensin-converting Enzyme from Rat Lung*

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(Received for publication, September 13, 1973)

SUMMARY

Angiotensin-converting enzyme was found in rat lung predominantly in association with membranous subcellular particles. The converting enzyme was solubilized from a particulate fraction of rat lung that sedimented between 775 and 54,000 × g with sodium deoxycholate and was subsequently purified with DEAE-cellulose and Sephadex G-200 chromatography. The fraction with converting enzyme activity obtained from Sephadex G-200 chromatography had an estimated molecular weight of 270,000 as observed by gel filtration. Analytical disc gel electrophoresis of this preparation showed a single band. The specific converting enzyme activity of the purified preparation was 17.6 μmoles per min per mg with hippurylhistidylleucine as substrate and 1.8 μmoles per min per mg with angiotensin 1 as substrate. This represented a greater than 100-fold purification of the activity of subcellular particles. Sodium chloride was required for activation of the enzyme, and it was inhibited by EDTA, bradykinin potentiating factor-nonapeptide, and angiotensin 2, but not by histidylleucine. The purified preparation was free of carboxypeptidase activity on angiotensin 1. The distribution of converting enzyme activity in subcellular particles from rat lung paralleled that of 5'-AMPase activity which has been associated with pinocytotic vesicles of endothelial membranes. Little or no converting enzyme activity was present in particles obtained from lung lavage.

* This investigation was supported by Grants HL06924 and HL14456 from the National Heart and Lung Institute, National Institutes of Health, United States Public Health Service.
† Recipient of Research Career Development Award, United States Public Health Service.
perfusion of the pulmonary artery with 60 ml of ice-cold 0.9% saline. The lungs were removed and homogenized in ice-cold 0.02 M potassium phosphate buffer, pH 8.3 (0.3 ml of buffer per set of lungs), for 1 min at a speed setting of 40 in a Virtis homogenizer. The homogenate was separated by differential centrifugation at 2g for 10 min at 37°C, the pellet after further centrifugation at 12,000g for 20 min; P3, the supernatant after the pellet at 12,000g for 20 min; P4, the supernatant after the pellet at 12,000g for 20 min. The pellets were rehomogenized in 0.02 M potassium phosphate, pH 8.3, and refrigerated at 2°C until used.

**Conversion of converting enzyme from particulate material—**

Cofocused fractions P2 and P3, which showed the highest converting enzyme activity and about 50% of the total lung converting enzyme activity, were used for purification procedures. For the usual preparation, fractions P2 and P3 were collected from a batch of 40 animals and were homogenized with a Virtis homogenizer in 250 ml of 0.02 M phosphate buffer, pH 8.3. Sodium deoxycholate was added to a final concentration of 0.10% (approximately 0.33 mg of sodium deoxycholate per mg of protein) was added to the homogenate. This was shaken well for several minutes and allowed to stand for 1 hour at 2°C. The mixture was centrifuged at 20,000g for 1 hour; and for removal of sodium deoxycholate, the supernatant was dialyzed overnight at 2°C against 6 liters of 0.02 M phosphate buffer, pH 8.3.

The dialyzed material was filtered through Whatman No. 1 paper, and the filtrate was pumped at 80 ml per hour onto a column of DEAE cellulose. This and the following chromatographic procedures were performed at room temperature. The column was washed with approximately 2 liters of 0.02 M phosphate buffer, pH 8.3, and was developed with 500 ml of a 0 to 200 mM linear gradient of KCl in phosphate buffer. Fractions which showed converting enzyme activity were pooled and concentrated to 8 ml with a BioMed UF-1 concentrator. The concentrated column pool was pumped at 8 ml per hour onto a Sephadex G-200 column. Four runs of about 8 ml each were performed, since a single run of 8 ml did not give adequate separation of the first two peaks. The column was developed by reverse flow with 0.02 M phosphate buffer, pH 8.3, at 8 ml per hour.

**Conversion of converting enzyme activity—**

Converting enzyme activity was determined for the most part by the spectrophotometric method of Cushman and Cheung (11) with synthetic Hp-His-Leu as substrate. The hydrolysis of hippuric acid from the substrate was determined for the most part by the spectrophotometric method of Cushman and Cheung (11) with synthetic Hp-His-Leu as substrate. The final concentration of the substrate and inhibitor prior to addition of enzyme.

**5'-AMPase activity—**

The 5'-AMPase activity of the subcellular fractions was determined by the method of Lowry et al. (20).

**Another assay for measuring converting enzyme activity utilized radioactive angiotensin I as substrate. Conditions for this assay were essentially the same as those for the Hp-His-Leu assay with the exceptions that the assay was carried out for 1 hour at 37°C, 0.38 µCi of [14C]angiotensin I was added as substrate, and the reaction was stopped with 1.5 ml of hot absolute ethanol. The mixture was centrifuged, and the supernatant was evaporated to dryness overnight in a desiccator. The residue was resuspended in 0.2 ml of medium used for high voltage electrophoresis (pyridine-acetic acid-water, 5:50:945). This was mixed with carrier angiotensin I, angiotensin 2, His-Leu, and leucine, and an aliquot was spotted for paper electrophoresis on Whatman No. 1 filter paper. Electrophoresis was carried out for 2½ hours at 2500 vols. The paper was dried and angiotensin I, angiotensin 2, His-Leu, and leucine were located with ninhydrin spray. The entire strip of paper was cut into sections, and their radioactivity was determined by liquid scintillation counting. Converting enzyme activity was determined from the area under standard assay conditions. The specific activity is expressed as the number of disintegrations per minute of enzyme that hydrolyzes 1 µmole of Hp-His-Leu per min at 37°C.

![Diagram](http://www.jbc.org/Downloaded from)

**Fig. 1. Structure of angiotensin I and site of hydrolysis by angiotensin converting enzyme.**
5 ml per wash, for each animal. Washes from the animals were pooled. An aliquot of the pool was kept and the remainder was centrifuged at 54,000 x g for 1 hour. The precipitate was resuspended in a small volume of 0.02 M potassium phosphate buffer, pH 8.3. The suspension was homogenized by hand with 30 strokes of a Thomas B hand homogenizer, and all samples were stored at 2° prior to the determination of converting enzyme activity. A Wright-Giemsa stain of a microscopic slide from the suspended particles prior to homogenization showed the presence of mononuclear cells; after hand homogenization, a similarly prepared slide showed only nonspecific particles and nuclei, but no intact cells.

Materials

Hippuryl-L-histidyl-L-leucine was synthesized by Fox Chemical Co. Angiotensin I, angiotensin II, bradykinin potentiating factor-nanopeptide, and [U-3H][Phe8]-[Asp4]-angiotensin I, specific activity 100 mCi per mM were from Schwarz-Mann. [4,5-3H]Leu10-[IleK]angiotensin I, specific activity 250 mCi per mM, was obtained from New England Nuclear Corp. L-Histidyl-L-leucine was from Mann Research Laboratories. L-Leucine and sodium cholate were from Fisher Scientific Co. Whatman DE52 microgranular DEAE-cellulose was obtained from Reeve-Angel. Sephadex G-200 and the molecular weight marker kit were obtained from Pharmacia Fine Chemicals, Inc. Triton X-100 and acrylamide were from Eastman Kodak Co. Sodium dodecyl sulfate was from Sigma Chemical Co.

RESULTS

Subcellular Distribution of Converting Enzyme Activity in Rat Lung—Subcellular fractionation of the lung showed that the major portion of converting enzyme activity was associated with particulate elements of the lung (Table I). Only 23% of the total enzymatic activity of the homogenate remained in the supernatant after centrifugation at 54,500 x g for 1 hour, while about 50% of the total protein of the homogenate was in the supernatant. With some preparations, enzymatic activity in the supernatant was so low that it could not be measured (e.g. Table IV). The highest specific activity in the subcellular fractions (0.2 μmoles per min per mg) was associated with the P3 fraction. Examination of this fraction by electron microscopy (Fig. 2) revealed that it contained vesicular membranous structures of various sizes; no mitochondria, nuclei, or other cellular organelles were identified.

Purification of Converting Enzyme from Combined Fractions P2 and P3—For purification procedures, combined Fractions P2 and P3 were used as the source of enzyme. Passage of the dialyzed sodium deoxycholate extract of these fractions through a column of DEAE-cellulose resulted in the recovery of several protein fractions (Fig. 3). Converting enzyme activity was eluted at 39 to 104 mM KCl with a continuous 0 to 200 mM KCl gradient in buffer. At this stage of purification, converting enzyme was stable for at least 3 months at 2°. The enzyme could withstand repeated freezing and thawing without loss of activity. Lyophilization, however, resulted in considerable loss of enzymatic activity.

| Subcellular fraction | Total protein (mg) | Specific activity (μmoles/min/mg) | Total activity (units) |
|----------------------|-------------------|-------------------------------|-----------------------|
| P1                   | 36.0              | 0.052                         | 1.87                  |
| P2                   | 12.2              | 0.084                         | 1.02                  |
| P3                   | 11.2              | 0.200                         | 2.25                  |
| S3                   | 57.5              | 0.027                         | 1.55                  |
| Whole lung homogenate| 117.5             | 0.054                         | 6.35                  |

With Sephadex G-200 column chromatography a single active peak was eluted shortly after the void volume, at 176 to 200 ml (Fig. 4). At this stage of purification, converting enzyme was stable for at least 3 months at 2°. Disc gel electrophoresis of
fraction with converting enzyme activity obtained from Sephadex G-200 chromatography showed the presence of a single heavy band over a 4-fold concentration range. Incubation of gel slices under standard assay conditions showed that only the slices corresponding to the single heavy band were enzymatically active (Fig. 5).

An assessment of the procedure for purification (Table II) showed that a greater than 100-fold purification of enzymatic activity from combined Fractions P2 and P3 had been achieved, and that the specific activity of converting enzyme with Hp-His-Leu as substrate was 17.6 µmoles per min per mg. The final pooled fraction from the Sephadex G-200 column contained about 0.2% of the original protein from the combined P2 and P3 fractions from lung homogenates (Table II). Only 25.6% of the total converting enzyme activity was recovered with the purification procedure. About one-half of the loss occurred with sodium deoxycholate extraction. It appeared that the loss represented failure of extraction rather than denaturation. Any loss on column chromatography could not be accounted for specifically. Since the enzyme was able to withstand exposure to room temperature readily, it did not seem that this was a factor in this loss. Small losses did occur in the BioMed UF-1 concentration.

Molecular Weight Determination—By calibration of the Sephadex G-200 column with proteins of known molecular weights, it was determined that the molecular weight of converting enzyme was approximately 270,000 (Fig. 6).

Carboxypeptidase Activity of Fractions with Converting Enzyme Activity during Purification—The sodium deoxycholate extract of combined Fractions P2 and P3 and the DEAE-cellulose and Sephadex G-200 chromatography fractions containing converting enzyme activity were checked for carboxypeptidase activity by incubation with angiotensin I labeled either in the terminal leucine position with [3H] or in the N-Phe position with [14C] (see Fig. 1). With [3H]leucine-labeled angiotensin I, radioactivity was found largely in the His-Leu position after incubation with the sodium deoxycholate extract and chromatographic separation (Fig. 7A). Radioactivity also was found in the leucine position with the sodium deoxycholate extract, indicating the presence of carboxypeptidase activity. However, there was little cleavage of the terminal leucine after the DEAE-cellulose step of purification and no cleavage by the fraction obtained from Sephadex G-200. A similar removal of carboxypeptidase activity with purification was observed when [14C]phenylalanine-labeled angiotensin I was used as substrate (Fig. 7B). It was presumed, in

![Fig. 4. Sephadex G-200 chromatography of the pooled fractions from DEAE-cellulose with angiotensin converting activity. Chromatography was carried out on Sephadex G-200 as described under “Methods.” The bracketed tubes were pooled for further evaluation.](image)

![Fig. 5. Polyacrylamide gel electrophoresis of the fraction with converting enzyme activity obtained from Sephadex G-200 chromatography. The fraction was subjected to electrophoresis as described under “Methods.” The gel was sliced along its long axis into two pieces. One-half was stained in Coomassie brilliant blue while the other half was cut into thin slices and incubated with Hp-His-Leu under standard assay conditions. Only the slices corresponding to the single heavy band showed converting enzyme activity.](image)

![Fig. 6. Molecular weight determination of converting enzyme by Sephadex G-200 chromatography. The following marker proteins (Kav) were used to calibrate the Sephadex G-200 column: ribonuclease A (0.685), chymotrypsinogen A (0.590), ovalbumin (0.422), and aldolase (0.228). Converting enzyme had an elution volume of 185 ml corresponding to Kav value of 0.123.](image)

![Fig. 7. Carboxypeptidase activity of fractions with converting enzyme activity during purification.](image)

### Table II

| Fraction                  | Total Protein | Total Activity | Specific Activity | Activity Recovered | Purification |
|---------------------------|---------------|---------------|------------------|--------------------|--------------|
| A. P2 + P3 homogenate     | 940           | 131           | 0.139            | 100                | 1            |
| B. Dialyzed P2 + P3       | 189           | 58            | 0.307            | 44                 | 2.2          |
| C. DEAE-cellulose         | 5.3           | 45            | 5.8              | 33                 | 42           |
| D. Sephadex G-200         | 1.9           | 33.5          | 17.6             | 25.6               | 127          |

Results are based on lungs from 40 animals.
this case, that radioactivity occurring in the phenylalanine position on paper chromatography was that from the cleaved terminal amino acid of angiotensin 2, phenylalanine, after angiotensin 1 was hydrolyzed. This does not exclude the possibility that a larger peptide cleavage product separated in the same position as phenylalanine. Although radioactivity predominated in this position on paper chromatography following incubation with the sodium deoxycholate extract and was present with the DEAE-cellulose-separated product containing converting enzyme activity, incubation with the Sephadex G-200 fraction which clearly had converting enzyme activity failed to produce any radioactivity in the phenylalanine position.

**Angiotensin-converting Enzyme Activity of Sephadex G-200 Fraction with Angiotensin 1 as Substrate—**Angiotensin 1, radioactively labeled in the terminal leucine position, was tested to determine the substrate saturation characteristics of converting enzyme. Maximum specific activity for the enzyme with angiotensin 1 as substrate was about 1.8 μmoles per min per mg (Fig. 8). This was about one-tenth the maximum activity when Hp-His-Leu was used as substrate with the Sephadex G-200 fraction (Table II).

**Activation and Inhibition of Angiotensin-converting Enzyme—**Converting enzyme from rat lung required NaCl for activation and the activity was inhibited in the presence of 10^{-4} M EDTA (Table III). Similar results have been observed for the enzyme from rabbit (11), calf (19), dog (24), hog (25), and guinea pig lung (25); from horse (1), hog (26), and human (16) plasma; and from bovine kidney cortex (7). Converting enzyme also was inhibited by angiotensin 2, and by a bradykinin potentiating peptide from the venom of Bothrops jararaca (Table III). The concentrations required for inhibition of 50% of the enzymatic activity (IC50) were 6 \times 10^{-5} M for angiotensin 2, and 2 \times 10^{-7} M for BPP_{6α}. Converting enzyme was not inhibited by His-Leu with concentrations up to 10^{-7} M.

**Location of Angiotensin-converting Enzyme—**The precise location of converting enzyme in the lung, as well as in other tissues, remains unknown. 5'-AMPase activity has been identified by electron microscopy as a marker associated with endothelial cell membranes and with pinocytes of these membranes (27, 28). For this reason, we thought it would be of interest to study the distribution of 5'-AMPase activity in the subcellular fractions obtained from the lung and to determine how closely this corresponds to the distribution of converting enzyme activity. Table IV demonstrates that there is a very close correspondence of converting enzyme and 5'-AMPase activities. The highest specific activity for both of these enzymes occurred in the particulate fraction which sedimented between 3,100 and 54,500 \times g, and
Subcellular fractionation was done as described under ‘Methods’ with lungs from two rats in each preparation. Measurements of converting enzyme activity (with Hp-His-Leu as substrate) and 5'-AMPase activities are described under ‘Methods.’

Table IV
Comparison of distribution of converting enzyme and 5'-AMPase activities in subcellular fractions of rat lung

| Fraction | Converting enzyme activity | 5'-AMPase |
|----------|----------------------------|-----------|
|          | Preparation 1 | Preparation 2 | Preparation 1 | Preparation 2 |
| P1       | 0.020         | 0.040       | 2.43          | 1.81          |
| P2       | 0.057         | 0.070       | 2.38          | 2.08          |
| P3       | 0.114         | 0.181       | 4.86          | 4.60          |
| SS       | 0             | 0.018       | 0.20          | 0.43          |

Table V
Converting enzyme activity of material from lung lavage

Lung lavage material was obtained and assays were carried out as described under ‘Methods’ with Hp-His-Leu as substrate.

| Material                                      | Converting enzyme activity | 5'-AMPase |
|-----------------------------------------------|----------------------------|-----------|
| Uncentrifuged lavage material                 |                            |           |
| 54,500 × g × 1 hour homogenized lavage sediment | 0.003                      |           |
| Control sodium deoxycholate extract from combined P2 + P3 fractions | 0.177                      |           |

the specific activities were considerably less for both in the supernatant.

Assessment of Material from Lung Lavage for Converting Enzyme Activity—Material obtained from lung lavage was tested for converting enzyme activity to determine if particles containing this enzyme could be removed from the lung in this manner. Mononuclear cells were obtained by this procedure and particles obtained from disruption of these cells and other components of the lavaged material failed to demonstrate the presence of converting enzyme activity (Table V).

Discussion

The general concept that many compounds including polypeptides, prostaglandins, amines, and nucleotides are altered during passage through the pulmonary circulation is now well established. The mechanisms for their alterations are variable. In the case of angiotensin I, a significant alteration occurs through hydrolysis of the terminal dipeptide with the formation of vasoactive angiotensin 2.

The location of the converting enzyme within the lung is not known. Our studies, as well as those of others (17, 29), indicate that the enzyme is primarily bound to subcellular membranous structures. Two lines of indirect evidence suggest that the enzyme is associated with membranes from endothelial cells. Firstly, when angiotensin I is perfused in the pulmonary circulation, the volume of distribution of it and its product, angiotensin 2, is that of the intravascular or extracellular compartment (29, 30). Secondly, 5'-AMPase activity has been found to be associated with pinocytotic vesicles of endothelial cells as determined by histochemical techniques (27–29), and our studies show that the subcellular distribution of 5'-AMPase activity following fractionation of lung tissue parallels that of angiotensin-converting enzyme activity. Also, the enzyme was not recovered in lung washes, suggesting that it is not associated with cells free in the alveolar or airway spaces, such as macrophages.

We utilized several methods in attempts to solubilize the enzyme from the combined P2 and P3 fractions. In addition to sodium deoxycholate extraction, these included the use of the bile salt sodium cholate, the ionic detergent sodium dodecyl sulfate, and the nonionic detergent Triton X-100. Although sodium cholate was as effective a solubilizer as sodium deoxycholate, its use resulted in some loss of enzymatic activity. Sodium dodecyl sulfate at 0.1% resulted in complete loss of enzymatic activity. Triton X-100 at 0.2% was an effective solubilizer and did not inactivate the enzyme; however, adequate removal of Triton X-100 could not be accomplished readily. Converting enzyme was found to leach into 0.02 m phosphate buffer at pH 8.3 when the combined P2 and P3 fractions were left standing at 2° for several days. Storing of the homogenized pellets at 2° for 30 days resulted in the leaching of almost all converting enzyme and protein into the phosphate buffer as could be obtained with sodium deoxycholate treatment of fresh preparations.

The converting enzyme obtained by DEAE-cellulose and Sephadex G-200 chromatography appears to be highly purified as determined by gel electrophoresis. Carboxypeptidase activity, which was associated with the crude lung subcellular particles, was removed during the purification process.

Our final preparation had a specific activity of 17.6 μmoles per min per mg. This approximates the value of 22 μmoles per min per mg reported for a preparation from rabbit lung by Cushman and Chung (9) and is somewhat higher than the value of 10 μmoles per min per mg reported by Dorer et al. (14) for converting enzyme from hog lung. Converting enzyme from rat lung has an activity with Hp-His-Leu as substrate about 10 times higher than that with angiotensin 1 as substrate. This is qualitatively in agreement with the Hp-His-Leu to angiotensin 1 ratio of 18 reported for the rabbit lung enzyme (9) and 2.7 reported for the calf lung enzyme (12), but differs from the value of 0.11 reported for the converting enzyme from dog lung (31).

The molecular weight of the converting enzyme from rat lung was estimated to be 270,000. This compares with the following values obtained from other species: hog lung, 300,000 (11); calf lung, 300,000 to 330,000 (12); human lung, 480,000 (15); human plasma, 150,000 (16); hog and guinea pig plasma and lung, 150,000 (25); and porcine plasma, 155,000 (26). The extent of molecular similarity of the enzyme from these various sources remains to be determined.

Our findings of activation of the enzyme by chloride and inhibition by EDTA are similar to those observed for the enzyme from rabbit (11), calf (12), dog (24), hog (25), and guinea pig (25) lung; from horse (1), hog (26), and human (16) plasma; and from bovine kidney cortex (7). The inhibition data for angiotensin 2 and BPPNa cannot be directly compared with values obtained for other converting enzymes, except as discussed below, because of differences in assay conditions, substrates, and substrate concentrations. Nevertheless, there seems to be a close correlation between the various reported values. The concentration required for inhibition of 50% of rat lung converting enzyme (I50) by angiotensin 2 was 2 × 10^-3 M. This is qualitatively similar to the I50 value of 10^-7 M obtained with [Asp',Val']angiotensin 2 on the enzyme from porcine plasma with angiotensin 1 as substrate reported by Lee et al. (29). These workers also observed that His-Leu at 10^-2 M did not inhibit the porcine plasma enzyme. The I50 for BPPNa with rat lung converting enzyme was 2 ×
10^{-7} \text{ M}. \text{ This value can be directly compared with an } I_{50} \text{ value of } 5.6 \times 10^{-7} \text{ M observed for the enzyme from rabbit lung, since the assay systems were identical (32). An } I_{50} \text{ of } 1.43 \times 10^{-7} \text{ also has been reported for the enzyme from an extract of dog lung using angiotensin 1 as substrate (33). BPPs, inhibits the conversion of angiotensin 1 to angiotensin 2 in the isolated lungs of guinea pig, dog, and rat (34).}

Acknowledgments—We thank Ms. Leslie Wells for her skillful technical assistance with these experiments. We thank Dr. Karen O’Hare for the electron microscopic evaluation of our subcellular preparation.

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*J. Biol. Chem.* 1974, 249:2312-2318.

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