Pulmonary Angiotensin-converting Enzyme

STRUCTURAL AND CATALYTIC PROPERTIES*

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Angiotensin-converting enzyme has been solubilized from a particulate fraction of rabbit lung and purified to apparent homogeneity in 11% yield by a procedure including fractionation with DEAE-cellulose and calcium phosphate gel, elution from Sephadex G-200, and lectin affinity chromatography. The molecular weight estimated by equilibrium sedimentation was approximately 129,000, either in the absence or presence of 6 M guanidine hydrochloride. A slightly higher value of 140,000 determined for the reduced, denatured protein by gel electrophoresis in the presence of sodium dodecyl sulfate and a much higher figure derived from gel filtration are probably due to the glycoprotein nature of the enzyme. Its oligosaccharide content accounted for 26% of the weight calculated from its amino acid and carbohydrate composition. The estimated content of sugar residues per mole was: galactose, 57; N-acetylglucosamine, 53; mannose, 43; N-acetyleneuraminic acid, 19; and fucose, 4. Threonine and alanine were identified, respectively, as NH₂-terminal and COOH-terminal residues by the dansylation procedure and by digestion with carboxypeptidase A. The enzyme was found to contain approximately 1 g atom of zinc per mol.

Kₘ values for hydrolysis of hippurylhistidylleucine and angiotensin I were 2.3 and 0.07 mM, and the corresponding turnover numbers were 15,490 and 792 mol/min/mol at 37°. Bradykinin was also a substrate, and release of its COOH-terminal dipeptide, Phe-Arg, was catalyzed at a comparable rate to that of His-Leu from the COOH terminus of angiotensin I. Enzyme activity required the presence of chloride ions and was inhibited by EDTA and by low concentrations of Bothrops bradykinin-potentiating peptides. In addition, hydrolysis of hippurylhistidylleucine was inhibited competitively by other defined peptides, including di- and tripeptides, which were not substrates.

Angiotensin-converting enzyme (kininase II, EC 3.4.15.1) is a mammalian COOH-terminal dipeptidyl hydrolase (for a recent review see Ref. 1). It was first detected in equine plasma (2) as a chloride ion-dependent, EDTA-sensitive activity catalyzing release of His-Leu from the COOH terminus of angiotensin I and thereby generating angiotensin II, a potent vasopressor octapeptide. Although converting activity is known to be present in many organs (3, 4), the level of angiotensin II in the systemic circulation is thought to be regulated by hydrolysis of angiotensin I in the lungs (5). The rapidity of this hydrolysis in vivo (5, 6) and the morphologic appearance of fractionated membranes with converting activity (7, 8) have suggested that the responsible enzyme may be a constituent of the capillary endothelial membrane in direct apposition to the pulmonary circulation.

We recently developed a method for obtaining small amounts of pure angiotensin converting enzyme after solubilization from rabbit pulmonary membranes (9). It was established that this enzyme was a glycoprotein and that it could also catalyze release of the COOH-terminal dipeptide, Phe-Arg, and adjacent (Ser-Pro) dipeptides from bradykinin, a hypotensive nonapeptide. Since bradykinin-potentiating peptides from Bothrops venom (10) were known to inhibit angiotensin conversion in vitro (11) and in vivo (12), these results indicated that the same membranous pulmonary enzyme might participate in the maintenance of systemic blood pressure by two mechanisms, i.e. by catalyzing both the formation of a pressor peptide, angiotensin II, and the inactivation of a depressor peptide, bradykinin.

Our procedure has now been adapted to the isolation of substantial amounts of apparently homogeneous enzyme. In this communication we report data suggesting that the glycoprotein possesses a molecular weight of approximately 129,000, that it contains a single polypeptide chain with threonine and alanine as the NH₂-terminal and COOH-terminal residues, and that it is associated with one molar equivalent of bound zinc. Some catalytic properties are also described.

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EXPERIMENTAL PROCEDURE

Materials

Frozen type III rabbit lungs were from Pel-Freeze. Commercial products used in the enzyme purification included Nonidet-P-40 (Shell Chemicals), DEAE-cellulose (Bio-Rad) which was cycled in 1 N HCl and 1 N NaOH prior to equilibration in buffer, calcium phosphate gel (Nutritional Biochemicals), and Sephadex G-200 (Pharmacia). Sephaose 2B (Pharmacia), containing approximately 4 mg of covalently bound ricin agglutinin per ml bed volume, was prepared by the method of Adair and Kornfeld (13) and was a gift from Dr. Stanley Nathenson of this institution.

Unlabeled angiotensin I and dihydro-ineptidil was Calbiochem products. Angiotensin I labeled with carbon-14 in the COOH-terminal leucine residue (333 μCi/μmol) was from New England Nuclear. Guanidine hydrochloride, (1-aspartate, 5-isoleucine) angiotensin II and Pyr-Lys-Trp-Ala-Pro ("Bradykinin-potentiating factor") were products of Schwarz/Mann. Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (SQ 20881) was a gift from Dr. Z. P. Horovitz of Squibb. Hippurylhistidylleucine (Hip-His-Leu) was from Research Plus. A standard kit of dansylated amino acids and bradykinin triadate were purchased from Sigma. Other peptides were obtained from Bachem or Cyclo Chemicals. Materials for disc gel electrophoresis were Fisher products and carboxypeptidase A was from Worthington.

Methods

Enzyme Assays and Kinetic Analyses—The standard assay was the spectrophotometric procedure described by Cushman and Cheung (14) using Hip-His-Leu as substrate. A unit of enzyme activity is the amount required to catalyze formation of 1.0 μmol of hippuric acid per min at 37° under their standard conditions.

Conversion of angiotensin I labeled with carbon-14 in the COOH terminal leucine residue was estimated by the release of radioactive peptide. Leucine was released by treatment of the substrate with Bradykinin

Gel Electrophoresis—Gel electrophoresis under denaturing conditions was performed on 5 to 20% acrylamide gradients with electrode buffer containing 0.1% sodium dodecyl sulfate in 0.025 M Tris·0.192 M LiCl, pH 8.5 (15). The proteins were initially reduced and denatured by boiling for 2 min in 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol. Slabs were stained for protein with Coomassie blue (16). For standard electrophoresis of native proteins the conditions were identical except for the absence of sodium dodecyl sulfate.

Sedimentation Equilibrium—Molecular weights were estimated at 20 °C in a Beckman model E ultracentrifuge equipped with interference optics and a temperature control unit. A 3-mm column of solution containing 0.3 mg of protein per ml was used in a 12-mm double sector aluminum coated Epon cell. FC-43 fluorocarbon was placed in the bottom of the cell. For determining the subunit value the dissociating conditions described by Marshall and Cohen (18) were employed.

Amino Acid Analysis—Amino acid analyses were performed according to the method of Spackman et al. (19). Aliquots of enzyme (90 μg) were hydrolyzed with constant boiling 5.7 N HCl in evacuated, sealed tubes for 24 and 72 hours at 110°. Methionine and half-cystine were estimated as methionine sulfoxide and cysteic acid, respectively, on a sample of the enzyme that had been oxidized with performic acid (20). Tryptophan was determined by a minor modification of the method of Liu and Chang (21). The enzyme was hydrolyzed with 4% methanesulfonylfluoride and the hydrolysate was applied to the short column of the amino acid analyzer. The usual procedure for short column runs was modified to achieve a good separation of tryptophan and glucosamine. Elution was carried out with 0.21 M sodium citrate, pH 5.4, at 40° and no buffer change was made. 1

Carbohydrate Composition—Analysis of the carbohydrate residues was carried out by gas-liquid chromatography as described by Crompton (22).

COOH-terminal Analysis—A sample of enzyme (4.5 mg) in 2.2 ml of 0.2 M N-ethylmorpholine acetate, pH 8.5 (23). Aliquots (0.5 ml) were removed at various time intervals, treated immediately with 0.13 ml of 1 N HCl to stop the reaction, and subjected to amino acid analysis.

NH₂-terminal Analysis—The dansylation procedure was carried out as described by Gray (24). After acid hydrolysis the dansyl derivative of enzyme was diazylated extensively with 20 mm Tris-HCl, pH 7.13. Determinations of zinc, manganese, and cobalt content were made at 215.1, 280.9, and 241.0 nm, respectively, using a 10X expansion scale. The absorbances were compared with a blank containing the dialysis buffer.

Protein Determination—The method of Lowry et al. (25) was employed using crystalline bovine serum albumin as the standard.

RESULTS

Enzyme Purification

A preparation from 7.8 kg of starting material is described below and summarized in Table I. Six batches of lungs, each approximately 1.3 kg, were processed separately through the DEAE-cellulose step and then combined prior to subsequent procedures. All operations were performed at 0-4°. Essentially identical results were obtained with two large scale preparations.

Preparation of Homogenate and 16,000 × g Fraction—Frozen rabbit lungs (1.3 kg) were chopped into small pieces and suspended in 2600 ml of a buffer containing 20 mm Tris-HCl, pH 7.8, 30 mm KCl, 5 mm magnesium acetate, and 0.25 mg sucrose. The suspension was homogenized 3 times for 45 sec in a Waring Blender with 1 min cooling intervals in a water-ice bath and then filtered through two layers of cheesecloth. The homogenate (3500 ml) was centrifuged at 16,000 × g in a GSA rotor for 1 hour and the pellet was dispersed in 10 mm Tris-HCl, pH 7.8. The dispersed pellet is called the 16,000 × g fraction.

Preparation of Nonidet-P-40 Extract—The 16,000 × g fraction (2100 ml) was made 0.5% with respect to Nonidet-P-40, agitated vigorously with a magnetic stirrer for 3 hours, and then centrifuged at 16,000 × g for 1 hour. The supernatant solution is referred to as the Nonidet-P-40 extract.

DEAE-cellulose Fractionation—The Nonidet-P-40 extract (1700 ml, 22.8 g of protein) was mixed with a slurry (4500 ml) of DEAE-cellulose (packed volume 1900 ml) equilibrated in 10 mm potassium phosphate buffer, pH 6.5, containing 0.5% Nonidet-P-40. After stirring for 45 min the suspension was filtered through 2 sheets of Whatman No. 1 filter paper and concentrated 1 liter of the filtrate containing 80 mg of protein on a Buchner funnel. The filter cake was suspended in 4 liters of the same phosphate buffer containing 0.05% Nonidet-P-40, stirred for 30 min then refiltered. Enzyme was then eluted with 0.2 M KCl by dispersing the filter cake in 2.5 liters of phosphate buffer containing 0.025% Nonidet-P-40 and 0.35 M KCl. After stirring for 45 min the mixture was filtered and elution was repeated with 2 liters of buffer containing 0.2 M KCl. The combined filtrates were concentrated approximately 8-fold by ultrafiltration in a Diaflo apparatus with a PM 10 filter and then dialyzed extensively against 1 mm potassium phosphate, pH 7.5, containing 0.025% Nonidet-P-40.

Calcium Phosphate Gel Fractionation—The DEAE-cellulose eluate was fractionated with calcium phosphate gel in two steps. The procedure depends on the fact that activity is not adsorbed to the gel in the first step, whereas a large fraction of


1 The abbreviation used is: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
TABLE I

Purification of angiotensin-converting enzyme from 7.8 kg of rabbit lungs

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
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<tbody>
<tr>
<td>Homogenate</td>
<td>210</td>
<td>721</td>
<td>0.99</td>
</tr>
<tr>
<td>16,000 g fraction</td>
<td>126</td>
<td>331</td>
<td>0.18</td>
</tr>
<tr>
<td>Nonidet-P-40 extract</td>
<td>102</td>
<td>137</td>
<td>0.42</td>
</tr>
<tr>
<td>DEAE-cellulose eluate (conc)</td>
<td>33.6</td>
<td>48.7</td>
<td>0.84</td>
</tr>
<tr>
<td>First calcium phosphate gel supernatant (conc)</td>
<td>3.00</td>
<td>4.30</td>
<td>7.7</td>
</tr>
<tr>
<td>Second calcium phosphate gel eluate (conc)</td>
<td>0.455</td>
<td>1.04</td>
<td>21</td>
</tr>
<tr>
<td>Sepharose G-200, peak II (conc)</td>
<td>0.400</td>
<td>0.118</td>
<td>67</td>
</tr>
<tr>
<td>Ricin-agglutinin-Sepharose eluate (conc)</td>
<td>0.240</td>
<td>0.080</td>
<td>89</td>
</tr>
</tbody>
</table>

FIG. 1. Sephadex G-200 gel filtration of the second calcium phosphate gel eluate. Details of the procedure are described in the text.

contaminating proteins are. After removal of these extraneous proteins in the first step, the enzyme is adsorbed to the gel in the second step. In each step different batches of gels behaved somewhat differently, and careful pilot experiments were critical in optimizing yield and purification.

Six batches of enzyme from the DEAE-cellulose step were combined (3360 ml, 48.7 g) and treated with a slurry (4640 ml) containing 375 g (dry weight as solids) of calcium phosphate gel in 1 mM potassium phosphate, pH 7.5. The mixture was stirred for 1 hour and then centrifuged for 30 min at 10,000 x g. The supernatant solution containing the enzyme was concentrated by ultrafiltration. This first calcium phosphate gel supernatant fraction (300 ml, 4.30 g) was then treated with a second slurry (900 ml) containing 75 g of gel in water. The suspension was stirred for 1 hour, then centrifuged at 16,000 x g for 30 min and the pellet was extracted similarly with 1200 ml of water. Activity was eluted from the washed pellet by extracting twice with 1000 ml of 10 mM potassium phosphate, pH 7.5. The active extracts were combined and concentrated to 200 ml by ultrafiltration through a PM 10 membrane. They were then diluted to 2000 ml with 10 mM Tris-HCl, pH 7.8, and concentrated 10-fold. This procedure was used to eliminate residual detergent and was performed a total of 6 times, until the filtrate no longer contained material absorbing at 230 nm when read against the Tris buffer. The eluate was finally concentrated to 46 ml and centrifuged at 16,000 x g for 1 hour to remove a small, loosely packing precipitate.

Gel Filtration on Sephadex G-200—The concentrated second calcium phosphate gel eluate was applied to a column (105 x 9 cm) of Sephadex G-200 which was equilibrated and developed with 10 mM Tris-HCl, pH 7.8. Fractions (20 ml) were collected at a flow rate of 40 ml per hour. The recovery of activity was quantitative; however, it was approximately equally divided into two well resolved protein components (Fig. 1). The first of these (Peak I) was present in the void volume of the column and was not significantly purified with respect to the input material. The second component, Peak II (2820 to 3220 ml, Vc/Vo ~ 1.3) was concentrated by ultrafiltration and dialyzed against 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl. The elution volume of this fraction corresponded to that of a protein of molecular weight approximately 300,000, as estimated by the method of Andrews (26).

Affinity Chromatography on Immobilized Ricin Agglutinin—The concentrated Peak II Sephadex filtrate was subjected to chromatography on a column (1 x 13 cm) of Sepharose 2B containing covalently bound ricin agglutinin and equilibrated in 10 mM Tris-HCl, pH 7.8/0.15 M NaCl. This lectin specifically recognizes galactose residues (27). Because the capacity of the column was only 800 enzyme units, chromatography was carried out 10 times with 4 ml of enzyme solution. After application the sample was allowed to equilibrate for 1 hour in the column which was then washed with 60 ml of starting buffer. Elution was performed with 0.1 M lactose in the Tris-NaCl solution. About 6 ml of eluting buffer were washed into the column and allowed to equilibrate for 2 hours to ensure complete dissociation of the enzyme lectin complex. The enzyme was then eluted with an additional 30 ml of the lactose-containing buffer. The combined lactose eluates were concentrated by ultrafiltration and dialyzed extensively against 10 mM Tris-acetate, pH 7.4. The concentrated ricin-agglutinin-Sepharose eluate appeared faintly yellow and possessed a specific activity similar to that of homogeneous enzyme obtained by our previously published procedure (9). It retained its full activity after storage at -20° for 6 months.

Physical and Chemical Characteristics of Purified Enzyme

Gel Electrophoresis—The purified enzyme exhibited a single protein band after gel electrophoresis under standard conditions (Fig. 2). Activities for the hydrolysis of Hip-His-Leu, angiotensin I, and bradykinin are known to reside in this protein, since they have previously been shown to be co-eluted from the gel slice in which it was present (9). The enzyme preparation also showed a single major protein component after denaturation and electrophoresis in the presence of sodium dodecyl sulfate. In confirmation of our previous results (9), the molecular weight of the reduced, denatured enzyme was estimated to be approximately 140,000 by this technique.

The behavior of the enzyme in the excluded Sephadex G-200 fraction (Peak I) was also examined (Fig. 2). It failed to enter the separating gel under standard conditions. When it was compared with an equal number of units of purified enzyme under reducing, denaturing conditions, its principal polypeptide possessed the same mobility, shape, and staining intensity as the enzyme protein. Only minor amounts of other Coomassie blue-reactive components were visible despite the fact that the specific activity of this fraction was only 20% that of the purified enzyme. A possible explanation for these results may be that this fraction contains the same enzyme glycopeptide complexed to other glycoproteins which fail to stain with...
constant of 7.9 S during glycerol gradient centrifugation (9). The native enzyme derived from a sedimentation rate composition. They agree well with our original estimate of 0.705 ml/g determined (29) from the amino acid and carbohydrate residues derived from these data were 129,000 and 128,500 respectively. The high latter value presumably reflects the large content of tryptophan residues in this protein (Table II). Optical Characteristics—The enzyme exhibited a typical protein spectrum in the ultraviolet range (not illustrated) with an absorption maximum at 280 and minimum at 250 nm. A solution containing 1 mg of enzyme protein per ml possessed optical densities of 0.78, 1.10, and 1.95 at 230, 260, and 280 nm, respectively. The high latter value presumably reflects the large content of tryptophan residues in this protein (Table II).

Molecular Weight—The plots of log fringe displacement against $r^2$ from the sedimentation equilibrium analyses under native or dissociating conditions (Fig. 3) showed good linearity, indicating a high degree of mass homogeneity. The molecular weights derived from these data were 129,000 and 128,500 under the native and denaturing conditions, respectively. These values were obtained using a partial specific volume of 0.705 ml/g determined (29) from the amino acid and carbohydrate composition. They agree well with our original estimate of 136,000 for the native enzyme derived from a sedimentation constant of 7.9 S during glycerol gradient centrifugation (9). Aromatic residues (phenylalanine, tyrosine, tryptophan) accounted for 11.7% of the total protein weight.

Amino Acid and Carbohydrate Composition (Table II) — Aromatic residues (phenylalanine, tyrosine, tryptophan) accounted for 11.7% of the total protein weight.
counted for 11.9% of the total amino acids, a value 45% higher than the average derived from a large number of sequenced proteins (30). The fraction of hydrophobic residues calculated according to Heller (31) was 44%, while the ratio of polar to apolar residues (32) was 1.2. These results indicate that this membrane-bound enzyme possesses a moderately high degree of hydrophobicity.

Sugar residues were found to constitute approximately 26% of the total weight of amino acid and carbohydrate. This oligosaccharide content is considerably higher than that which we reported originally (9). Part of the discrepancy is probably due to the fact that our early value was based on the dry weight of a very small sample of the glycoprotein. However, the content of N-acetylneuraminic acid relative to the other sugars is clearly higher in the present preparation than in that obtained by our previous procedure. It is not unlikely that further study will disclose some heterogeneity in the oligosaccharide moiety of the enzyme.

COOH-terminal Analysis—The kinetics of amino acid appearance during digestion with carboxypeptidase A (Fig. 4) indicated the presence of a COOH-terminal alanine residue. Serine and leucine were found in the next two positions; however, the results did not permit an unequivocal assignment of their sequence. No additional amino acids were found when carboxypeptidase B was included in the reaction mixture. For the data shown in Fig. 4, the amount of alanine after digestion for 1 hour corresponded to only 0.63 residue per mol of enzyme, as estimated using the assumptions described in Table II. However, when the digestion was performed for the same time period at 37°C in buffer containing sodium dodecyl sulfate (33), the release of alanine, serine, and leucine corresponded, respectively, to 1.09, 1.02, and 1.16 residues per mol. Under these conditions several other amino acids were found in smaller quantities.

NH2-terminal Analysis—Dansyl-threonine, N'-dansyl-lysine and o-dansyl-tyrosine were the only derivatized amino acids detected on polyamide thin layer chromatograms after hydrolysis of the dansylated protein.

Heavy Metal Analyses—The zinc content of the enzyme, as determined by atomic absorption spectroscopy, was 10.0 nmol per mg of protein, corresponding to 1.19 g atoms per mol of enzyme. No manganese or cobalt was detected.

Catalytic Properties of Purified Enzyme

$K_m$ values for Hip-His-Leu and angiotensin I were 2.3 and 0.07 mM. The corresponding $V_{max}$ values were 130 and 6.67 μmol/min/mg of protein. These results correspond to $k_{cat}$ values of 15,430 and 792 mol/min/mol of enzyme using the assumptions described in Table II. The paper electrophoresis technique for detecting ninhydrin-reactive products of bradykinin degradation (9) is not sufficiently quantitative to permit precise measurement of those parameters. However, a peptide analyzer was employed to establish that the enzyme liberated 4.6 μmol of the COOH-terminal dipeptide Phe-Arg/min/mg of protein from 1.6 mM bradykinin.3 Bradykinin is thus hydrolyzed almost as rapidly as angiotensin and its $k_{cat}$ value can be calculated as 546 mol/min/mol of enzyme assuming the determination to represent a maximal rate. Both bradykinin and angiotensin I were found to inhibit enzymatic release of hippuric acid from Hip-His-Leu. Their respective concentrations required to yield a 50% decrease in the rate of hydrolysis of 5 mM Hip-His-Leu were 9 and 50 μM. Dorer et al. (35) have directly determined $K_m$ and $V_{max}$ values of 0.85 μM and 1.4 μmol/min/mg of protein for bradykinin using a highly purified enzyme preparation from porcine lungs. The corresponding figures reported by them for angiotensin I were 30 μM and 2.1 μmol/min/mg.

Activity with Hip-His-Leu as substrate was more than 90% dependent upon the presence of NaCl and was markedly inhibited by 0.1 mM EDTA and by low concentrations of bradykinin-potentiating peptides (Table III). The NaCl requirement could be met with MgCl2 but not with Na2SO4. p-Hydroxymercuribenzoate (0.1 mM) did not alter the reaction velocity. Similar data have been described for angiotensin-converting enzyme from many sources (1); however, in contrast to the results of Cheung and Cushman (36), we found the nonapeptide to be slightly more inhibitory than the pentapeptide. Since the enzyme contains 13 half-cystine residues, its diminished activity in the presence of sulfhydryl compounds may be due to reduction of an important intrachain disulfide bridge rather than to heavy metal chelation, as has previously been suggested (14).

Phe-Arg, Ser-Pro, and angiotensin II, which are produced during the hydrolysis of bradykinin and angiotensin I, were found to inhibit the enzyme at fairly low concentrations...
whereas His-Leu, the COOH-terminal dipeptide of angiotensin I, was less effective. A more systematic investigation of the action of small peptides was carried out with the compounds shown in Table IV. Kinetic analyses indicated that all of these peptides were competitive inhibitors of enzyme activity. In the series Ala-Ala, Ala-Ala-Ala, Ala-Ala-Ala-Ala there was no alteration in the \( K_i \) value with increasing chain length. However, only the tetrapeptide was found to be substantially hydrolyzed when 750 nmoles of peptide were incubated with 20 milliunits of enzyme for 30 min and examined by paper electrophoresis. Acetylation of the \( \alpha \)-NH\(_2\) group of the peptide decreased its inhibitory activity while simultaneously rendering it more susceptible to the action of the enzyme. For example, N-acetyl-Ala-Ala-Ala was extensively hydrolyzed, yet possessed a considerably higher \( K_i \) value than the unsubstituted tripeptide. Amongst the dipeptides the lower \( K_i \) values for Lys-Ala, Arg-Ala, and Tyr-Ala compared to those for Asp-Ala, Glu-Ala, and Leu-Ala suggest that the affinity of the enzyme for basic and aromatic peptides may be higher than that for peptides with acidic and branched side chains.

**DISCUSSION**

The present procedure for isolating angiotensin-converting enzyme after solubilization from lung particles has provided us with substantial amounts of material which is sufficiently pure for structural studies. The molecular weight of 129,000 estimated for the native enzyme by equilibrium sedimentation agrees well with the value obtained by gelatin gradient centrifugation (9) and provides additional evidence that the higher figure derived by gel filtration is due to the large oligosaccharide content of this protein (9). Our results are therefore consistent with those of others who have determined molecular weights for the lung enzyme of 270,000 to 300,000 based on elution volumes from Sephadex G-200 (8, 39, 40).

The molecular weight of the enzyme under denaturing conditions determined by equilibrium sedimentation or disc gel electrophoresis was found to be comparable to that of the native protein. The slightly higher figure with gel electrophoresis is probably due to the oligosaccharide moiety, since this technique (39), like gel filtration (26), is known to yield anomalously high values for glycoproteins. Only threonine and alanine were detected as NH\(_2\)-terminal and COOH-terminal by-products. The molecular weight of 129,000 for Lys-Ala, Arg-Ala, and Tyr-Ala compared to those for Asp-Ala, Glu-Ala, and Leu-Ala suggest that the affinity of the enzyme for basic and aromatic peptides may be higher than that for peptides with acidic and branched side chains.

**Table IV**

<table>
<thead>
<tr>
<th>Peptide inhibitors of angiotensin-converting enzyme</th>
<th>( K_i^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Ala</td>
<td>0.08</td>
</tr>
<tr>
<td>Ala-Ala-Ala</td>
<td>0.08</td>
</tr>
<tr>
<td>Ala-Ala-Ala-Ala</td>
<td>0.08</td>
</tr>
<tr>
<td>N-acetyl-Ala-Ala</td>
<td>4.0</td>
</tr>
<tr>
<td>N-acetyl-Ala-Ala-Ala</td>
<td>0.30</td>
</tr>
<tr>
<td>N-acetyl-Ala-Ala-Ala-Ala</td>
<td>0.13</td>
</tr>
<tr>
<td>Lys-Ala</td>
<td>0.05</td>
</tr>
<tr>
<td>Arg-Ala</td>
<td>0.06</td>
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<tr>
<td>Asp-Ala</td>
<td>0.50</td>
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<tr>
<td>Glu-Ala</td>
<td>1.30</td>
</tr>
<tr>
<td>Leu-Ala</td>
<td>0.40</td>
</tr>
<tr>
<td>Tyr-Ala</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Values were determined by the method of Dixon (37) using Hip-His-Leu as substrate.*

of molecular weight determinations in suggesting that the enzyme contains a single, large polypeptide chain. It thus appears to differ from a soluble porcine pulmonary angiotensin-converting enzyme which was reported to be composed of three polypeptide subunits, each of molecular weight 70,000 (41), but to resemble somewhat a membranous converting enzyme from porcine kidneys thought to possess a molecular weight of 185,000 and to contain a single polypeptide chain with about 8% neutral sugars (42).

Angiotensin-converting enzyme has been suspected to be a metalloenzyme since its discovery (2). Our finding that it contains a stoichiometric amount of tightly bound zinc is consistent with the observation that manganese, cobaltous, and zinc ions, respectively, restored 40, 160, and 100% of activity to a rabbit lung acetone powder extract which had been inactivated by dialysis against EDTA (14). This COOH-terminal dipeptidyl carboxypeptidase thus resembles other exopeptidases, such as carboxyptidase A (43), carboxyptidase B (44), and leucine aminopeptidase (45), in its content of zinc.

When relatively crude preparations of angiotensin-converting activity have been subjected to gel filtration, two or more peaks of activity, one of which is in the void volume, have often been observed (41, 46, 47). In at least one instance, enzyme in the excluded fraction has differed from the retained activity in not catalyzing bradykinin degradation (49). In our preparation the two activity peaks in the gel filtration step do not appear to represent different polypeptide chains. Enzyme in the excluded fraction, like the purified enzyme, was active on bradykinin. It was inhibited by antibody prepared against the purified enzyme and it migrated on gels in the reduced, denatured form identically to the purified enzyme. However, it failed to penetrate standard gels. A possible explanation for this activity is that it is due to the same glycopolypeptide enzyme, which in this fraction is tightly bound to one or more other proteins. Conceivably such a complex might derive from topographical relationships in the original membrane structure and its nonenzymatic components might play an important role in regulating activity in vivo.

Little is known about the substrate specificity of angiotensin-converting enzyme other than the fact that it does not act upon peptides with protected COOH termini and does not catalyze hydrolysis of peptide bonds containing the imino group of a prolyl residue (1). Our studies indicate that a large number of peptides which need not necessarily be substrates can act as competitive inhibitors of the enzyme. The results suggest a low affinity for peptides containing acidic or branched chain amino acids. The lower affinity for angiotensin I as compared with bradykinin (35), which does not contain acidic or branched chain amino acids, may be explicable on this basis. Similarly, we have found that human fibrinopeptide A, which contains a high proportion of acidic amino acids (49), inhibits the enzyme only at very high concentrations (9, 1.1 mM) and is not a substrate.

Bothrops peptide inhibitors of angiotensin-converting enzyme have been shown to transiently alleviate experimental hypertension in rats thought to be due to renin-dependent overproduction of angiotensin I (50). Encouraging clinical results have recently been obtained with the nonapeptide in human disease (51). Since the physiologically important activity is thought to be luminal in the pulmonary vascular bed (5, 6), it should be accessible to the action of circulating macromolecules. In this context it is worth noting that we have obtained antiserum with anticatalytic activity from goats.
immunized with the purified enzyme. It will be interesting to determine whether this antiserum can protect rabbits against the hypertensive effect of intravenously administered angiotensin I.

Acknowledgments—We are grateful to Dr. John Codington, Department of Biological Chemistry, Harvard Medical School, for performing the carbohydrate analysis and to Dr. Lewis Greene, Brookhaven Laboratories, for determining the specific activity of the enzyme with bradykinin as substrate. In addition, we thank Dr. Robin Briehl of this institution, Dr. C. Y. Lai, Roche Institute of Molecular Biology, and Dr. Roberta Colman, Department of Chemistry, University of Delaware, for helping us in their laboratories with equilibrium sedimentation, amino acid analysis, and determination of heavy metal content, respectively.

REFERENCES

Pulmonary angiotensin-converting enzyme. Structural and catalytic properties.
M Das and R L Soffer


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