Molecular and Catalytic Properties of Rabbit Testicular Dipeptidyl Carboxypeptidase*

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Rabbit testicular dipeptidyl carboxypeptidase activity was purified by a procedure exploiting its affinity for N-[α-[1-(S)-carboxy-3-phenylpropyl]-l-lysyl-l-proline. The molecular, catalytic, and immunological properties of the testicular enzyme are presented and compared with the corresponding properties of pulmonary angiotensin-converting enzyme. Although catalytically similar and immunologically related to pulmonary dipeptidyl carboxypeptidase, the testicular enzyme has a molecular weight (100,000) which is lower by a factor of about one-third and differs in its NH₂ and COOH termini. Furthermore, we present evidence that the testicular enzyme is not a post-translational product of the pulmonary type enzyme. These data suggest that testicular and pulmonary dipeptidyl carboxypeptidase are two distinct proteins which are catalytically similar and immunologically closely related.

COOH-terminal dipeptidyl carboxypeptidase activity (angiotensin-converting enzyme, peptidyldipeptide hydrolase, EC 3.4.15.1) is present in the vascular endothelial cells of virtually all mammalian organs (1). The enzyme plays a key role in the control of blood pressure by converting angiotensin I to angiotensin II, a powerful vasoconstrictor, and by inactivating bradykinin, a vasodilator (reviewed in ref. 2). The lung is believed to be the most important physiological site of this conversion (3). Dipeptidyl carboxypeptidase activity in the testis, however, differs from that in the lung, first, because it increases dramatically at puberty (1, 4) and is thus probably hormonally controlled, and second, because antipulmonary enzyme antibodies can discriminate between it and the pulmonary enzyme (5, 6).

As a first step toward establishing the physiological function of the testicular enzyme and the biochemical basis for the structural and regulatory differences between it and the pulmonary enzyme, we have now isolated and characterized the pure testicular enzyme. The purification has been facilitated by a step which takes advantage of its high affinity for N-[α-[1-(S)-carboxy-3-phenylpropyl]-l-lysyl-l-proline, a recently described (7) potent inhibitor of pulmonary angiotensin-converting enzyme.

This paper presents the molecular and catalytic properties of the purified testicular enzyme and their comparison with the properties of the enzyme from lung.

EXPERIMENTAL PROCEDURES

Materials

Pure angiotensin-converting enzyme (90 units/mg) from rabbit lung was prepared as described previously (8). The angiotensin-converting enzyme inhibitor, N-[α-[1-(S)-carboxy-3-phenylpropyl]-l-lysyl-l-proline (7), both unlabeled and containing 14C at positions 2 and 3 and with 14C at positions 2 and 3, was generously provided by Dr. A. Patchett of Merck. Captopril (3-mercaptop-2-methyl-6-propionyl-l-proline) was a gift from Dr. Z. Horovitz of Squibb. 1.4-Butanediol diglycidyl ether was a Sigma product, and Sepharose 4B was purchased from Pharmacia. [3H]Methionine (1185 Ci/mmol) was from Amersham Corp. Other products were those described in previous publications (5, 8).

Methods

Enzyme Assays—Dipeptidyl carboxypeptidase activity was determined using as substrate His-Leu according to the method of Cushman and Cheung (9). A unit of activity is the amount which catalyzes release of 1.0 pmol of hippuric acid per min at 37 °C under the conditions described by them. Activity on angiotensin I containing a [14C]Leu residue at its COOH terminus was quanitated by paper electrophoresis (9).

Preparation of Inhibitor-Sepharose Gel—Sepharose CL-4B was activated and coupled to the amino group of the inhibitor through a bisoxirane spacer arm as described by Sundberg and Porath (11). Washed Sepharose CL-4B (100 ml packed gel, approximately 67 g) was stirred for 8 h at 23 °C in a solution made from 100 ml of 1.4-butanol diglycidyl ether (approximately 70% in 100 ml of 0.6 M NaOH containing 2 mg of sodium borohydride per ml). The activated Sepharose was then washed on a sintered glass funnel with 10 liters of water, and the filter cake, approximately 67 g, was stirred for 3 days at 37 °C with 100 ml of 0.5 M K₂CO₃ (pH 11) containing 2.5 mM inhibitor. Residual epoxide was then blocked by...
treatment with 1 m glycerol, pH 10, for 12 h at 37 °C. The gel was washed thoroughly with 0.5 M NaCl and water and was finally stored at 4 °C in 0.1 M NaHCO3 (pH 8.0) containing 1 M NaCl. The concentration of covalently bound inhibitor estimated using the radioactive compound, was 1.0 μmol in the packed gel.

Preparation of Radioactive Pulmonary Angiotensin-Converting Enzyme—Fresh rabbit lungs (4.4 g) were minced and shaken in 200 ml of 10 mM Tris-HCl, pH 7.5. After passage through two Ringer bicarbonate (14) which contained 2 mg of glucose per ml of 300 μCi of L-[14C]methionine, and 40 μM of the other L-amino acids. Incubation was for 6 h at 37 °C in an atmosphere of 5% CO2 and 95% O2, after which the tissue was homogenized and its angiotensin-converting enzyme was assayed by treatment with Nonidet P-40 (18). The enzyme was subjected to affinity chromatography as described below for testicular dipeptidyl carboxypeptidase. The resulting eluate (70 munits/μg) contained 1.95 × 106 cpm/μg and exhibited a single radioactive and Coomassie blue-reactive band after slab gel electrophoresis in the reduced denatured state.

Amino Acid Analyses—Amino acid analyses were performed by the method of Spackman et al. (15) on samples of the enzyme which had been hydrolyzed with constant boiling 5.7 N HCl in evacuated sealed tubes for 24 and 72 h in 110 °C. Methionine and half-cystine were estimated as methionine sulfoxide and cysteic acid on an aliquot of enzyme that had been oxidized with performic acid (16). Trypophan was measured after hydrolysis in 4 N methanesulfonic acid (17).

Carbohydrate Analysis—The content of N-acetylgalcosamine and N-acetylgalactosamine was determined on the amino acid analyzer after hydrolysis of the enzyme with 4 N HCl at 100 °C for 5 h. The amino sugars were separated by elution at a flow rate of 44 ml/h with sodium citrate buffer, pH 5.2 (0.15 M Na+ and 0.01 M Na2SO4) at 80 °C for 1 h as described by Aminoff (19). Other carbohydrate residues were quantitated by gas-liquid chromatography (20).

Protein Determinations—Protein concentrations were determined by the method of Lowry et al. (21) using bovine serum albumin as the standard.

Isolation of mRNA and Cell-free Protein Synthesis—Male New Zealand White rabbits were killed by air embolism, and total RNA was isolated from the excised testes using the guanidine thiocyanate procedure described by Chirgwin et al. (22). RNA prepared in this manner had an absorbance at 1.8-2.0. Poly(A)-containing RNA was then isolated by chromatography on oligo(dT)-cellulose (Type T-3, Collaborative Research, Waltham, MA) (23) and was estimated by using an A260 nm value of 25.0. This RNA was translated for 90 min at 30 °C in a micrococcal nuclease-treated rabbit reticulocyte lysate system (24). The assay mixture (180 μl, pH 7.4) contained 60 μl of lysate (Bethesda Research Laboratories), 25 mM Hepes, 10 mM creatine phosphate, 48 mM KCl, 87 mM potassium acetate, 1.2 mM MgCl2, 15 μg/ml of creatine kinase, a mixture of unlabeled amino acids (without methionine), 50 μM each, 200 μl of [14C]methionine (1100 Ci/mmol, New England Nuclear), and 30 μg of poly(A)-containing RNA per ml. After cell-free translation, the reaction mixture was adjusted to 2% SDS, boiled for 3 min, and diluted 4-fold with 2.5% Triton X-100, 190 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 6 mM EDTA. Goat anti-pulmonary angiotensin-converting enzyme antiserum (15 μl) was then added, followed by 100 μl of protein A-Sepharose. The total mixture was incubated overnight with end-to-end rotation. The immune complex-protein A-Sepharose was collected by centrifugation and was used for centrifugation and washing with 28 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 0.1% SDS, and 0.5% Nonidet P-40. The antigen was eluted by boiling in 5 mM Tris-HCl, pH 6.8, containing 20% glycerol, 10 mM dithiothreitol, 2% 2-mercaptoethanol, and 5% SDS. The Sepharose was removed by centrifugation, and the supernatant was analyzed by electrophoresis on 7.5% polyacylamide slab gels in the presence of SDS (25) and by fluorography using E:N.HANCE (New England Nuclear).

RESULTS

Enzyme Purification—one of three similar preparations which yielded comparable results is described below and is summarized in Table I. All operations were performed at 0-4 °C.

Frozen rabbit testes (100 g) were cut into small pieces and homogenized for 90 s in a chilled Waring Blender containing 200 ml of 10 mM Tris-HCl, pH 7.5. After passage through two layers of cheesecloth, the homogenate (276 ml) was centrifuged at 16,000 × g for 1 h. The resulting pellet was suspended in 200 ml of Tris buffer, and Nonidet P-40 was added to a final concentration of 0.5% (v/v). The suspension was stirred for 4 h and centrifuged at 16,000 × g for 1 h. To the supernatant solution (Nonidet P-40 extract, 200 ml) was added 1 g of streptomycin sulfate. This treatment was found to be essential for removing nucleic acid which was present in the testicular Nonidet P-40 extract in high quantity and interfered in the subsequent affinity column procedure. After stirring for 20 min, the turbid solution was centrifuged at 16,000 × g, and the streptomycin supernatant (150 ml) was adjusted to 0.1 M Hepes, pH 7.5, 0.3 M KCl, and 0.1 mM ZnCl2. It was then applied at a flow rate of 20 ml per h to an affinity column (1.5 × 60 cm) containing 1 μmol of bound inhibitor per ml of packed volume and equilibrated in the same Hepes buffer. The column was then washed first with 300 ml of buffer containing 0.5% Nonidet P-40 and then with buffer lacking detergent until the absorbance at 280 nm was below 0.01. Enzyme was finally eluted with buffer containing 0.1 mM inhibitor. Fractions were monitored by their absorbance at 280 nm, and those containing protein were pooled, concentrated by ultrafiltration through a PM-10 filter, and dialyzed for 12-h intervals, first against 3 changes of 4 liters of 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, and then against 4 changes of the same buffer lacking EDTA but supplemented with 100 μM ZnCl2. After these dialyses, the affinity eluate was concentrated by ultrafiltration to a final volume of 1.0 ml and was subjected to gel filtration through a column (1.0 × 100 cm) of Sephadex G-200 which was equilibrated and developed with 10 mM Tris-HCl, pH 7.5. Fractions were collected at a flow rate of 20 ml per h, and those having a specific activity greater than 160 were combined, concentrated, and stored frozen at −20 °C.

Two protein components were bound by the affinity column and specifically eluted by the inhibitor (Fig. 1). The minor protein (5%) probably corresponds to the pulmonary type of dipeptidyl carboxypeptidase (angiotensin-converting enzyme), which is found in various endothelial cells throughout the body (26, 27), since it migrated identically with that enzyme during gel electrophoresis in the reduced denatured form with a mobility suggesting a molecular weight of 140,000 and since (not shown) both it and the major protein component were immunospecifically removed from solution by treatment with goat antipulmonary enzyme antibodies and donkey antigoat gamma globulin. When radioactive pulmonary enzyme, which had been purified by the same affinity chromatographic procedure used for testicular dipeptidyl carboxypeptidase, was added to a crude testicular homogenate and the preparation was subjected to the standard isolation procedure, the radioactive protein was not converted to a smaller species (Fig. 1). This suggests that the testicular type of dipeptidyl carboxy-
peptidase does not arise by post-translation proteolytic cleavage of the larger molecule (see below).

During affinity chromatography, approximately 25% of the applied enzyme activity was recovered. The loss of activity was almost certainly not due to residual inhibitor in the eluted enzyme since the specific activity was so high and since pilot experiments indicated that radioactive inhibitor could be completely removed by the dialysis procedure described above. Furthermore, increasing the concentration of inhibitor 5-fold during the elution procedure did not augment the recovery of activity. The affinity column, which had a capacity of 50 μg of purified enzyme/ml of homogenate (50 μg of purified enzyme/ml of homogenate), taken through the streptomycin fractionation step of the isolation procedure, and then incubated at 4 °C for 48 h. Protein molecular weight markers (positions indicated on the left side) included β-galactosidase, phosphorylase b, and bovine serum albumin.

Physicochemical Properties—The testicular dipeptidyl carboxypeptidase exhibited a mobility corresponding to a molecular weight of 100,000 after slab gel electrophoresis in the reduced denatured state (Fig. 1). Under the same conditions, the molecular weight of the pulmonary enzyme was calculated to be 140,000. The molecular weight of native pulmonary and testicular dipeptidyl carboxypeptidases was estimated by centrifugation of a mixture of the two enzymes on a glycerol gradient using the method of Martin and Ames (28) (Fig. 2). Molecular weight values of 145,000 and 94,000 were calculated for the native pulmonary and testicular enzymes, respectively.

Arginine (0.6 mol/mol of enzyme) was identified as the NH₂-terminal residue of testicular dipeptidyl carboxypeptidase by the Edman procedure (29) followed by acid hydrolysis of the anilinothiazolinone (30). Serine (0.6 mol/mol of enzyme) was identified as the COOH-terminal residue by hydrazinolysis (29). Analysis also yielded the expected corresponding threonyl and alanyl residues from pulmonary angiotensin-converting enzyme, confirming results obtained previously by the dansylation technique and by digestion with carboxypeptidase A (8).

The amino acid compositions of the testicular and pulmonary enzymes are presented in Table II. The higher content of tryptophanyl residue (68 μg/mg) probably accounts for the extraordinarily high absorbance value at 280 nm of 2.9 displayed by a solution containing 1.0 mg of enzyme protein per ml.

The carbohydrate component of the testicular enzyme (Table II) accounted for about 20% of the weight of its combined aminoacyl and sugar residues. The composition differed from that of the pulmonary glycoprotein particularly in its relatively large content of galactosamine and in the absence of fucose.

Immunological studies indicate a high degree of similarity between the two protein chains despite the structural differences described above. In the case of studies using antipulmonary enzyme antibodies, immune preparations from both goat and mouse were employed and yielded essentially identical results. The data obtained with mouse preparations (Figs. 3 and 4) were chosen for presentation since that species was the only one immunized with the pure rabbit testicular enzyme. In a competition radioimmunoassay using antipulmonary enzyme antibodies and radioiodinated pulmonary enzyme,
Fig. 3. Competition radioimmunoassays. The assays were carried out as described under “Experimental Procedures.” Results presented in A were obtained using the radioiodinated pulmonary enzyme as displaceable antigen and antipulmonary enzyme antiserum (1:2200). Those in B were not present in the testicular polypeptide. In striking contrast (Fig. 3B), when the radioimmunoassay was performed using antitesticular enzyme antibodies and radioiodinated testicular enzyme, identical displacement curves were generated with the preparations from lung and testis, i.e., the antitesticular enzyme antibodies did not recognize any determinants of the testicular protein which were not also present in the pulmonary species. These data suggest that the enzyme from testis is immunologically closely related to the pulmonary polypeptide.

Catalytic Properties—The basic catalytic properties of the pulmonary and the testicular enzyme are presented in Table III. Although the kinetic parameters of the pulmonary enzyme have been reported previously (8), they were redetermined in this study so that meaningful comparisons of the two enzymes, assayed under identical conditions, could be made. The data obtained for both enzymes plotted according to the method of Lineweaver and Burk are presented in Fig. 5. Both enzymes were found to have the same $K_m$ for Hip-His-Leu and angiotensin I. Although the testicular enzyme displayed much higher $V_{max}$ values for both substrates, the corresponding turnover numbers, based on a molecular weight of 100,000 and a weight of 1.21 mg of enzyme per mg of protein measured by the Lowry procedure for pulmonary and testicular enzyme, respectively, were not present in the testicular polypeptide. In striking contrast (Fig. 3B), when the radioimmunoassay was performed using antitesticular enzyme antibodies and radioiodinated testicular enzyme, identical displacement curves were generated with the preparations from lung and testis, i.e., the antitesticular enzyme antibodies did not recognize any determinants of the testicular protein which were not also present in the pulmonary species. These data suggest that the enzyme from testis is immunologically closely related to the pulmonary polypeptide.

| Table II: Amino acid and carbohydrate composition of rabbit testicular and pulmonary dipeptidyl carboxypeptidase |
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| Amino acid or carbohydrate | Testis | Lung |
| Lysine | 53.2 | 37.7 |
| Histidine | 41.3 | 31.4 |
| Arginine | 60.1 | 61.5 |
| Aspartic acid | 101.0 | 83.1 |
| Threonine | 57.9 | 36.0 |
| Serine | 42.4 | 34.3 |
| Glutamic acid | 128.6 | 106.1 |
| Proline | 29.4 | 52.0 |
| Glycine | 25.5 | 27.2 |
| Alanine | 49.1 | 40.4 |
| Half-cysteine | 10.3 | 8.0 |
| Valine | 49.7 | 38.4 |
| Methionine | 34.9 | 22.0 |
| Leucine | 41.4 | 32.7 |
| Tyrosine | 81.2 | 78.9 |
| Phenylalanine | 42.6 | 55.0 |
| Tryptophan | 49.9 | 50.5 |
| Fucose | <1 | 5.7 |
| Mannose | 47.2 | 65.0 |
| Galactose | 66.5 | 77.0 |
| N-Acetylgalactosamine | 69.7 | 109.0 |
| N-Acetylgalactosaminic acid | 31.5 | 32.7 |

$^*$: All values are the average of two separate runs.
$^\dagger$: Based on a molecular weight of 140,000 and 100,000 for pulmonary and testicular enzyme, respectively.
$^\ddagger$: Corrected for decomposition by extrapolation of 24- and 72-h values to zero time.
$^\S$: Determined as cystine acid.
$^\S\S$: Determined after hydrolysis with 4-N-methanesulfonic acid (16).
$^\S\S\S$: Determined as galactosamine and glucosamine after acid hydrolysis and verified to be derived from N-acetylgalactosamine and N-acetylglucoaminic acid, respectively, by gas-liquid chromatography (29).
Testicular Dipeptidyl Carboxypeptidase activity in the male reproductive system, unlike elsewhere in the body, appears to be hormonally regulated (1, 4). The catalytic properties of a partially purified fraction of this exopeptidase from semen were found to be similar to those of pulmonary angiotensin-converting enzyme (34). This observation provided the rationale for adapting the recently developed competitive inhibitor of converting enzyme, N-[2-carboxy-3-phenylpropyl]-L-lysyl-L-proline (7), to purification of the testicular dipeptidyl carboxypeptidase, and this strategy has enabled us to obtain the enzyme in pure form.

The polypeptide responsible for dipeptidyl carboxypeptidase activity in most organs and body fluids is structurally and catalytically similar to pulmonary angiotensin-converting enzyme (2). However, our results indicate that the responsible testicular polypeptide is much shorter and contains different NH2- and COOH-terminal residues than the pulmonary enzyme. In view of these structural differences and because there is no known function of angiotensin II in the male reproductive system, it seems appropriate to refer to this enzyme as testicular dipeptidyl carboxypeptidase rather than angiotensin-converting enzyme.

Despite these structural differences, our data obtained with antibodies against the two pure glycoproteins indicate that they are closely related. Thus their active sites are probably very similar, since antipulmonary enzyme antibodies inhibit the activity of both enzymes identically and since they exhibit indistinguishable requirements, substrate specificities, kinetic properties, and inhibitor profiles. That antipulmonary enzyme antibodies can recognize determinants of the pulmonary protein which are not present in the testicular enzyme is to be expected since the pulmonary polypeptide is so much larger. However, antitesticular enzyme antibodies failed to identify determinants on the testicular enzyme which are absent from the pulmonary glycoprotein. The data from the in vitro translation experiment suggests that the testicular enzyme is not generated post-translationally by proteolysis of pulmonary type angiotensin-converting enzyme. Furthermore, mRNA isolated from lung and translated in the same reticulocyte lysate system used for the in vitro synthesis of the testicular enzyme, produced an immunoprecipitable translation product with an Mr of 129,000 (35). Thus it is likely that the difference in size of the two enzymes may be attributed to the existence of two related genes, a difference in the transcription of the same gene or a difference in the processing of the RNA transcript. It will be especially interesting to determine whether the biosynthetic mechanism responsible for the difference in structure of the testicular dipeptidyl carboxypeptidase also mediates its atypical regulatory properties.
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