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CANINE PULMONARY ANGIOTENSIN-CONVERTING ENZYME

PHYSICOCHEMICAL, CATALYTIC AND IMMUNOLOGICAL PROPERTIES

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Summary

Antiontensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) has been solubilized from canine pulmonary particles and purified to apparent homogeneity. A value of approx. 140000 was estimated for the molecular weight of the native and the reduced, denatured forms of the enzyme. No free NH_2-terminal residue was detected by the dansylation procedure. Carbohydrate accounted for 17% of the weight of the enzyme, and the major residues were galactose, mannose and *N*-acetylglucosamine with smaller amounts of sialic acid and fucose. Removal of sialic acid residues with neuraminidase did not alter enzymatic activity. The enzyme contained one molar equivalent of zinc. Addition of this metal reversed stimulation and inhibition of activity observed in the presence of Co^{2+} and Mn^{2+}, respectively.

Immunologic homology of pure dog and rabbit enzymes was demonstrable with goat antisera. Fab fragments and intact IgG antibodies displayed similar inhibition dose vs. response curves with homologous enzyme, whereas the fragments were poor inhibitors of heterologous activity compared to the holoantibodies. The canine glycoprotein was much less active than the rabbit preparation in catalyzing hydrolysis of Hip-His-Leu. In contrast, the two enzymes exhibited comparable kinetic parameters with angiotensin I as substrate.

Introduction

Angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) catalyzes cleavage of His-Leu from the COOH-terminus of angiotensin I to yield angiotensin II [1], the pressor agent of the renin-angiotensin system [2]. The enzyme is an important therapeutic target in experimental [3] and human [4]

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renovascular hypertension. Since it is localized on the luminal surface of vascular endothelial cells in direct apposition to the circulation [5,6], we have recently begun to explore the possibility that anticatalytic antibody may be useful for inhibiting its activity in vivo. Antibody prepared against the pure rabbit enzyme was lethal to rabbits [7], but was tolerated by rats and prevented their vasopressor response to angiotensin I [8]. The dog is particularly well suited for studying the evolution of renin-dependent hypertension [9,10]. Therefore, in order to extend our studies on a possible immunologic approach to the control of blood pressure, we have purified and characterized canine pulmonary converting enzyme and antibodies raised against it.

Materials and Methods

Materials

Dog lungs were from Pel-Freez Biologicals, Rogers, Ark. Ricin-agglutinin-agarose containing 1.1 mg ricin agglutinin per ml bed volume was purchased from Miles Laboratories, Inc., Elkhart, Ind. Pure rabbit pulmonary angiotensin-converting enzyme (90 units/mg) and goat antibodies directed against it were prepared as described elsewhere [8,11]. Other materials and reagents were obtained from sources listed in previous publications from this laboratory [8,11–14].

Methods

Enzyme assays. The standard assay was that described by Cushman and Cheung [15] using Hip-His-Leu as substrate. A unit of activity corresponds to the amount required to catalyze formation of 1.0 μmol of hippuric acid per min at 37°C. Conversion of angiotensin I labelled with ^{14}C in the COOH-terminal leucyl residue was estimated by release of radioactive His-Leu [13,16]. The enzymatic hydrolysis of bradykinin was examined qualitatively by paper electrophoresis [11].

Enzyme purification. The procedure is adapted with certain important modifications from that which we developed for isolation of the rabbit pulmonary enzyme [11,12]. A typical preparation starting with 3 kg of tissue is briefly described. All operations were performed at 0–4°C.

The frozen lungs were thawed and homogenized with 6 l of 10 mM Tris · HCl (pH 7.4) in a Waring blendor. The crude homogenate was centrifuged at 100000 × *g* for 1 h. The pellet was taken to 5700 ml in the same buffer, stirred overnight in the presence of 0.5% Triton X-100 and centrifuged at 16000 × *g* for 1 h. The supernatant fraction (Triton extract) was mixed for 30 min with 10 l of a slurry containing DEAE-cellulose (packed volume 5 l) in 10 mM K_2HPO_4 (pH 6.5)/0.5% Triton X-100 and then filtered on a Buchner funnel. The cake was washed with 10 l of the same buffer containing 0.025% detergent, and activity was then eluted by two extractions each with 8 l of 50 mM K_2HPO_4 (pH 6.5)/0.025% Triton X-100. The eluates were combined, concentrated by ultrafiltration and dialyzed against 1 mM K_2HPO_4 (pH 7.5)/0.025% Triton X-100. The DEAE-fraction was mixed for 1 h with 2250 ml of a slurry containing 150 g calcium phosphate gel (as dry weight) in 1 mM K_2HPO_4 (pH 7.5). The suspension was centrifuged, and the active supernatant fraction was

concentrated and made to 10 mM Tris · HCl (pH 7.5)/0.15 M NaCl. It was then adsorbed to a column (6 × 30 cm) of ricin-agglutinin-agarose which was washed with 6 l of starting buffer to remove residual detergent and eluted with the same buffer containing 0.1 M lactose. The eluate was concentrated to 8.3 ml by ultrafiltration and then subjected to gel filtration through a column (3.6 × 100 cm) of Sephadex G-200 in 10 mM Tris · HCl (pH 7.5). Activity was resolved equally into two peaks, one emerging with the void volume, the other at a V_e/V_o value of 1.3. Only the latter was significantly purified. This Sephadex G-200 fraction was concentrated and centrifuged through a 5—20% sucrose gradient (30 ml) in 10 mM Tris · HCl (pH 7.5) for 24 h at 25000 rev./min. Peak fractions were collected, dialyzed against 1 mM K_2HPO_4 (pH 7.5) and adsorbed to a column (1 × 20 cm) of hydroxyapatite in the same buffer. The enzyme was eluted as a peak of constant specific activity at 6—10 mM K_2HPO_4 with a linear gradient (300 ml) from 1 to 25 mM K_2HPO_4 (pH 7.5). Active fractions were pooled, concentrated and dialyzed against 10 mM Tris/acetate (pH 7.4).

Gel electrophoresis. Slab gel electrophoresis of reduced, denatured proteins was carried out on 5—20% polyacrylamide gradients in the presence of 0.1% sodium dodecyl sulphate (SDS) at pH 8.3 [12]. Protein bands were developed with Coomassie Blue [17].

Amino acid analyses. Aliquots of enzyme were hydrolyzed with 5.7 M HCl in vacuo for 36 and 86 h at 110°C and analyzed according to Spackman et al. [18]. Methionine and half-cystine were estimated as methionine sulfone and cysteic acid after oxidation of the enzyme with performic acid [19]. For determination of tryptophan, hydrolysis was performed with 4 M methane sulfonic acid, and the amino acid was resolved from glucosamine on the short column of the amino acid analyzer [12].

Analysis of NH_2-terminal residue. Derivatized amino acids were separated by polyamide thin-layer chromatography after acid hydrolysis of the dansylated enzyme [20].

Carbohydrate analysis. Carbohydrate residues were identified by gas chromatography as described previously [14].

Digestion with neuraminidase. Pure enzyme (45 μg) was incubated with or without 10 μg of neuraminidase in 200 μl 50 mM Tris/acetate (pH 6.0)/1 mM $CaCl_2$ at 37°C for 5 h. After assaying for activity, the solutions were extensively dialyzed against water and analyzed for sialic acid.

Estimation of zinc. Analyses were carried out at 213.7 nm using a Perkin-Elmer Model 360 atomic absorption spectrophotometer equipped with an HGA-2100 graphite furnace.

Antibody preparations. Anti-dog enzyme antibodies were raised in two goats by monthly intradermal injections of purified canine enzyme (100 μg per animal) in 10 mM Tris · HCl (pH 7.4)/0.15 M NaCl emulsified with an equal volume of complete Freund's adjuvant. IgG antibodies were isolated by heat treatment, $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography [8]. Fab fragments were prepared from the IgG fraction (1 g) by incubating with 10 mg pepsin for 16 h at 37°C in 20 ml 0.2 M sodium acetate (pH 4.5). The pH was adjusted to 7.0 with Tris base and digestion was continued for 16 h with 10 mg papain in the presence of 10 mM cysteine and 1 mM EDTA. The Fab fragments (approx. 400 mg) were separated from enzymes and undigested

antibody by gel filtration on a column (2.5 × 200 cm) of Sephadex G-100 in 10 mM Tris · HCl (pH 7.4)/0.15 M NaCl. The molecular weight of the unreduced Fab fragments was 45000 as estimated by polyacrylamide gel electrophoresis in the presence of 0.1% SDS. As expected, the fragments did not form a precipitin band with enzyme during double diffusion in agar, but did exhibit immune-specific ability to inhibit formation of such a band between enzyme and holoantibody.

Immunodiffusion. Double diffusion in agar was performed according to Ouchterlony [21].

Radioimmunoassays. The procedure was carried out as described previously [8] except that buffers did not contain non-ionic detergents. The pure radioiodinated dog and rabbit enzymes contained 69000 and 67000 cpm/ng protein.

Protein determinations. For enzyme fractions, the method of Lowry et al. [22] was used with bovine serum albumin as the standard. For antibody preparations, an $E^{1\%}_{280\,nm}$ value of 14.0 was employed.

Results

Physicochemical properties

The isolation procedure is summarized in Table I. The enzyme was purified 1800-fold with a yield of 11%. After slab gel electrophoresis in the reduced, denatured state, the purified preparation exhibited a single polypeptide band of molecular weight 140000 (Fig. 1). Equivalent units of the excluded Sephadex fraction and the pure enzyme yielded a band of similar mobility and intensity, suggesting that the same protein accounts for the two activity peaks resolved by the gel filtration step of the procedure. The sedimentation coefficient of the native enzyme during sucrose gradient centrifugation was estimated at 7.9 S by comparison with bovine liver catalase. The molecular weight determined from this figure by the method of Martin and Ames [23] was approx. 140000, and the correspondence of this value with that obtained for the reduced, denatured protein indicates that the enzyme contains a single polypeptide chain. The paradoxically early elution of the purified preparation from Sephadex G-200 (V_e/V_o = 1.3) has also been noted with converting enzymes from other species and presumably reflects their glycoprotein nature [24].

TABLE I

PURIFICATION OF ANGIOTENSIN-CONVERTING ENZYME FROM 3 kg OF DOG LUNGS

Fraction	Volume (ml)	Protein (mg)	Specific activity (units/mg protein)
Homogenate	7500	300 000	0.014
Triton X-100	3800	50 000	0.070
DEAE-cellulose (conc.)	750	9 830	0.21
Calcium phosphate gel (conc.)	250	3 730	0.47
Ricin-agglutinin-agarose (conc.)	8.3	280	4.7
Sephadex G-200 (conc.)	2.4	34.7	18
Sucrose gradient	14	19.9	22
Hydroxyapatite (conc.)	4	17.9	25

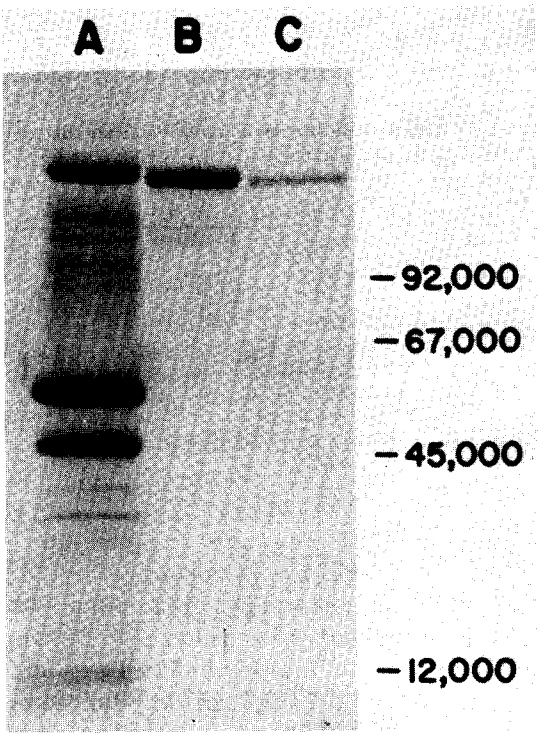


Fig. 1. Polyacrylamide slab gel electrophoresis under reducing, denaturing conditions. Each lane contained 45 munits of activity. A, fraction excluded from Sephadex G-200; B, purified canine enzyme; C, purified rabbit enzyme. The position of molecular weight markers (phosphorylase *A*, albumin, ovalbumin and lysozyme) run simultaneously is indicated by the arrows.

The content of hydrophobic amino acids [25] in the purified enzyme was 42%, and the ratio of polar to apolar residues [26] was 1.25 (Table II). The relative abundance of aromatic amino acids (12%) accounts for a high absorbance ($E^{1\%}_{280\,nm}$ = 22.0) in the ultraviolet region. The dansylation procedure [20] failed to reveal an NH_2-terminal residue, whereas a control determination on the rabbit protein yielded the expected [12] threonyl residue.

Oligosaccharide accounted for 17% of the weight of the enzyme derived from the sum of its aminoacyl and carbohydrate components (Table II). The activity of the preparation was unchanged after release of more than 97% of its sialyl residues by treatment with neuraminidase.

The zinc content of the enzyme was 7.6 nmol per mg protein, corresponding to a value of 0.94 g atoms per mol. Enzymatic activity was stimulated by inclusion of Co^{2+} in reaction mixtures and inhibited by the presence of Mn^{2+} (Table III). These effects were largely eliminated by simultaneous addition of Zn^{2+} at low molar ratios with respect to added Co^{2+} (1 : 3) or added Mn^{2+} (1 : 10).

Catalytic properties

The pure canine and rabbit enzymes differed markedly in their action on Hip-His-Leu. Their respective specific activities under standard assay conditions were 25 and 90 μmol/min per mg protein. The lower activity of the

TABLE II

AMINO ACID AND CARBOHYDRATE COMPOSITION OF CANINE PULMONARY ANGIOTENSIN-CONVERTING ENZYME

Values for amino acids are those obtained after 86 h hydrolysis. The figures in parentheses are the comparable values calculated from our previous data [12] on the rabbit enzyme.

Amino acid or carbohydrate	μg/mg protein	Mol%	
Lysine	51.3	5.0	(4.2)
Histidine	36.5	3.2	(3.1)
Arginine	69.3	5.4	(5.0)
Aspartic acid	95.6	10.2	(10.3)
Threonine *	38.7	4.8	(4.5)
Serine *	36.5	5.2	(5.9)
Glutamic acid	123.1	11.7	(10.5)
Proline	45.2	5.7	(6.0)
Glycine	25.8	5.5	(5.6)
Alanine	40.6	7.0	(8.5)
Half-cystine **	9.0	1.1	(1.6)
Valine	48.0	5.9	(5.9)
Methionine ***	28.3	2.9	(3.1)
Isoleucine	34.4	3.8	(3.9)
Leucine	97.3	10.6	(10.0)
Tyrosine	65.0	4.9	(4.5)
Phenylalanine	56.4	4.8	(4.7)
Tryptophan †	35.0	2.3	(2.7)
Total	935.7	100.0	(100.0)
Fucose	8.1	4.8	(2.3)
Mannose	46.7	26.4	(24.4)
Galactose	52.1	29.6	(32.4)
N-Acetylglucosamine	77.1	34.4	(30.1)
N-Acetylneuraminic acid	14.2	4.8	(10.8)
Total	198.2	100.0	(100.0)

* Corrected for decomposition by extrapolation of 86 and 36 h values to zero time.
** Determined as cysteic acid.
*** Determined as methionine sulfone.
† Determined after hydrolysis with 4 M methane sulfonic acid.

TABLE III

EFFECT OF ADDED METAL IONS ON ANGIOTENSIN-CONVERTING ENZYME ACTIVITY

The complete system was that used in the standard assay and included 7.3 munits of purified canine or rabbit enzyme. Additions were in the form of cobaltous sulfate or zinc acetate.

Addition	Dog lung enzyme activity (%)	Rabbit lung enzyme activity (%)
None	100	100
10 nmol Co^{2+}	267	138
10 nmol Co^{2+} + 3 nmol Zn^{2+}	147	111
10 nmol Co^{2+} + 50 nmol Zn^{2+}	113	103
30 nmol Mn^{2+}	68	61
30 nmol Mn^{2+} + 3 nmol Zn^{2+}	94	99
30 nmol Mn^{2+} + 50 nmol Zn^{2+}	102	102
3 nmol Zn^{2+}	100	103
50 nmol Zn^{2+}	99	104

canine glycoprotein was associated with a higher K_m value (5.9 vs. 2.3 mM) and a lower V value (45 vs. 130 μmol/min per mg). These results contrasted with the similar kinetic properties of the two enzymes on angiotensin I. Each exhibited a K_m value of 0.09 mM for this physiologic substrate, and the respective V values were 5.4 and 5.9 μmol/min per mg (Fig. 2). Like rabbit [11] and porcine [28] converting enzymes, the pure canine preparation possessed bradykininase activity. Bradykinin (80 nmol) was completely degraded after incubation with 20 milliunits of enzyme for 60 min at 37°C. Phe-Arg and Ser-Pro, the COOH-terminal and penultimate dipeptidyl residues, were identified as reaction products. Additional catalytic properties (Table IV) were also similar to those determined previously for the rabbit enzyme [12].

Immunologic properties

Double diffusion in agar with purified dog and rabbit preparations revealed reactions of partial identity with antisera against either enzyme (not shown). In competition radioimmunoassays (Fig. 3), the weight of rabbit enzyme needed to displace 50% of the radioiodinated dog enzyme from its complex with anti-dog enzyme antibody was 120 times the required weight of un-

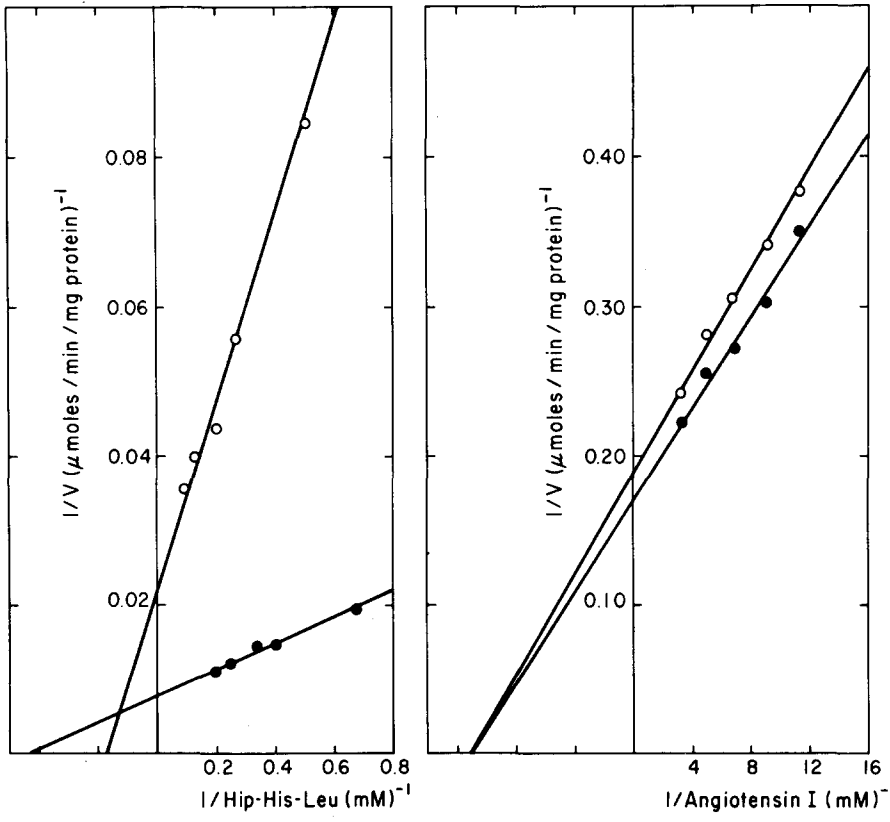


Fig. 2. Relationship of reaction velocity and substrate concentration. The data were analyzed by the method of Lineweaver and Burk [27]. ○, canine enzyme; ●, rabbit enzyme.

TABLE IV

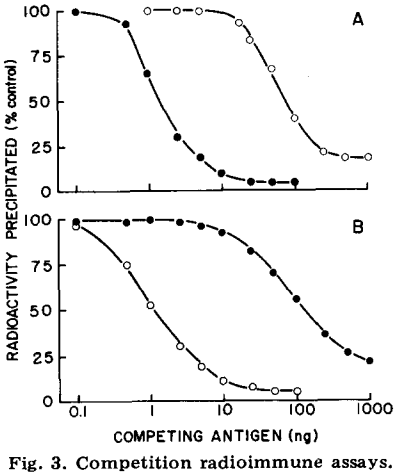
CATALYTIC PROPERTIES OF THE CANINE ENZYME

The complete system was that employed for the standard enzyme assay and included 7.3 munits of purified enzyme.

Addition or deletion	Inhibition (%)
—NaCl	72
+ 0.1 mM phenylmethylsulfonylfluoride	0
+ 0.1 mM EDTA	96
+ 1.0 mM dithiothreitol	99
+ 0.75 μM bradykinin-potentiating nonapeptide *	98
+ 0.04 mM angiotensin II	32
+ 1.0 mM His-Leu	32
+ 1.0 mM Phe-Arg	50
+ 0.1 mM Ser-Pro	41

* Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro.

labelled canine enzyme. A 65-fold excess of dog compared to rabbit enzyme was necessary to displace 50% of the radioactive rabbit glycoprotein from its homologous immune complex. The degree of homology indicated by these values is substantially lower than that previously estimated from the ability of Hip-His-Leu enzyme units in a crude canine pulmonary extract to compete with the pure rabbit glycoprotein for binding to anti-rabbit enzyme antibody [8]. This is because the earlier estimate was based on the assumption that the two enzymes possessed an identical specific activity.



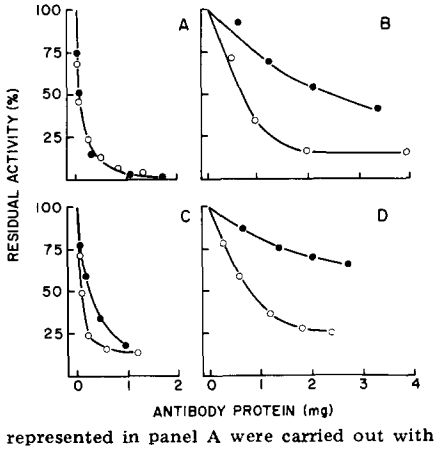


Fig. 3. Competition radioimmune assays. The experiments represented in panel A were carried out with diluted (1 : 2100) anti-rabbit enzyme serum and 1.0 ng of pure radioiodinated rabbit enzyme. Those shown in panel B were performed with diluted (1 : 1000) anti-dog enzyme serum and 1.0 ng of pure labelled canine enzyme. Competing proteins were pure canine (○) and rabbit (●) enzyme.

Fig. 4. Anticatalytic activity of IgG and Fab fragments. The upper panels show the anticatalytic activity of anti-rabbit enzyme antibody proteins against (A) rabbit enzyme and (B) dog enzyme. The lower panels depict the anticatalytic activity of anti-dog enzyme antibody proteins against (C) dog enzyme and (D) rabbit enzyme. The antibody proteins used were IgG (○) and Fab fragments (●). Reaction mixtures (100 μl) contained IgG or Fab fragments and 2.5 μg of purified dog or rabbit lung enzyme in 10 mM Tris · HCl (pH 7.4)/0.15 M NaCl. After 30 min incubation at 37°C, aliquots were assayed for residual enzyme activity with Hip-His-Leu as substrate.

IgG antibodies directed against either the dog or rabbit enzyme inhibited both homologous and heterologous activities (Fig. 4). However, the amount of antibody required to inhibit 50% of the activity associated with an equal weight of enzyme protein was almost an order of magnitude less for the homologous combination, and the maximum inhibition reached at high antibody concentrations was also greater under these circumstances. The anticatalytic action of Fab fragments on homologous activity was comparable on a weight basis to that of the holoantibody. In contrast, the effect of the fragments on heterologous activity was much less than that of the intact IgG molecules as reflected in both the slope of the inhibition dose vs. response curves and the maximum inhibitions.

Discussion

Canine pulmonary angiotensin-converting enzyme has not previously been purified and characterized, although the importance of the lung in angiotensin conversion was initially recognized in the dog [29], and pulmonary particles from this species represented one of the first subcellular preparations in which converting activity was detected [30]. Our results indicate that the dog enzyme closely resembles that which we previously characterized from rabbit lung [11,12]. Both contain a single glycopolypeptide chain of comparable molecular weight, and they possess similar amino acid and carbohydrate compositions and display immunologic homology by several criteria. Each is associated with a molar equivalent of zinc which can apparently exchange with Co^{2+} or Mn^{2+}, although these divalent ions appear to be bound with lower affinity. The NH_2-terminal residue is inaccessible and presumably blocked in the canine glycoprotein, whereas it is threonine in that of the rabbit.

It is noteworthy that the pure canine enzyme catalyzes hydrolysis of Hip-His-Leu much less efficiently than does the rabbit enzyme, although the two preparations exhibit almost identical kinetic parameters with angiotensin I. This is consistent with a previous speculation [24] that evolutionary conservation of an unnatural hydrolytic activity in this enzyme need not necessarily parallel that for its physiologic substrate. The observation is important because it indicates that comparisons of converting activity in different species based on hydrolysis of a model substrate may not accurately reflect the relative content of either angiotensin-converting enzyme molecules or catalytic potential for angiotensin I.

Since univalent Fab fragments appear to be effective inhibitors of homologous converting enzyme, they may be useful for blocking the renin-angiotensin system in vivo provided that they do not generate the lethal reaction which occurs after administration of intact anti-enzyme antibodies to homologous recipient animals [7]. If this poorly understood reaction is mediated by complement, then presumably it may be circumvented with Fab fragments. The unexpected finding that the fragments are much poorer inhibitors of heterologous activity than are holoantibodies suggests that they will not yield the high degree of immunologic blockade which has been achieved with intact antibodies in heterologous recipients [8].

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