Pulmonary Angiotensin-Converting Enzyme Antienzyme Antibody†

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ABSTRACT: A method has been developed for quantitating anticatalytic activity in antibody preparations made in goats against pure solubilized angiotensin-converting enzyme from rabbit pulmonary membranes. Anticatalytic activity was purified about 90-fold from a single batch of serum by a procedure including diethylaminoethylcellulose chromatography and elution from Sepharose columns containing covalently bound pure enzyme. Antiholoenzyme antibody was fractionated with respect to charge and binding affinity; however, these different populations each inhibited enzymatic hydrolysis of hippurylhistidylleucine, angiotensin I, and bradykinin. The inhibition dose–response curves were similar for hydrolysis of hippurylhistidylleucine and angiotensin I despite the difference in molecular weight of these substrates. Evidence is presented suggesting that a single molecule of antibody can bind two molecules of enzyme and that at least 18% of the total antiholoenzyme antibody population is directed against determinants which influence catalytic activity. A competitive immunoassay was developed with radioiodinated pulmonary enzyme as displaceable antigen. The anticatalytic and radioimmune assays were used to examine immunological properties of converting enzymes in various rabbit organs and fluids. Kidney, brain, and serum were found to contain converting enzymes which were immunologically identical with that in rabbit lung. Converting enzyme in seminal plasma was similar to the lung enzyme in the anticatalytic assay, but showed lower immunoreactivity in the radioimmune assay.

Angiotensin-converting enzyme (EC 3.4.15.1) is a COOH-terminal dipeptidyl peptidase (for a recent review see Soffer, 1976). It catalyzes cleavage of His-Leu from angiotensin I (Lentz et al., 1956) to yield the vasopressor octapeptide angiotensin II, which is the biologically active component of the renin–angiotensin system (Helmer, 1957). The same glycoprotein COOH-terminal dipeptidyl peptidase catalyzes sequential release of Phe-Arg and Ser-Pro from bradykinin, thereby inactivating this powerful vasodepressor nonapeptide (Soffer et al., 1974; Dorer et al., 1974). Peptidic inhibitors of angiotensin-converting enzyme isolated from snake venom (Ferreira et al., 1970; Ondetti et al., 1971) prevent initiation and development of experimental renin-dependent hypertension (Greene et al., 1972; Miller et al., 1972), implicating the enzyme as a therapeutic target in this disease. The lung is the most important site for generation of angiotensin II destined for the systemic circulation (Ng and Vane, 1967, 1968); however, conversion of angiotensin I occurs in many organs (Oparil et al., 1970; Kreye and Gross, 1971; DiSalvo and Montefusco, 1971; Oates and Stokes, 1974) and converting activity is widespread (Huggins and Thampi, 1968; Cushman and Cheung, 1971a). Lung converting enzyme is a constituent of the vascular endothelial membrane and is present on the luminal surface in direct contact with the pulmonary circulation (Ryan et al., 1975). Extrapulmonary enzyme is also generally localized in vascular endothelial cells (Caldwell et al., 1976a). We recently isolated and characterized pure converting enzyme from rabbit pulmonary membranes after solubilization with a nonionic detergent (Soffer et al., 1974; Das and Soffer, 1975). Since this plasma membrane enzyme is probably directly accessible to the action of circulating macromolecules, it may be possible to regulate its activity in vivo by immunologic means. In this communication we describe the preparation, assay, fractionation, and some properties of goat antibody. We have developed an operational quantitative expression for the anticatalytic effect of these antibodies in vitro which is independent of their mechanism of action. Studies using the antibodies for detection, measurement, and characterization of extrapulmonary enzymes are also presented.

Experimental Procedures

Materials

Unlabeled angiotensin I was from Calbiochem. Angiotensin I labeled with carbon-14 in the COOH-terminal leucine residue (333 μCi/μmol) was obtained from New England Nuclear. Hip-His-Leu was from Research Plus. Bradykinin triacetate was a Sigma product. ^{125}I, carrier-free, was from Amersham/Searle. Ampholine carrier ampholytes were purchased from LKB and other materials for isoelectric focusing were obtained from Bio-Rad. Cyanogen bromide activated Sepharose 4B was purchased from Pharmacia Fine Chemicals and DEAE[1]-cellulose (Whatman DE-52) was from Reeve-Angel. Complete Freund's adjuvant was a Difco product. Nonidet P-40 was from Shell Chemicals. All rabbit organs were from Pel-Freez.

Methods

Enzyme Preparation. Angiotensin-converting enzyme from rabbit pulmonary membranes was purified as described earlier (Das and Soffer, 1975). The purity of the glycometalloenzyme was confirmed by polyacrylamide gel electrophoresis and sedimentation equilibrium (Das and Soffer, 1975). Crude solubilized enzyme preparations from rabbit lung, kidney, brain, and liver were obtained by extraction of the 16 000*g* pellet in the presence of Nonidet P-40 as described for the

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Temed, *N,N,N',N'*-tetramethylethylenediamine; Boc, *tert*-butyloxy; Bis, *N,N'*-methylenebisacrylamide.

initial three steps in the purification of the rabbit lung enzyme (Das and Soffer, 1975).

Immunization of Goats. Antienzyme antiserum was produced by monthly immunization of goats with 150-μg doses of pure enzyme dissolved in 1 ml of 0.15 M NaCl–10 mM Tris-HCl, pH 7.5, and emulsified with an equal volume of complete Freund's adjuvant. Injections were given intramuscularly and intradermally at multiple sites. Sera (heat-treated at 56 °C for 30 min) were tested for anticatalytic activity at regular intervals and reached a plateau after 2 months. The heat-treated sera had no converting enzyme activity.

Enzyme Assay. The spectrophotometric assay using Hip-His-Leu as substrate was done according to the method of Cushman and Cheung (1971b). A unit of enzyme activity is the amount required to catalyze formation of 1 μmol of hippuric acid per min at 37 °C. The specific activity of pure rabbit pulmonary angiotensin-converting enzyme under the standard conditions was 89 units/mg protein.

Release of radioactive His-Leu from angiotensin I labeled with carbon-14 in the COOH-terminal leucine residue was determined as described by Lee et al. (1971). Reaction mixtures (30 μl) contained 50 mM Tris-HCl, pH 7.8, 30 mM NaCl, 0.27 mM angiotensin I (4 μCi/μmol), and enzyme (about 8 ng). After incubation at 37 °C for 30 min, 5 μl of 6 N HCl was added to stop the reaction. Then 1 μl of 50 mM His-Leu (unlabeled) was added as carrier and an aliquot (25 μl) of this mixture was analyzed by paper electrophoresis. Under these conditions the specific activity of pure rabbit lung angiotensin-converting enzyme was 5.0 μmol of angiotensin I hydrolyzed min^{-1} (mg protein)$^{-1}$. This assay was not suitable for crude enzyme preparations due to the presence of dipeptidase and other exopeptidases.

Assay of Anticatalytic Activity. The standard reaction mixture (100 μl) contained 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 μg of pure rabbit lung angiotensin-converting enzyme, and antibody (1–2 munit). After incubation at 37 °C for 30 min, an aliquot of the reaction mixture was assayed for residual enzyme activity with either Hip-His-Leu or angiotensin I as substrate. In assays with Hip-His-Leu, 2 μl of the reaction mixture was used. For assays with angiotensin I, the reaction mixture was diluted sixfold with 0.15 M NaCl and 1 μl of the diluted mixture was used. The reaction of enzyme with antibody reached equilibrium within 30 min and there was no dissociation of enzyme–antibody complex under the conditions of enzyme assay. The inactivation of enzyme with increasing concentrations of antibody was linear up to about 50% inhibition. We define a unit of anticatalytic activity as the amount of antibody required to inhibit completely the catalytic activity of 1.0 mg of pure rabbit lung angiotensin-converting enzyme on a given substrate under these conditions as extrapolated from the linear portion of the inhibition dose–response curve.

The inhibitory effect of antibody on the bradykininase activity of the enzyme was studied only qualitatively. Incubation of enzyme with antibody was carried out under the standard conditions as described above. An aliquot (5 μl) was then added to a solution (50 μl) containing 50 mM Tris-HCl, pH 7.8, 30 mM NaCl, and 1.6 mM bradykinin. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 10 μl of 6 N HCl. The mixture was subjected to paper electrophoresis in pyridine–acetic acid–water (1:10:89), pH 3.5, for 2 h with a potential gradient of 50 V/cm. The paper was developed with 1% ninhydrin in 0.09% cadmium acetate in acetone–water–acetic acid (100:10:2). The color intensity of the Phe-Arg spot was an indicator of residual enzyme activity.

Preparation of Enzyme–Sepharose. Freeze-dried CNBr-activated Sepharose 4B (10 g; Pharmacia) was swollen and washed with 1 l. of 1 mM HCl in a sintered-glass funnel to remove the lactose. The activated Sepharose was then cooled in ice and rapidly washed with 2 volumes of cold 0.1 M $NaHCO_3$, pH 9.0. Then it was dispersed in a solution (50 ml) of rabbit lung angiotensin-converting enzyme (0.6 mg/ml) in 0.1 M $NaHCO_3$, pH 9.0. The coupling was allowed to proceed overnight at 4 °C before filtering and washing with 0.1 M $NaHCO_3$. The filtrate was virtually devoid of A_{280nm} absorbing material, indicating that coupling of the enzyme to Sepharose was virtually complete. The washed enzyme–Sepharose was dispersed in 1 M ethanolamine hydrochloride, pH 8.0, and the suspension was stirred for 2 h to ensure complete blocking of the unreacted active groups. The enzyme–Sepharose was then washed with 10 mM Tris-HCl, containing 0.15 M NaCl, pH 7.5, and stored as a suspension at 4 °C. Sodium azide (0.02%) was added to prevent microbial growth.

Isoelectric Focusing in Polyacrylamide Gel Slabs. Isoelectric focusing was performed in a slab gel apparatus patterned after that of Reid and Bieleski (1968). The gels were 1.5-mm thick, 130-mm wide, and 92-mm high and were formed by pumping in a solution containing 7.5% acrylamide, 0.2% Bis, 0.025% ammonium persulfate, 0.036% Temed (v/v), and 2% ampholine carrier ampholytes (pH range 3.5–10). Sample wells 8-mm wide and 1.5-mm apart were cast with a comb-like plexiglass spacer. The 7.5% slab gel with sample wells was overlaid with a solution of 3% acrylamide, 0.08% Bis, 0.05% ammonium persulfate, 0.025% Temed (v/v), and 2% ampholine carrier ampholytes (pH range 3.5–10). Immediately after this solution was put in and before it set into a gel, 20 μl of each sample (in 10% glycerine and 2% ampholine carrier ampholytes, pH range 3.5–10) was layered directly into the bottom of each sample well under the 3% acrylamide solution with a microsyringe. After the overlaid 3% acrylamide solution had set into a gel, the cathode solution (0.24 M NaOH) and the anode solution (0.15 M H_3PO_4) were put into the lower and upper electrode chambers, respectively. The isoelectric focusing was carried out at 4 °C for 24 h (final potential 300 V). The gel slabs were stained for 1 h with 0.1% Coomassie blue in ethanol–water–acetic acid (9:9:2) and then were destained in ethanol–water–acetic acid (6:13:1).

Immunodiffusion. Double immunodiffusion in agar was performed according to the method of Ouchterlony (1958).

Radioimmune Assay. Pure rabbit lung angiotensin-converting enzyme (5 μg) was labeled with ^{125}I, as described by Hunter (1967). The specific activity of the radiolabeled enzyme was about 1.4×10^8 cpm/μg of protein. For radioimmune assay, all incubations were performed in a final volume of 200 μl and the dilutions were made in 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, bovine serum albumin (2 mg/ml), 0.1% Nonidet P-40. The reaction mixture contained preimmune goat serum (5 μl), ^{125}I-labeled rabbit lung angiotensin-converting enzyme (0.2 ng), the competing antigen, and the goat antiserum (final dilution 1:35 000). The amount of antibody used was sufficient to precipitate about 50% of the radiolabeled enzyme in the absence of any competing antigen. After incubation at 37 °C for 8 h, 50 μl containing 180 μg of donkey anti-goat IgG was added to the reaction mixture to precipitate the enzyme–antibody complex, and incubation was continued for an additional hour at 37 °C and then for 2 h at 4 °C. The precipitate was collected by centrifugation at 4 °C and washed twice with 1 ml of cold 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and its radioactivity was determined in a γ counter.

Protein Determination. Enzyme protein was determined by

TABLE I: Purification of Anticatalytic Activity from 400 ml of Immune Serum.

Fraction	Protein (mg)	Total Units[a]	Units/mg Protein
Heat-treated serum	33 000	110	0.0033
0–50% saturated $(NH_4)_2SO_4$ fraction	8 790	105	0.012
DEAE-Cellulose (total)	3 290	93.1	0.028
Fraction I	1 580	52.8	0.033
Fraction II	1 090	27.8	0.026
Fraction III	620	12.5	0.020
Enzyme-Sepharose[b] (total)	316	90.3	0.29
pH 4.0 eluate, DEAE-cellulose Fraction I (4.0 I)	104	26.0	0.25
pH 2.5 eluate, DEAE-cellulose Fraction I (2.5 I)	93.5	27.1	0.29
pH 4.0 eluate, DEAE-cellulose Fraction II (4.0 II)	40	9.6	0.24
pH 2.5 eluate, DEAE-cellulose Fraction II (2.5 II)	38.5	16.2	0.42
pH 4.0 eluate, DEAE-cellulose Fraction III (4.0 III)	16.5	4.13	0.25
pH 2.5 eluate, DEAE-cellulose Fraction III (2.5 III)	23.5	7.29	0.31

[a] Determined using Hip-His-Leu as substrate as described in Methods. [b] Recovery of protein and anticatalytic activity have been corrected to include utilization of the entire DEAE-cellulose fractions I, II, and III.

the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard. One milligram of protein as estimated by this technique corresponds to 8.43 nmol of pure enzyme (Das and Soffer, 1975).

Antibody proteins were estimated by absorbance at 280 nm. An $A_{280nm}^{0.1\%}$ value of 1.41 for γ-globulins was used in calculating the amounts of protein present in the purified affinity column eluates, the DEAE-cellulose fractions, and the 0–50% saturated $(NH_4)_2SO_4$ fraction. For unfractionated serum, a value of 0.90 for the $A_{280nm}^{0.1\%}$ was used in calculations.

Results

Purification of Anticatalytic Activity. Antisera from four goats yielded a single precipitin band after immunodiffusion both against the purified enzyme and a crude extract of rabbit lung. The immune sera also inhibited the enzymatic hydrolysis of hippurylhistidylleucine and the enzymatic conversion of angiotensin I to angiotensin II. Preimmune sera did not show any of these characteristics. Purification of anticatalytic activity was carried out on a large batch of immune serum from a single animal and is summarized in Table I. All operations were performed at 0–4 °C unless otherwise stated.

Heat Treatment. A 400-ml portion of antiserum was heated at 56 °C for 30 min to destroy complement and any angiotensin-converting activity.

Ammonium Sulfate Fractionation. The heat-treated serum was subjected to fractionation by the addition of solid $(NH_4)_2SO_4$ up to 50% saturation. The precipitate obtained after centrifugation at 16 000 *g* for 20 min was dialyzed against 15 mM potassium phosphate, pH 8.0. The dialyzed fraction was centrifuged at 16 000*g* for 20 min to remove a small precipitate.

DEAE-Cellulose Column Chromatography. The ammonium sulfate fraction was applied to a column (31 × 9 cm) of DEAE-cellulose which was equilibrated and developed with

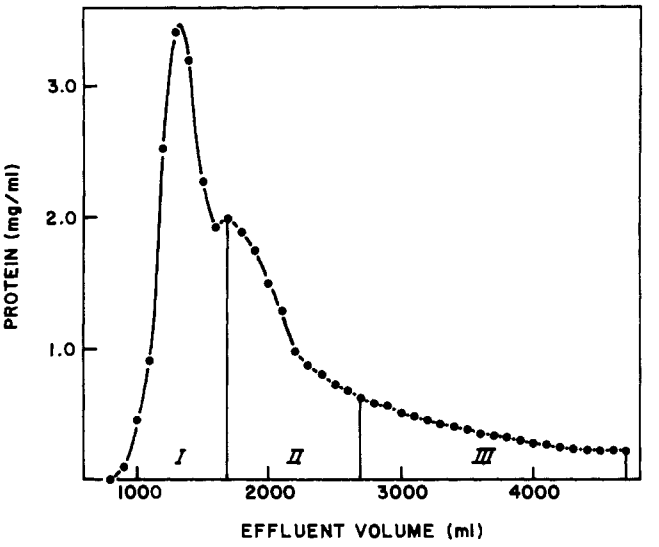

FIGURE 1: DEAE-Cellulose fractionation of the 0–50% saturated $(NH_4)_2SO_4$ fraction. Details of the procedure are described in the text.

15 mM potassium phosphate, pH 8.0. Fractions (20 ml) were collected at a flow rate of 50 ml per hour. The immune globulin peak was asymmetrical with a sharp emerging front and was divided into three major fractions, as shown in Figure 1. Each of these fractions was concentrated by ultrafiltration with Amicon PM 10 filters, then dialyzed against 10 mM Tris-HCl–0.15 M NaCl, pH 7.5, and centrifuged at 16 000*g* for 20 min to remove small amounts of precipitate.

Affinity Chromatography. Each of the DEAE-cellulose fractions was purified further by affinity chromatography using columns of Sepharose 4B containing covalently bound angiotensin-converting enzyme. Since a large amount of pure enzyme was necessary for this step, and since the capacity of the affinity columns was low (about 0.5 anticatalytic unit bound per mg of immobilized enzyme), only small aliquots of the DEAE-cellulose fractions (containing about 4–5 anticatalytic units) were subjected to affinity purification.

The antibody binding capacities of the affinity columns were higher at 37 than at 4 °C and, therefore, the columns (12 × 1 cm) of enzyme–Sepharose, each containing about 10 mg of pure enzyme, were equilibrated at 37 °C with 10 mM Tris-HCl–0.15 M NaCl, pH 7.5. Then aliquots of the DEAE-cellulose fractions I, II, and III, containing respectively 120, 256, and 281 mg of protein, were applied to the individual columns. After application of the samples, the columns were allowed to equilibrate at 37 °C for 1 h and were then washed with 150 ml of starting buffer at this temperature. These washes contained no anticatalytic activity and did not yield a precipitin band after immunodiffusion against the enzyme.

The elutions were carried out at 4 °C with two acidic buffers of different pH. The first eluting buffer was 10 mM Tris-acetate–1 M NaCl, pH 4.0. About 10 ml of this buffer was washed into each column and allowed to equilibrate for 1 h. Then an additional 30 ml of this buffer was used for eluting a fraction (35–50%, depending on the type of DEAE-cellulose fraction used) of the anticatalytic activity. The remaining bound anticatalytic activity was completely eluted with 20 mM glycine hydrochloride–1 M NaCl, pH 2.5. All eluates were neutralized immediately after elution with 2 M Tris-HCl, pH 7.5, and were then dialyzed against 10 mM Tris-HCl–0.15 M NaCl, pH 7.5. The dialyzed preparations were concentrated by ultrafiltration with Amicon PM 10 filters. After concen-

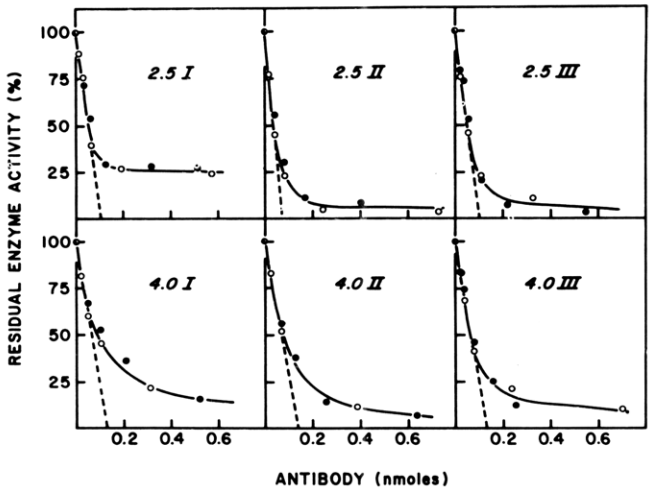

FIGURE 2: Inhibition of enzyme activity by purified antibody fractions. Incubation of enzyme (0.042 nmol) with various amounts of antibody was carried out under the standard conditions described under Methods. Residual enzyme activity was measured with both angiotensin I (○—○) and Hip-His-Leu (●—●) as substrate.

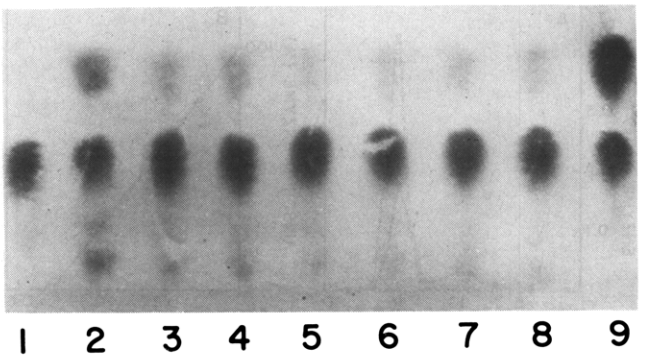

FIGURE 3: Inhibition of bradykininase activity. Angiotensin-converting enzyme (5 μg) was incubated in the absence and presence of 90 μg of antibody under the standard conditions, and aliquots were then examined for residual bradykinase activity by the procedure described under Methods. (1) No enzyme blank; (2) reaction mixture with active enzyme; (3, 4, 5, 6, 7, and 8) reaction mixtures with enzyme and antibody fractions 4.0 I, 2.5 I, 4.0 II, 2.5 II, 4.0 III, and 2.5 III, respectively; (9) paper electrophoresis standards Phe-Arg (top) and bradykinin (bottom).

tration small amounts of insoluble material were removed by centrifugation at 16 000*g* for 20 min. The concentrated affinity chromatography step fractions were stored at −20 °C and retained full anticatalytic activity for at least 6 months. After immunodiffusion against the pure enzyme or a crude extract of rabbit lung, all six fractions yielded a single precipitin line of identity. Recovery of anticatalytic activity was over 95% in this affinity purification step. There was no detectable binding (<0.1%) of protein by these columns from preimmune IgG fractions under conditions in which as much as 15% of the protein in immune IgG preparations was retained. After a single usage the affinity columns lost most of their capacity to bind specific antibody.

Heterogeneity of Purified Antibody Fractions. Analytical isoelectric focusing of the six purified antibody fractions revealed a high degree of heterogeneity (not shown). The p*I* values of the protein bands varied from 6.1 to 8.5. The affinity column eluates derived from DEAE-cellulose fraction I had the most basic proteins with values between 7.6 and 8.5. Those from DEAE-cellulose fraction III yielded antibodies having the lowest values which ranged between 6.1 and 6.7. The immune-specific material obtained from DEAE-cellulose fraction II contained at least four protein bands with p*I* values between 6.9 and 7.3 and, in addition, contained other proteins present in the affinity column eluates of both DEAE-cellulose fractions I and III. The focusing patterns for pH 4.0 and 2.5 eluates derived from a particular DEAE-cellulose fraction showed similar protein bands but in different relative proportions.

Inhibition of Enzyme Activity by Purified Antibody. When enzyme was incubated with antibody at 37 °C, inhibition was observed within 2 min and remained unchanged during the entire incubation period. The rapid attainment of equilibrium precluded studies on kinetics of the association process. The total affinity column eluate contained 0.29 anticatalytic unit per mg of antibody protein. The specific anticatalytic activities of the six fractionated populations comprising the total antiholoenzyme antibody ranged from 0.24 to 0.42 U/mg (Table I). In calculating molar ratios from such data, it should be noted that an anticatalytic unit is defined relative to 1.0 mg of enzyme as estimated by the Lowry procedure. This quantity of enzyme (M_r 129 000) corresponds to 8.43 nmol on the basis of its weight (1.087 mg) derived from amino acid and carbohydrate analysis (Das and Soffer, 1975). Therefore, a figure of 0.29 anticatalytic unit per mg of antibody protein (M_r 150 000) indicates that 2.73 molecules of total specific antiholoenzyme antibody are required to completely inhibit a single enzyme molecule. Calculated similarly the corresponding molar values for the six subfractions ranged from 3.30 to 1.88.

The inhibition dose–response curves for these fractions on the enzymatic hydrolysis of Hip-His-Leu and angiotensin I are shown in Figure 2. Inhibition was independent of the type of substrate used for enzyme assay and was a linear function of antibody concentration up to about 50%. At high antibody–enzyme ratios more than 99% inhibition was obtained with all the antibody fractions, except with the pH 2.5 eluate of DEAE-cellulose fraction I, which did not inhibit beyond 80% of the enzyme activity. At low antibody–enzyme ratios the pH 2.5 eluates inhibited more strongly than the pH 4.0 eluates. All of the six antibody fractions also inhibited the bradykininase activity of the enzyme (Figure 3).

Binding of Enzyme by Purified Antibody. In order to estimate the number of moles of enzyme which could be bound at limiting antibody concentrations, mixtures containing various proportions of the two components were incubated and analyzed by sucrose gradient centrifugation (Figure 4). The activity peak at 7.9 S (Soffer et al., 1974) was used as a measure of unbound enzyme. The kinetic characteristics of this enzyme peak were the same as those of the pure enzyme. In the presence of a molar excess of enzyme, the percentage of initial total activity represented in this residual fraction was a linear inverse function of the concentration of antibody. Graphical extrapolation of this percentage against the molar ratio of antibody to enzyme showed an intercept value of approximately 0.5 at 0% unbound enzyme. We interpret these data as suggesting that a single antibody molecule can bind two molecules of enzyme. This interpretation is consistent with the position of the second peak of activity in these gradients which corresponded to a molecular weight of 398 000 as calculated by the method of Martin and Ames (1961). The expected value for an aggregate containing two molecules of enzyme (M_r 129 000 (Das and Soffer, 1975)) and one molecule of antibody would be about 408 000.

Immunological Analysis of Converting Enzymes from Various Rabbit Tissue Extracts and Fluids. The results of

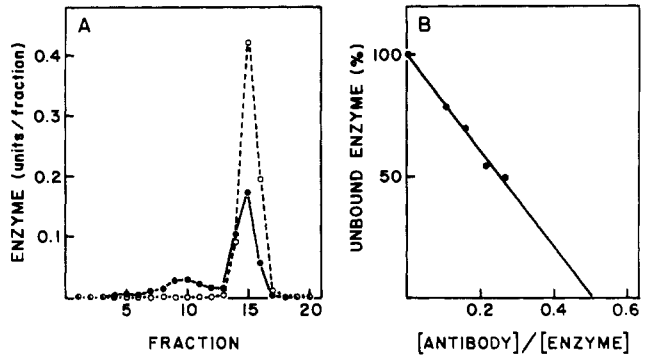

FIGURE 4: Equilibrium study of enzyme binding by the pH 2.5 eluate of DEAE-cellulose fraction II. Angiotensin-converting enzyme (126 pmol) was incubated at 37 °C for 20 min in the absence and presence of varying amounts of antibody (13.6, 20.4, 27.2, and 34.0 pmol) in 300 μl of 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5. The reaction mixtures were then centrifuged to remove any precipitate formed. Aliquots (200 μl) of the supernatants were centrifuged at 4 °C for 4 h at 50 000 rpm in a SW 50.1 swinging bucket rotor through 5–20% sucrose gradient (4.8 ml) in 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5. The tubes were then punctured at the bottom and 250-μl fractions were collected and analyzed for enzyme activity with Hip-His-Leu as substrate. (A) Sucrose gradient centrifugation of reaction mixtures containing no antibody (O- - -O) and 34 pmol of antibody (●—●). (B) Enzyme binding as a function of antibody/enzyme molar ratio. Unbound enzyme was calculated from the enzyme activity present in sucrose gradient fractions 13–17.

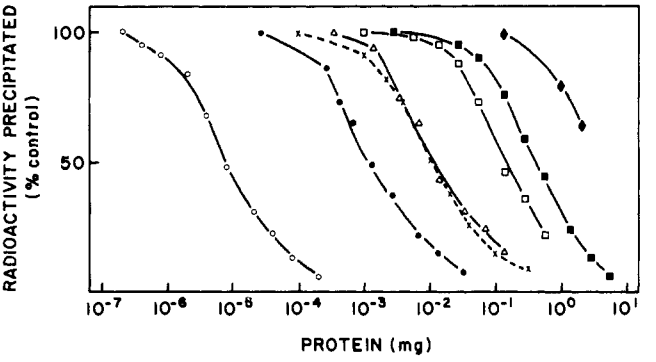

FIGURE 5: Competition of rabbit tissue extracts and fluids with ^{125}I-labeled pure rabbit lung angiotensin-converting enzyme for antibody binding. The competition radioimmune assays were performed as described under Methods. The competing proteins were: (O—O) pure rabbit lung enzyme; (●—●) rabbit lung Nonidet P-40 extract; (X- - -X) rabbit seminal plasma; (△—△) rabbit kidney Nonidet P-40 extract; (□—□) rabbit brain Nonidet P-40 extract; (■—■) rabbit serum; (♦—♦) rabbit liver Nonidet P-40 extract.

radioimmunoassay are shown in Figure 5 and Table II. Pure rabbit lung angiotensin-converting enzyme (1–100 ng) competed with radioiodinated enzyme for antibody binding in a concentration-dependent manner. Crude preparations of converting enzyme from lung, kidney, brain, serum, and seminal plasma were also capable of competing with ^{125}I-labeled enzyme in a parallel manner. For lung, kidney, brain, and serum, the ratio of catalytic activity to milligrams of competing antigen was about equal to the specific activity of the pure lung enzyme, suggesting a close molecular similarity between these converting enzymes. However, for the converting enzyme from seminal plasma this ratio was much higher, indicating that this enzyme is not completely identical with the lung converting enzyme. The competition exhibited by the liver extract was very small, as would be expected from its low catalytic activity. It has previously been shown that the hepatic enzyme is located in vascular endothelial cells (Caldwell et al., 1976a).

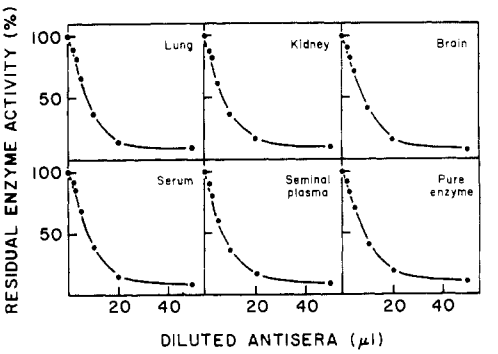

FIGURE 6: Anticatalytic effect of antibody on converting enzyme activity in various tissue extracts and fluids of rabbit. Tissue extract or fluid (containing 3 munit of enzyme activity) was incubated at 37 °C in the absence and presence of the indicated amounts of goat antisera (diluted 1:140) in 150 μl of 10 mM Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 0.1% Nonidet P-40. After incubation for 1 h, 100 μl of a solution containing 12.5 mM hippurylhistidylleucine, 0 25 M potassium phosphate buffer, pH 8.3, and 0.525 M NaCl was added to the reaction mixtures to assay the residual enzyme activity by the method of Cushman and Cheung (1971b).

TABLE II: Immunoreactivity and Catalytic Activity of Converting Enzyme from Various Rabbit Tissue Extracts and Fluids.

Tissue Extract or Fluid	Catalytic Act.[a] (munit/mg protein)	μg/mg Protein[b]	Unit/mg[c]
Lung Nonidet P-40 extract	545	5.89	93
Kidney Nonidet P-40 extract	59	0.617	95
Brain Nonidet P-40 extract	4.6	0.049	94
Serum	1.8	0.0174	103
Seminal plasma	219	0.676	324
Liver Nonidet P-40 extract	0.09		

[a] Assayed using hippurylhistidylleucine as the substrate as described in Methods. [b] Determined by radioimmune assay (μg of enzyme per mg of protein). [c] Units of catalytic activity per mg of competing antigen.

Anticatalytic effects of antibody on converting enzyme activity in various tissues and fluids is shown in Figure 6. The inhibition profiles are all strikingly similar to those observed with the lung converting enzyme.

Immunodiffusion analysis was carried out with lung, kidney, and seminal plasma converting enzymes (not shown). When equivalent amounts of converting activity were tested, the lung and kidney extracts gave single precipitin bands which were identical with and of equal intensity as the band produced by the pure lung enzyme. The seminal plasma preparation gave a precipitin line of lower intensity. However, at a threefold higher seminal plasma enzyme concentration a single sharp immunoprecipitin line of identity was observed.

Discussion

Angiotensin-converting enzyme is a surface glycoprotein which is directly accessible to the circulation and which can be isolated as a pure water-soluble antigen. It therefore provides an unusual opportunity for examining the possibility of regulating the action of an enzyme in vivo by immunologic means. The possibility is particularly important in the case of converting enzyme because of its critical role in the genesis of renovascular hypertension. Preliminary experiments have suggested (Caldwell et al., 1976b) that intravenously administered antienzyme antibody can inhibit the vasopressor re-

sponse to angiotensin I and potentiate the vasodepressor effect of bradykinin in rabbits. However, the antibody was also found to be toxic by a mechanism unrelated to its anticatalytic action. A quantitative formulation of anticatalytic action may therefore be useful in studies aimed at dissociating the different effects of antibody and developing a rational scheme for its use in vivo.

These studies have characterized antiholoenzyme antibody isolated by immunoadsorbent chromatography as a heterogeneous molecular population. However, various fractions separated on the basis of charge or binding affinity were found to inhibit enzyme activity. The mechanism(s) by which antibody inhibits the action of enzymes is not definitely established. Antibodies directed against determinants far from the active site may inhibit by inducing conformational changes to inactive states, whereas those reacting with determinants near the catalytic site may block substrate access. It has been shown for a number of enzymes that inhibition by antibody is greater with increasing substrate molecular weight (Brown et al., 1959; Fazekas and Groth, 1963; Lepow, 1963) and this has been interpreted to indicate that antibody prevents substrate access by steric hinderance. However, we observed the same degree of inhibition regardless of whether Hip-His-Leu (M_r 430) or angiotensin I (M_r 1297) was used as substrate. Our results are in contrast to those of Oshima et al. (1974), who prepared rabbit antibody against a partially purified fraction of porcine kidney angiotensin-converting enzyme. These authors reported a marked difference in the inhibitory action of a single concentration of immune globulin on the hydrolysis of Hip-His-Leu, angiotensin I, and Boc-Phe(NO_2)-Phe-Gly.

Although IgG antibodies are known to be bivalent, binding of a large antigen molecule at one site may impose steric interference to the binding of another antigen molecule at the second site. Studies by Sachs et al. (1972) using antibody prepared against staphylococcal nuclease (M_r 17 000) indicate that such interference may be possible in enzyme–antibody interaction. Our sedimentation analyses (Figure 4) suggest that a single antibody molecule can bind two molecules of converting enzyme, a much larger protein (M_r 129 000). We found that 2.73 molecules of antiholoenzyme antibody were required to completely inhibit one molecule of enzyme as extrapolated from the linear portion of an inhibition dose–response curve. If a single inhibitory antibody molecule inhibits two molecules of enzyme, then the fraction of inhibitory molecules in the antiholoenzyme antibody population is 0.5/2.72 or 18%. This high value may indicate that the active site and other sequence(s) of rabbit converting enzyme which influence catalysis are unusually antigenic because they are exposed on the outside of the glycoprotein, or because they have less homology with the goat enzyme than other parts of the molecule.

Ryan et al. (1975) have used antibody conjugated with microperoxidase to establish that pulmonary angiotensin-converting enzyme is located on the luminal aspect of vascular endothelial cells in direct apposition to the circulation. Our previous studies (Caldwell et al., 1976a) with fluorescein-labeled antibody prepared against the lung glycoprotein have indicated that extrapulmonary enzyme is also largely localized in vascular endothelial cells. These results suggest that most converting enzyme is directly accessible to the blood and, therefore, to circulating antibody. Our present studies establish that most extrapulmonary enzyme is susceptible to inhibition by antibody against the pulmonary enzyme and is immunologically indistinguishable from the lung glycoprotein. This is of special interest in the case of the kidney since it suggests that enzyme known to occur in parenchymal brush border cells (Ward et al., 1975; Caldwell et al., 1976a) is similar to that in vascular endothelium. The exception to immunological identity which we have found is the seminal plasma enzyme. A maturation-dependent increase in testicular fluid converting activity was first described by Cushman and Cheung (1972). The fact that seminal plasma activity is much greater than expected from its content of competing antigen suggests that certain antigenic sites present in the lung enzyme are absent, different, or masked in that of the seminal plasma. These sites probably do not influence catalysis since seminal plasma activity, like all others examined in this work, is inhibited by antibody identically with that in the lung.

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Synthesis, Structure Determination, Spectral Properties, and Energy-Linked Spectral Responses of the Extrinsic Probe Oxonol V in Membranes†

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ABSTRACT: The direct synthesis of bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol, which is shown to be the fluorescent probe OX-V (formerly MC-V), is described. The emission lifetime (0.9 ± 0.1 ns) and the spectral properties of this dye in a number of systems are presented as well as the relative polarizations associated with the transition moments of the observable electronic transitions. The structure of OX-V was determined using elemental analysis and infrared and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The use of the contact shift reagent, Eu(fod)$_3$-d_{27}, greatly facilitated the interpretation of the NMR results. In aqueous media, the anionic form of OX-V is present virtually exclusively due to the low solubility of the neutral species; formation of the latter species occurs when ethanol or methanol solutions of OX-V are acidiied. Both neutral and anionic dye forms can be detected in chloroform–ethanol solvents. The fluorescence intensity from excitation of the neutral species is an order of magnitude weaker than that from excitation of the anionic form and may result from the formation of excited anions due to the loss of a proton by the neutral species in the excited state. Polarization results indicate that the visible absorption of the dye is due to a single electronic transition. OX-V has been employed as a probe primarily in beef heart submitochondrial particles, reconstituted ATPase vesicles, and pigeon heart mitochondria. The energy-linked spectral changes of the probe in these preparations are described and mechanisms proposed for the spectral effects.

Dyes of the merocyanine and related classes have proved to be useful as probes of membrane-related phenomena. Cohen et al. (1974) have evaluated a large number of dyes as probes of changes associated with the action potential in squid giant axons. The merocyanine MC-I (also known as M-540) proved to be among the most sensitive probes of membrane changes occurring during the action potential. Ross et al. (1974) have also demonstrated that a large change in the absorption spectrum of MC-I occurs during the action potential in giant axons. Tasaki et al. (1976) have recently studied crab nerve membranes labeled with several fluorescent probes including M-540. The dyes MC-I, MC-II, and OX-V[1] (formerly MC-V) have also been used extensively as probes (Chance, 1975). A red shift in the absorption spectrum of these dyes is observed when submitochondrial particles stained with these dyes are energized. In particular, the dye OX-V has been given extensive use. Changes in the absorption and fluorescence spectrum of this dye have been demonstrated when *R. rubrum* chromatophores are energized with 860-nm light (Chance and Baltscheffsky, 1975). Cohen et al. (see Chance, 1975) have also demonstrated that the fluorescence of OX-V undergoes changes similar to those observed with MC-I in squid axons, but the former dye is not as sensitive in this system as the latter

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[1] Abbreviations used: NMR, nuclear magnetic resonance; OX-V, (formerly MC-V), bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol; Eu(fod)$_3$-d_{27}, tris(heptofluorodimethyloctanedionato)-d_{27}-europium; TLC, thin-layer chromatography; ir, infrared; uv, ultraviolet; SMP, submitochondrial particles; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; PHM, pigeon heart mitochondria; Mopes, 3-(*N*-morpholino)propanesulfonic acid.