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Characterization of Angiotensin Converting Enzyme by [^{3}H]Captopril Binding

STEPHEN M. STRITTMATTER and SOLOMON H. SNYDER

Departments of Neuroscience, Pharmacology and Experimental Therapeutics, and Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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SUMMARY

We demonstrate that [^{3}H]captopril selectively labels angiotensin converting enzyme (EC 3.14.15.1) (ACE) and employ this technique to probe enzyme-inhibitor interactions. [^{3}H]Captopril binding sites copurify with ACE activity from rat lung or rat brain. At each stage of the purification the V_{max}/B_{max} ratio, or k_{cat} is 17,000 min^{-1} with hippuryl-L-histidyl-L-leucine as substrate. The specificity of [^{3}H]captopril binding is apparent in the similar pharmacologic profile of inhibition in crude and pure enzyme preparations. Furthermore, binding sites and enzyme activity comigrate in gel filtration and sucrose gradient sedimentation experiments. Equilibrium analysis of [^{3}H]captopril binding to purified ACE reveals a B_{max} of 6 nmol/mg of protein (K_D = 2 nM), demonstrating the presence of one inhibitor binding site per polypeptide chain. The kinetics of [^{3}H]captopril binding are characterized by monophasic association and dissociation rate constants of 0.026 nM^{-1} min^{-1} and 0.034 min^{-1}, respectively. The affinity of ACE for both [^{3}H]captopril and enalaprilat is greater at 37° than at 0°, demonstrating that these interactions are entropically driven, perhaps by an isomerization of the enzyme molecule. The ionic requirements for [^{3}H]captopril binding and substrate catalysis differ. Chloride and bromide ion, but not fluoride, are about 100-fold more potent stimulators of binding than catalysis. When the active site Zn^{2+} ion is replaced by Co^{2+}, catalysis was stimulated 2-fold, whereas binding activity was decreased by 70%.

ACE (EC 3.1.4.15.1) is present on the luminal surface of endothelial cells, especially in the lung, where the dipeptidyl-carboxypeptidase is responsible for the conversion of circulating angiotensin I to the hypertensive peptide angiotensin II (1, 2). The enzyme also degrades the hypotensive peptide bradykinin. Captopril, a potent and selective ACE inhibitor, is used clinically to lower blood pressure on the basis of these physiological roles for ACE (2, 3).

High levels of ACE are also found in specific regions of the brain and the male reproductive system (4, 5). Neuronal ACE is present in a striatonigral pathway where angiotensin and bradykinin are not found (6–9). We recently demonstrated that the neuronal enzyme is a distinct isozyme of ACE with unique catalytic specificity for substance P and substance K, which are amidated striatal peptides and may serve as endogenous substrates for this isozyme (10, 11). The testicular ACE is also distinguishable from endothelial ACE found in the lung, and it has an anatomical distribution suggesting a substrate other than angiotensin or bradykinin (12–14).

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We have recently described the binding of the radioactive ACE inhibitor, [^{3}H]captopril, to crude tissue preparations with a pharmacologic profile and tissue distribution strongly suggesting that the compound labels ACE (15). To confirm this suggestion, we have here monitored [^{3}H]captopril binding during the purification of ACE catalytic activity to homogeneity. We have also conducted further studies of the mechanism of inhibitor-enzyme interaction.

Materials and Methods

[Prolyl-3,4-^{3}H]*S*-acetylcapropril, 48 Ci/mmol, was supplied by Dr. S. Hurt, New England Nuclear, and converted to [^{3}H]captopril by treatment with 0.1 M NaOH as described (15), but was neutralized with phosphoric acid in place of HCl. Unlabeled *S*-acetylcaptopril was also obtained from New England Nuclear. Hip-His-Leu was purchased from Sigma. Frozen rat brain and lung were obtained from Pel Freeze. MK-422 and MK-522 were kindly provided by Dr. A. Patchett of Merck, Sharp and Dohme Research Laboratories. The source of other reagents is as described previously (12).

[^{3}H]Captopril binding assay. The filtration assay for membrane-bound ACE has been described previously (15); it involves incubation of sample and [^{3}H]captopril (2 nM) at 4° for 1 hr in 50 mM Tris·HCl, pH 7.9, 100 mM NaCl prior to filtration. The assay for soluble and

ABBREVIATIONS: ACE, angiotensin converting enzyme; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; MK-422, enalaprilat, *N*-(1(S)-carboxy-3-phenyl-propyl)-L-alanyl-L-proline; MK-522, *N*-(1(S)-carboxy-3-phenyl-propyl)-L-lysyl-L-proline; EDTA, ethylenediaminetetraacetate; BPP_{9a}, < Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro; BPP_{5a}, < Glu-Lys-Trp-Ala-Pro; MGTA, 2-mercaptomethyl-3-guanidinothiopropionic acid.

purified binding sites is identical except that polyethylenimine-treated filters are employed (16). For purified ACE, 5 ng of protein was employed. Specific [^{3}H]captopril binding is the difference between total binding and that obtained in the presence of 1 μM unlabeled captopril. Scatchard analysis of saturation data with [^{3}H]captopril concentrations varying by factors of 2 from 0.5–32 nM yielded B_{max} and K_D values. Various concentrations of inhibitors were included in the 1-hr incubation to determine their potency.

Hip-His-Leu assay. ACE was assayed by a modification (12) of the method of Yang and Neff (5). After incubation of enzyme and substrate at 37° in 2 mM Hip-His-Leu, 50 mM Tris·HCl, pH 7.6, 250 mM NaCl, the His-Leu product was detected by condensation with *o*-phthaldialdehyde.

ACE purification. ACE was purified from frozen rat lung and rat brain corpus striatum as described in detail (10). The method involves homogenization, membrane isolation, detergent solubilization, concanavalin A-Sepharose chromatography, and affinity chromatography on an MK-522 affinity resin.

Gel filtration of purified ACE. One ml of affinity purified ACE was applied to a 50-ml Agarose A1.5M column equilibrated with 50 mM Tris, pH 7.9, 300 mM NaCl, 4°, and eluted at a flow rate of 5 ml/hr. The elution positions of catalase (bovine liver), human serum albumin, and cytochrome *c* (bovine liver) were detected by protein determination. The position of blue dextran was monitored by absorbance at wavelength 700 nm.

Sucrose gradient centrifugation of purified ACE. One-half ml of purified ACE was applied to a 25-ml linear gradient of sucrose, 10–40% in 25 mM Tris, pH 7.9, 4°. The tubes were centrifuged for 7 hr at 58,000 rpm in a Beckman 60Ti rotor (239,000 × g_{av}). Fractions were collected from the bottom of the gradient. Standards were monitored by protein determination.

Anionic regulation. Purified ACE was dialyzed for 3 days at 4° with six changes of 25 mM sodium phosphate, pH 7.5. The dialyzed sample was assayed as described above but with phosphate as the assay buffer. Anions of interest were added as their sodium salts to the incubation buffer.

Cation dependence. EDTA (1 mM) was added to purified ACE and the preparation was dialyzed for 3 days at 4° with six changes of 25 mM sodium phosphate, pH 7.5, 5 μM EDTA. The dialyzed material was assayed after adding the chloride salts of divalent cations in the standard assay buffer.

Thermodynamic measurements. Saturation analysis of [^{3}H]captopril binding to purified ACE was performed with incubations at 0°, 10°, 23°, and 37° for 1 hr. Inhibition of the binding of 2 nM [^{3}H]captopril by MK-422, 0.5–256 nM, was also examined at each temperature. The K_D value for [^{3}H]captopril binding at a particular temperature was used to calculate the K_i of MK-422 from the concentration of MK-422 required to achieve 50% inhibition of [^{3}H]captopril binding. The pH of the Tris buffer was adjusted so that a pH of 7.9 was maintained during the incubation. Similar results were also obtained with a 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid buffer system (not shown).

Protein determination. Protein was assayed by the method of Lowry *et al.* (17) except for purified ACE, for which the fluorescamine method was employed (18). Bovine serum albumin was the protein standard.

Results

Specificity of [^{3}H]captopril binding. We have purified ACE from rat lung and corpus striatum to apparent homogeneity by concanavalin A chromatography and affinity chromatography utilizing MK-522, an inhibitor of ACE. The purity of the final preparation has been demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in another publication (10). [^{3}H]Captopril binding to detergent-solubilized ACE preparations could be detected by filtration over filters pretreated with polyethylenimine which retains protein by ionic interactions (16). The ratio of specific to nonspecific binding of 2 nM [^{3}H]captopril in this assay is about 10:1 for soluble binding sites (Fig. 1A) and for membrane-associated sites (15). The Scatchard plot for [^{3}H]captopril binding to purified ACE demonstrates one class of binding sites with a K_D of 2 nM (Fig. 1B).

The ratio of the maximal catalytic (V_{max}) to binding (B_{max}) activity is essentially identical at each stage of the purification (Table 1). Thus, all of the binding sites in crude homogenates can be accounted for by the amount of ACE present in those preparations. This V_{max}/B_{max} ratio is equal to the turnover number if one [^{3}H]captopril molecule binds to one active site. The B_{max} of the purified preparations (6 nmol/mg of protein; Fig. 1, Table 1) is identical to that predicted for one [^{3}H]captopril binding per purified ACE protein of M_r = 170,000.

To demonstrate further the specificity of [^{3}H]captopril for ACE, we evaluated the effect of a series of inhibitors on [^{3}H]captopril binding and catalytic activity of particulate enzyme, detergent-solubilized enzyme, and purified enzyme. Throughout the purification procedure the potencies of nearly all of these inhibitors are the same for brain and lung whether assayed by [^{3}H]captopril binding or Hip-His-Leu degradation (Table 2, Fig. 2). The potencies agree with reports for other

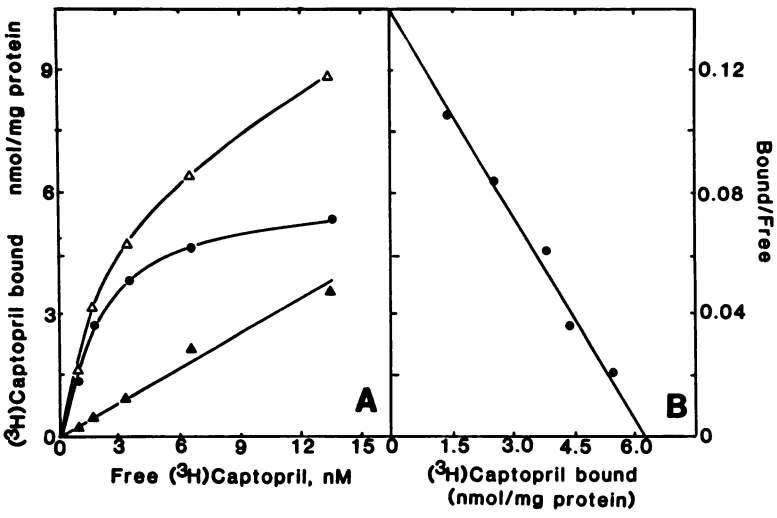

Fig. 1. Saturation of specific [^{3}H]captopril binding to purified ACE. A. Binding of increasing concentrations of [^{3}H]captopril to purified rat lung ACE. The binding of the indicated concentrations of [^{3}H]captopril to 5 ng of purified ACE was determined as in Materials and Methods. Nonradioactive captopril at 1 μM was employed to assess nonspecific binding. Specific binding (●) is the difference between total (△) and nonspecific binding (▲). Data represent a typical experiment which was replicated four times. B. Scatchard plot of saturation of specific [^{3}H]captopril binding. Specific binding for Fig. 1A is replotted. Similar experiments with purified striatal ACE yielded a K_D of 4 nM and a B_{max} of 6 nmol/mg of protein.

TABLE 1
Copurification of [^{3}H]captopril binding and ACE catalytic activity
ACE was purified from 25 g of rat lung and 25 g of corpus striatum as in Materials and Methods. The values below represent the total activity at each step of the purification. The numbers in parentheses are the specific activities (total activity/mg of protein). The purification was repeated five times with results varying less than 20%; the data are from a represenative experiment.

Preparation	[^{3}H]Captopril B_{max}	Hip-His-Leu V_{max}	V_{max}/B_{max} (1000 min^{-1})
	pmol	*μmol of His-Leu/min*	
Lung			
Homogenate	5700 (1.0)	81 (0.015)	14
Particulate	4200 (2.6)	79 (0.049)	19
Detergent soluble	4200 (6.7)	68 (0.11)	16
Concanavalin A	2600 (37)	45 (0.64)	17
Affinity	1200 (6000)	23 (115)	19
Corpus striatum			
Homogenate	1500 (0.21)	25 (0.0036)	17
Particulate	1400 (0.75)	25 (0.013)	17
Detergent soluble	1000 (0.60)	18 (0.010)	17
Concanavalin A	410 (2.7)	5.0 (0.033)	12
Affinity	120 (6000)	2.3 (115)	19

enzyme preparations (1, 2, 3, 15, 19), except for *S*-acetylcaptopril, which is substantially more potent in particulate preparations of brain than in lung or than in purified preparations of brain and lung ACE. Brain membranes can hydrolyze *S*-acetylcaptopril to the more potent captopril.[1]

Molecular sieving of purified ACE on an Agarose A1.5M column provides sharp peaks of [^{3}H]captopril binding and Hip-His-Leu degradation which are superimposable and close to the elution position of catalase (Fig. 3A). Sucrose gradient sedimentation also affords a sharp and superimposable elution of [^{3}H]captopril binding and Hip-His-Leu hydrolysis, with an elution position further from the bottom of the tube than catalase (Fig. 3B). The coelution of [^{3}H]captopril binding and Hip-His-Leu hydrolysis again demonstrates that [^{3}H]captopril labels ACE. The Stokes radii and sedimentation coefficients for ACE catalytic activity are reported elsewhere (10).

Kinetics of [^{3}H]captopril binding. The association and dissociation of [^{3}H]captopril to ACE are monoexponential func-

[1] S. Strittmatter, unpublished observation.

TABLE 2
Effects of ACE inhibitors on [^{3}H]captopril binding and ACE catalytic activity
ACE preparations from the indicated steps in purification of brain and lung enzyme were assayed for [^{3}H]captopril binding and Hip-His-Leu degradation with inhibitor concentrations which varied by factors of 2 from at least 10-fold above the reported K_i to 10-fold below. The concentration of inhibitor to produce 50% inhibition was determined graphically and converted to a K_i assuming competitive inhibition, a K_D for [^{3}H]captopril of 4 nM, and a K_m for Hip-His-Leu of 2 mM. The values are the average of two to five determinations which varied less than 30%. Examples are shown in Fig. 2. The K_D for [^{3}H]captopril was determined as described in Materials and Methods. EDTA was examined at only one concentration and the percentage of inhibition observed is reported.

Drug	[^{3}H]Captopril binding						Hip-His-Leu degradation					
	Particulate		Detergent soluble		Purified		Particulate		Detergent soluble		Purified	
	Lung	Brain	Lung	Brain	Lung	Brain	Lung	Brain	Lung	Brain	Lung	Brain
K_i (nM)												
Captopril	2	3	3	3	4	4	5	3	4	4	3	4
S-Acetylcaptopril	700	30	600	200	3,000	3,000	1,200	300	1,000	600	1,000	1,300
BPP_{5a}	700	700	1,000	700	1,000	1,000	26	27	130	28	200	150
BPP_{9a}	250	220	230	300	280	300	18	21	12	20	9	8
MK-422	7	11	5	5	25	20	0.3	0.5	0.4	0.5	0.5	0.4
MK-522	10	15	8	6	30	19	0.3	0.4	0.4	0.3	0.3	0.3
Thiorphan	40,000	40,000	40,000	40,000	30,000	40,000	4,000	5,000	3,000	3,000	3,000	3,000
K_D (nM)												
[^{3}H]Captopril	3	2	4	3	4	4						
Percentage of inhibition												
EDTA (1 nM)	100	100	100	100	100	100	100	100	100	100	100	100

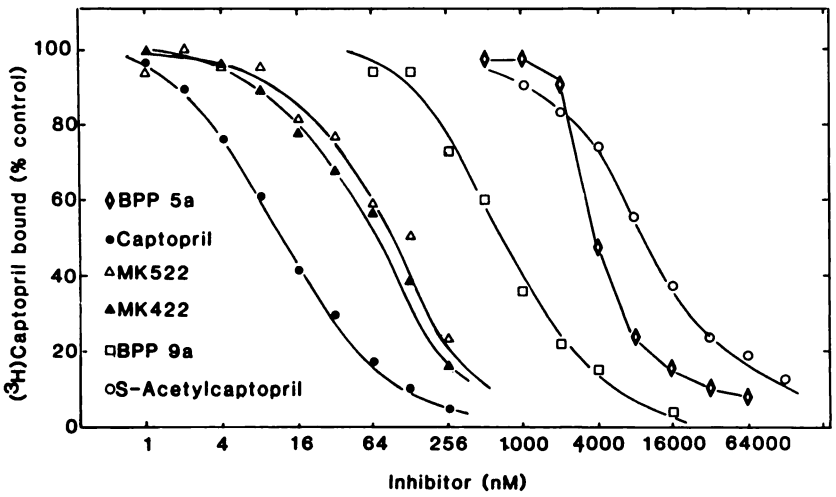

Fig. 2. Inhibition of [^{3}H]captopril binding to purified rat lung ACE. The binding of 2 nM [^{3}H]captopril to 5 ng of purified rat lung ACE was determined in the presence of the indicated concentration of several inhibitors. The data are expressed as a percentage of the value obtained with no inhibitor present. Similar curves were obtained for all of the fractions listed in Table 2.

tions of time (Fig. 4). The ratio of the dissociation constant to the association constant, 0.034 min^{-1}/0.026 nM^{-1} min^{-1} equals 1.3 nM, which is close to the K_D determined in equilibrium studies (Table 2). The monophasic behavior is that predicted for a one-step association of [^{3}H]captopril with a single set of noninteracting binding sites.

Influence of ions on ACE. ACE catalytic activity is dependent upon chloride ion (1, 2, 20), and recent studies show that inhibition of catalytic activity also requires chloride (21, 22). However, the concentration dependence and ion selectivity of inhibitor binding have not been described. We observe differences in the anion dependence of [^{3}H]captopril binding and Hip-His-Leu degradation in both the concentration of anion required for stimulation and the maximal level of stimulation. The responses of lung and brain ACE to different anions are identical. Both [^{3}H]captopril binding and enzymatic activity are negligible with sodium phosphate (Fig. 5). Hip-His-Leu hydrolysis is half-maximal at 80 mM NaCl and plateaus at 300–500 mM NaCl. [^{3}H]Captopril binding is stimulated by much lower levels of NaCl with half-maximal stimulation (EC_{50}) at 0.5 mM NaCl. We wondered whether the varying NaCl sensitivity reflects temperature differences between the two assays. When [^{3}H]captopril binding is assayed at 37°, the EC_{50} for chloride is still 0.5–1.0 mM (data not shown). Bromide ions are also substantially more potent stimulators of [^{3}H]captopril binding than of Hip-His-Leu degradation (Table 3). However, fluoride stimulates binding of [^{3}H]captopril slightly less potently than enzymatic activity.

There are differences in the maximal effects of anions on catalytic activity and [^{3}H]captopril binding. Thus, maximal enhancement of [^{3}H]captopril binding by bromide is almost as great as that by chloride. Conversely, bromide provides less than one-half as much stimulation of catalytic activity as chloride. The stimulatory efficacy of fluoride is nearly equal for

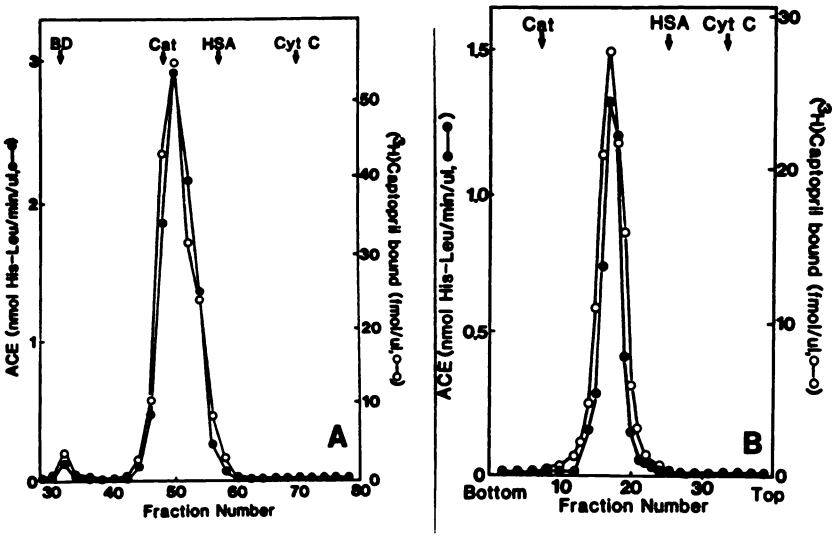

Fig. 3. Molecular properties of ACE. A. Gel filtration of lung ACE was performed as described in Materials and Methods and the fractions were assayed for [^{3}H]captopril binding and Hip-His-Leu degradation. Similar profiles were obtained for brain ACE. The markers are from separate chromatography on the same column: *BD*, blue dextran; *Cat*, catalase; *HSA*, human serum albumin; and *Cyt C*, cytochrome *c*. B. Sucrose gradient fractionation by lung ACE was performed as described in Materials and Methods and fractions were analyzed as indicated. Similar profiles were obtained for brain ACE. The markers are from a separate gradient centrifuged simultaneously.

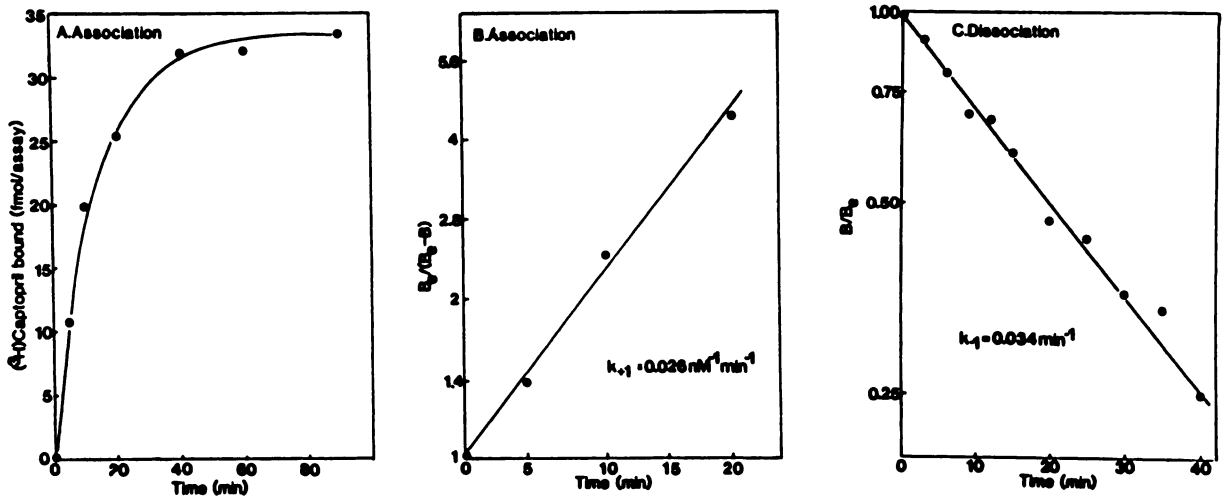

Fig. 4. Kinetics of [^{3}H]captopril binding to brain membranes. A. Association of [^{3}H]captopril to rat brain homogenates. Specific binding of 1 nM [^{3}H]captopril was determined after incubation at 4° for the indicated times. B. Association of [^{3}H]captopril to brain membranes. Data from A are replotted. [^{3}H]Captopril bound after 60 min divided by the quantity, binding at 60 min minus binding at the indicated time, is plotted as a function of time. The association constant (K_{+1}) is calculated from the equation $k_{+1} = (K_{obs} - k_{-1})/[^3H]$captopril. C. Dissociation of [^{3}H]captopril from rat brain membranes. Membrane fractions were incubated with 2 nM [^{3}H]captopril for 1 hr. The amount of specific binding remaining was determined at the indicated times after the addition of 5000-fold molar excess of unlabeled captopril (10 μM) over [^{3}H]captopril. The ratio of [^{3}H]captopril bound at the indicated time divided by binding measured without the addition of unlabeled captopril is plotted as a function of time. Data are from one of four experiments with similar results performed for brain and lung membrane fractions and for purified brain and lung ACE preparations.

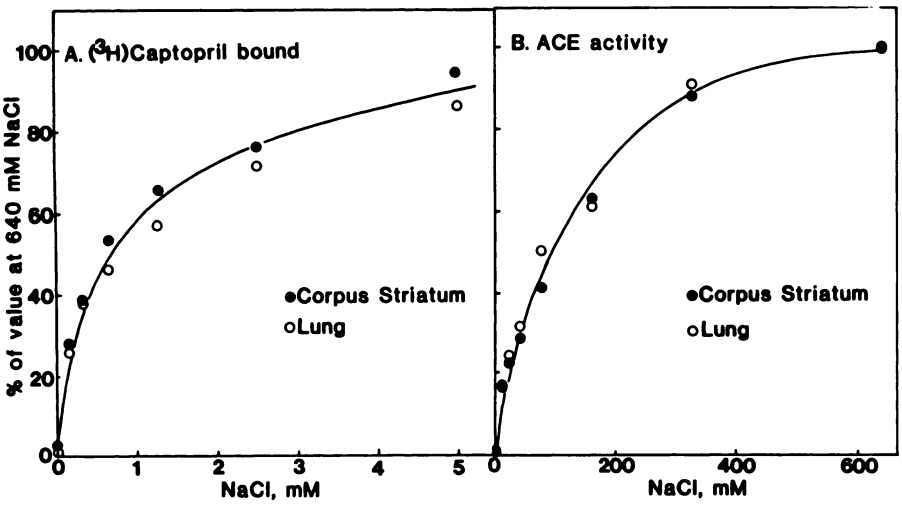

Fig. 5. Chloride dependence of ACE. Purified ACE from brain and lung was dialyzed to remove endogenous chloride and then assayed in the presence of the indicated amounts of NaCl for [^{3}H]captopril binding and Hip-His-Leu degradation. Data are from a typical experiment, replicated three times.

TABLE 3
Anion dependence of [^{3}H]captopril binding and ACE catalytic activity
The data are the means of three experiments of the type shown in Fig. 5 with the indicated anions. The concentration required to achieve 50% of the maximal ACE activity is reported (EC_{50}) with the maximal level achieved by that anion. Iodide, acetate, and perchlorate were not tested above 800 mM.

	[^{3}H]Captopril binding				Hip-His-Leu degradation			
	EC_{50}		Maximal activity		EC_{50}		Maximal activity	
	Lung	Brain	Lung	Brain	Lung	Brain	Lung	Brain
	mM		%		mM		%	
NaCl	0.5	0.6	100	100	75	85	100	100
NaBr	0.4	0.8	85	85	40	25	45	45
NaF	500	400	75	60	300	350	50	45
NaI (800 mM)			<5	<5			<1	<1
Sodium acetate (800 mM)			<5	<5			<1	<1
Sodium perchlorate (800 mM)			<5	<5			<1	<1

the two assays. Iodide, acetate, and perchlorate all fail to stimulate [^{3}H]captopril binding or Hip-His-Leu hydrolysis. Since all ions were examined as their sodium salt, the stimulatory effects of sodium chloride, bromide, and fluoride are clearly related to the anion.

Pure ACE has one zinc ion per mole of enzyme (1, 2). To compare other cations with zinc, we dialyzed ACE purified from brain and lung with EDTA to remove zinc and then examined the effects of added cations on [^{3}H]captopril binding and catalytic activity (Table 4). No major differences are apparent in the effects of divalent cations on lung and brain ACE. Manganese is as effective as zinc in restoring both [^{3}H]captopril binding and enzymatic activity. Other cations markedly differentiate the two ACE parameters. For instance, cobalt maximally increases Hip-His-Leu degradation 2-fold greater than zinc, whereas it is only 25–30% as effective as zinc in stimulating [^{3}H]captopril binding. Copper is 20–25% as active as zinc in augmenting catalytic activity but completely inactive in stimulating [^{3}H]captopril binding. Magnesium, strontium, barium, and nickel do not restore either [^{3}H]captopril binding or Hip-His-Leu degradation.

Thermodynamics of inhibitor binding to ACE. To determine thermodynamic constants, we measured the equilibrium binding of [^{3}H]captopril to lung and brain ACE and the inhibition of [^{3}H]captopril binding by the inhibitor MK-422 as functions of temperature (Fig. 6). As temperature is increased from 0° to 37°, the affinities of captopril and MK-422 for ACE increase similarly for brain and lung ACE, except that at all temperatures MK-422 is slightly more potent against the brain

TABLE 4
Divalent cation regulation of ACE
The effect of cations on ACE catalytic activity and [^{3}H]captopril binding was determined after dialysis of lung and brain enzyme with EDTA as described in Materials and Methods. Each compound was tested at concentrations that varied by a factor of 4 from 1 μM to 4 mM. The results are the means of two to four determinations which varied by less than 20%.

Compound	Maximal activity as % of $ZnCl_2$ level			
	[^{3}H]Captopril binding		Hip-His-Leu-degradation	
	Lung	Brain	Lung	Brain
None	3	1	1	1
$ZnCl_2$	100	100	100	100
$MnCl_2$	106	93	98	114
$CoCl_2$	25	30	191	196
$CuCl_2$	3	3	21	23
$MgCl_2$ (3 mM)	3	3	1	1
$SrCl_2$ (3 nM)	6	5	1	1
$BaCl_2$ (3 mM)	3	5	1	1
$NiCl_2$ (3 nM)	8	6	1	1

enzyme. Between 0° and 37° the K_D of [^{3}H]captopril for ACE decreases about 3-fold from 4.5 to 1.5 nM, whereas the K_i of MK-422 for inhibition of [^{3}H]captopril binding decreases nearly 15-fold from 20–25 to 1.5–1.8 nM. No changes in the B_{max} for [^{3}H]captopril binding occur at different temperatures (not shown). The van't Hoff plots for these relationships are linear, permitting a calculation of ΔH° and ΔS°. The ΔH° values for captopril and MK-422 interactions with ACE are positive and between 4 and 12 kcal/mol (Table 5). The −TΔS°

TABLE 5
Thermodynamics of ACE-inhibitor interaction
Thermodynamic parameters for ACE were calculated from Fig. 6, using the relationship: $\ln(K_A) = \left(\frac{-\Delta H}{RT}\right) + \frac{\Delta S}{R}$

Enzyme source	Ligand	ΔH°	ΔS°	−TΔS° (37°)
		kcal/mol	cal/mol · °k	kcal/mol
Lung	Captopril	+4.6	+55	−17.1
Brain	Captopril	+4.6	+55	−17.1
Lung	MK-422	+11.9	+81	−25.0
Brain	MK-522	+12.2	+83	−25.6

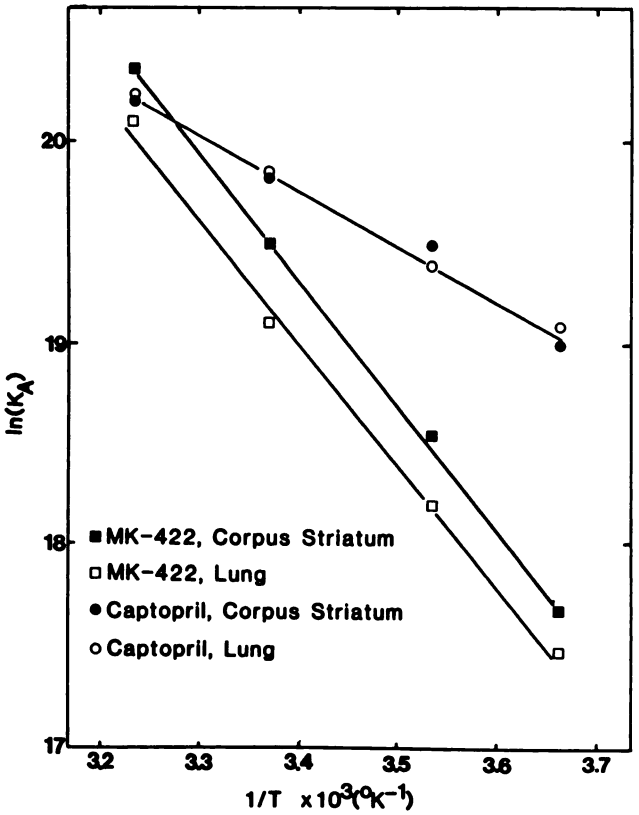

Fig. 6. v'ant Hoff plot for [³H]captopril and MK-422 binding to ACE. The K_D for [³H]captopril binding and the K_i for MK-422 against [³H]captopril binding were determined as described in Materials and Methods at 0°, 10°, 23°, and 37°. The data are the average of two to three determinations which varied by less than 20%.

values for captopril and Mk-422 binding to ACE are about 17–25 kcal/mol at 37°.

Discussion

In the present study we demonstrate the specificity of a [³H]captopril filter binding assay and then employ this technique to study enzyme-inhibitor interaction. The copurification, the comigration, and the pharmacology of [³H]captopril binding sites and ACE activity unambiguously define the specificity of the binding assay. These studies also demonstrate the existence of one [³H]captopril site per enzyme molecule, just as there is one zinc per enzyme molecule (1, 2).

The high affinity binding of [³H]captopril has permitted thermodynamic evaluation of inhibitor-enzyme interaction. In contrast, such evaluation for most substrate-enzyme interactions is quite difficult because detecting the binding event is often complex, and catalytic decomposition of the substrate interferes with detection of binding. We find that binding of both captopril and MK-422 to ACE is entropically driven at 37°; enthalpy changes are unfavorable. This suggests that there is a conformational change in ACE associated with inhibitor binding or perhaps that tightly bound water is released from the enzyme. The lower K_i of BPP_{5a} and BPP_{9a} in the enzymatic assay at 37° as compared to the [³H]captopril binding assay at 4° (Table 2) suggests that the interaction of these compounds with ACE may also be entropically driven.

Recent studies examining the onset of inhibition of ACE by enalapril, lisinopril, and captopril have suggested a "minimally" two-step interaction of inhibitor with enzyme (21, 22). The first step is a fast, low affinity association of inhibitor and enzyme followed by a slower conformational change of the enzyme to a high affinity form. Our demonstration of a favorable entropy change associated with inhibitor binding provides a separate piece of evidence for such a conformational change. The two steps were inferred (21, 22) by observing the association of inhibitor with enzyme at inhibitor concentrations well above the inhibitor K_i. The filtration step in our assay requires 30 sec to 1 min and thus prevents the accurate measurement of the rapid association rates of inhibitor with enzyme at high inhibitor concentrations. Therefore, our data do not distinguish between a mechanism whereby the conformational change occurs concomitant with inhibitor binding and one in which it occurs after inhibitor has bound to enzyme.

[³H]Captopril binding demonstrates an anion requirement for inhibitor-ACE association different from the anion dependence of ACE catalysis. Chloride and bromide, but not fluoride, are nearly 100-fold more potent in augmenting [³H]captopril binding than Hip-His-Leu hydrolysis. Effects of chloride on ACE catalysis may vary with the substrate employed. In an extensive evaluation of anion effects on a large number of rabbit lung ACE substrates, Shapiro *et al.* (20) identified some substrates whose degradation was stimulated half-maximally with as little as 3–5 mM NaCl (still above the levels required for [³H]captopril binding). Unlike captopril, these substrates all had positively charged amino acids in the ultimate or penultimate position. For substrates whose chemical properties more closely resemble those of captopril, by terminating in Ala-Pro, about 20 mM NaCl was required for half-maximal stimulation of enzymatic activity (20). Thus, the differential sensitivity to chloride of [³H]captopril binding and catalytic activity cannot be attributed simply to our use of Hip-His-Leu. Moreover, there are differences in anion selectivity for [³H]captopril binding and catalytic activity, since bromide is almost as effective as chloride in augmenting [³H]captopril binding but only one-half as active in stimulating Hip-His-Leu degradation. These findings can be explained in several ways. Conceivably, distinct anion sites exist for recognition of inhibitor and either the recognition or hydrolysis of substrates. Alternatively, our observations may reflect a different conformational state of the enzyme when it interacts with inhibitor as contrasted to substrate. The evidence for an enzyme conformation change upon inhibitor binding makes the latter possibility more likely.

Divalent cations also affect [³H]captopril binding and catalytic activity differently. For instance, with cobalt, Hip-His-Leu degradation rate is 2-fold greater than with zinc, whereas cobalt is less than a third as effective as zinc in restoring [³H]captopril binding. These differential effects may be explained by comparison to similar studies of enkephalin convertase

(carboxypeptidase E) (23, 24). The carboxypeptidase B-like activity of enkephalin convertase is enhanced 10-fold by cobalt. Enkephalin convertase is inhibited with nanomolar potency by MGTA which, like captopril, possesses a sulfhydryl group that interacts with the zinc of the enzyme. When the zinc of enkephalin convertase is replaced by cobalt, the affinity of the enzyme for MGTA is decreased about 10-fold (23, 24). These results were interpreted as reflecting the lesser affinity of sulfhydryl than carboxyl groups for cobalt relative to zinc (24, 25). Similarly, although substrate catalysis is enhanced by cobalt, [^{3}H]captopril interacts less effectively with the cobalt-containing form of ACE.

Despite our previous findings of catalytic differences between the brain and lung ACE isozymes (10), we have detected no differences between the isozymes in the kinetics of [^{3}H]captopril binding, the thermodynamics of inhibitor binding, or the ionic regulation of ACC activity.

Characterization of an enzyme such as ACE by ligand binding has several applications. Ligand binding techniques can provide the number of enzyme molecules in crude homogenates, and the turnover number can be calculated when measurements of binding and catalysis are combined. This permits an assessment of changes in enzyme protein concentration and turnover number as a function of altered physiological, pathological, or pharmacologic status. Ligand binding also facilitates the microscopic localization of an enzyme, as has proved feasible with ACE in the brain (6) and the male reproductive system (12, 26).

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