Effect of temperature and chloride on steady-state inhibition of angiotensin I-converting enzyme by enalaprilat and ramiprilat

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The kinetics of the steady-state inhibition of angiotensin I-converting enzyme (EC 3.4.15.1) at 25 °C and 37 °C with enalaprilat and ramiprilat can be simulated, assuming only one inhibitor-binding site, consistent with a 1:1 stoichiometry if the protein concentration was determined by amino acid analysis. In this temperature range the apparent inhibition constants for ramiprilat and enalaprilat were roughly doubled by a decrease in the chloride concentration from 0.300 M to 0.120 M.

INTRODUCTION

Angiotensin I-converting enzyme (ACE, peptidyl-dipeptide hydrolase, EC 3.4.15.1) has a central role in the regulation of blood pressure [1]. This zinc exopeptidase catalyses the removal of the C-terminal dipeptide from the vasodilating nonapeptide bradykinin and the formation of the vasoconstricting octapeptide angiotensin II from the decapeptide angiotensin I.

Recently the genes for human kidney and mouse kidney ACE have been cloned and their complete amino acid sequences identified [2,3]. ACE consists of a single peptide chain, organized into two domains with considerable similarity of sequence [4]. In each of the domains there is a putative metal-ion-binding site with an almost identical amino acid sequence, indicating a possibility for two active sites. However, only one Zn^{2+} ion is bound per molecule of ACE [5,6], suggesting that ACE contains only one active site.

Synthetic ACE inhibitors have been developed that are currently used in the treatment of hypertension and congestive heart failure [7]. In specific inhibitors, such as enalaprilat and ramiprilat (Fig. 1), a carboxylate group probably co-ordinates with the Zn^{2+} ion [8]. In kinetic studies these two powerful inhibitors have been classified as reversible, competitive and slow tight-binding inhibitors [8,9].

In the present work we quantify the number of inhibitor-binding sites by performing enzyme kinetic titrations with pure rabbit lung ACE and enalaprilat or ramiprilat. We also re-evaluate the strength of the steady-state inhibition, comparing the generally used assay conditions (0.3 M-NaCl and 25 °C) with more physiological temperature and chloride concentrations (0.12 M-NaCl and 37 °C).

MATERIALS AND METHODS

Chemicals

BSA (radioimmunoassay grade) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-(2-Furylacryloyl)-L-Phe-Gly-Gly (Fa-FGG) was from either Bachem Feinkemikalien

A.G. (Bubendorff, Switzerland) or Sigma Chemical Co. Enalaprilat {N-[1(S)-carboxy-3-phenylpropyl]-L-Ala-L-Pro} was a gift from Merck, Sharp and Dohme (Rahway, NJ, U.S.A.) and ramiprilat (2-{N-[(S)-1-ethoxycarbonyl-3-phenylpropyl]-L-Ala}-(3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid) was a gift from Hoechst A.G. (Frankfurt, Germany). All chemicals were of analytical grade. Deionized and ultrafiltered water (Elgastat Spectrum, Lane End, Tring, Herts., U.K.) was used for all solutions. Buffer solutions were filtered again through a 0.45 μ m-pore-size ME 25 membrane (Schleicher und Schüll, Dassel, Germany) before use.

Enzyme

Pure rabbit lung ACE was a gift from Peter Bünning (Hoechst A.G.). ACE was purified essentially as previously described [10]. Two different enzyme batches were used. Batch I was found to be electrophoretically homogeneous by SDS/PAGE with Phast-System (Pharmacia-LKB Biotechnology, Lund, Sweden). Batch II contained 88 % ACE, obtained from staining with Coomassie Brilliant Blue and quantified with a PhastImage densitometer (Pharmacia-LKB Biotechnology). Concentrated ACE was stored in small portions in standard assay buffer containing 0.300 M-NaCl, and no BSA, at -70 °C.

Amino acid analysis was performed by Aminosyraanalys-centralen, University of Uppsala (Uppsala, Sweden), according to standard techniques [11]. Tryptophan and cysteine were not determined. The protein concentration was determined from amino acid analysis, by using M_r 147 000 [2,3] and from the concentrations of 16 individual amino acids deduced from the amino acid composition [3].

Enzyme assay

ACE activity was measured with the chromogenic synthetic substrate Fa-FGG at 50 μ M [12]. Hydrolysis was monitored at 328 nm with $\Delta\epsilon$ determined to be $-2.54 \times 10^3 \pm 0.02 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$, on a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH, U.S.A.) or an Ultrospec II 4050 spectrophotometer (LKB Biochrom, Cambridge, U.K.).

Abbreviations used: ACE, angiotensin I-converting enzyme (EC 3.4.15.1); [E]_t, total enzyme concentration; [E]_{stoen}, total inhibitor-binding-site concentration; Fa-FGG, 3-(2-furylacryloyl)-L-Phe-Gly-Gly.

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Fig. 1. Chemical structures of ACE inhibitors

The standard assay buffer contained 50.0 mM-Hepes, 1.0 μ M-BSA, 1.0 μ M-ZnCl₂ and either 0.120 M- or 0.300 M-NaCl, adjusted to pH 7.50 with NaOH.

The temperature of the assay was measured inside a reference cuvette with a thermocouple. The variation in the assay temperature was less than ± 0.3 °C.

Enzyme, in the presence or in the absence of inhibitor, was preincubated in the standard assay buffer for 16–24 h at 25 °C. Hydrolysis was initiated by the addition of 10–20 μ l of Fa-FGG solution to 0.8–1 ml of preincubated enzyme/inhibitor solution. For measurements at temperatures higher than 25 °C, enzyme and inhibitor were first incubated at 25 °C for 16–24 h and then warmed to the required temperature and incubated at that temperature for 15 min–2.5 h before assay.

The Fa-FGG concentration was calculated from initial absorbance with ϵ_{328} determined to be $1.082 \times 10^5 \pm 0.002 \times 10^5$ M⁻¹·cm⁻¹. The variation of the Fa-FGG concentration within each assay series had a relative s.d. of 0.7–2%.

Kinetic evaluation

The kinetic steady-state method for the study of slow tight-binding inhibitors is based on eqn. (1), which, with certain limitations, is valid for any steady-state 1:1 interaction of enzyme and inhibitor [13,14]:

$$v = k_{\text{obs}} \cdot 0.5 \{ [E]_{\text{stoich.}} - [I]_t - K_1^{\text{app}} + ([I]_t + K_1^{\text{app}} - [E]_{\text{stoich.}})^2 + 4[E]_{\text{stoich.}} \cdot K_1^{\text{app}} \}^{\frac{1}{2}} \quad (1)$$

$$v = k_{\text{obs}} \cdot ([E]_t - [I]_t) \quad (2)$$

Where $[E]_t$ represents the total enzyme concentration, $[E]_{\text{stoich.}}$ represents the total inhibitor-binding-site concentration, assuming only one inhibitor-binding site on ACE, $[I]_t$ represents the total inhibitor concentration, K_1^{app} is the apparent inhibition constant and k_{obs} is related to the uninhibited mechanism [13]. If a simple Michaelis-Menten mechanism is assumed, $k_{\text{obs}} = k_0 [\text{Fa-FGG}]/(K_m + [\text{Fa-FGG}])$. Eqn. (2) is the asymptote to eqn. (1) at large $[E]_t/[I]_t$ ratios.

Initial velocities (v) were measured from the first 15%, or less, of hydrolysis. Randomly sampled initial-rate values were used for parameter determination by weighted regression analysis using the Marquardt algorithm [15].

$[E]_{\text{stoich.}}$ can be determined from the semi-linear plots of initial rates against total enzyme concentration, $[E]_t$, with different $[I]_t$ values, and from plots of initial rates against $[I]_t$ with different $[E]_t$ values. The appearance of these plots is diagnostic for tight-binding inhibitors. In the plot of the initial rate versus $[I]_t$ the tangent to the curve at $[I]_t = 0$ results in an intersection point on the abscissa where $[I]_t$ is proportional to the sum of $[E]_{\text{stoich.}}$ and K_1^{app} . In the plot of the initial rate versus $[E]_t$ in the presence of a tight-binding inhibitor, the asymptote to the curve when $[E]_t > [I]_t$ has an intersection point on the abscissa where $[E]_t$ is equal to the added $[I]_t$, i.e. $[E]_t = [E]_{\text{stoich.}}$. Under these conditions, when $[E]_t > [I]_t$, eqn. (2) gives an estimation of the inhibitor-binding-site concentration independent of enzyme/inhibitor stoichiometry.

Outliers were defined as rates that deviate between calculated and observed values, by at least 2.5 times the experimental s.d., defined as (residual sum of least squares)/(number of observations – numbers of parameters) [16,17].

RESULTS

Enzyme stability

The enzyme activity in the absence of inhibitor was not significantly changed during the preincubation at 25 °C, as also observed by others [18]. It was confirmed that the concentrated enzyme was stable for months at 4 °C in a standard assay buffer containing 0.300 M-NaCl, and no BSA [19]. Preincubation of diluted enzyme at 37 °C in the standard assay buffer containing 0.120 M-NaCl for 16–24 h resulted in approx. 15% decrease in activity. However, preincubation at 25 °C followed by 2.5 h at 37 °C did not significantly change the activity.

Enzyme concentration

The amino acid composition of rabbit lung ACE from batches I and II (results not shown) was consistent with the amino acid composition deduced from cloned mouse kidney ACE [3]. The total protein concentration, according to the total (and in-

dividual) amino determination, was 3.2 ± 0.5 μ M in batch I and 2.7 ± 0.4 μ M in batch II. However, according to the u.v. absorbance at 280 nm, using ϵ_{280} 2.0×10^5 – 2.3×10^5 M⁻¹·cm⁻¹ [5,18,20], the protein concentration in batch I should be approx. 8 μ M (A_{280} 1.8) and in batch II approx. 6 μ M (A_{280} 1.3).

The number of inhibitor-binding sites ($[E]_{\text{stoich.}}$) in batch I was determined from a fit to eqn. (1) with different ramiprilat-ACE and ACE-ramiprilat titrations, including points with excess ACE and excess ramiprilat. With 64 points, and assuming 1:1 stoichiometry, we found $k_{\text{obs}} = 86 \pm 6$ s⁻¹, $K_1^{\text{app}} = 12 \pm 2$ pM and $[E]_{\text{stoich.}} = 3.6 \pm 0.2$ μ M (Table 1) in the standard assay buffer containing 0.300 M-NaCl at 25 °C. In the fit to eqn. (1) no clear residual trends were present between enzyme concentration or inhibitor concentration and the initial-rate residuals.

In enzyme batch II, the number of inhibitor-binding sites was determined by titration of several concentrations of inhibitor with excess enzyme (Table 1). The results from selected ramiprilat-ACE titrations, fitted to eqn. (1), are shown in Figs. 2(a) and 2(b). Titration of enalaprilat with ACE gave the same $[E]_{\text{stoich.}}$ as found with ramiprilat when eqn. (1) was used.

Temperature-dependence of substrate hydrolysis

Fig. 3 shows that the rate of Fa-FGG hydrolysis by ACE increases linearly in the narrow temperature range. The rate of hydrolysis was identical in the standard assay buffer containing either 0.120 M- or 0.300 M-NaCl in the temperature range 25–37 °C.

Table 1. Titration of ramiprilat with ACE

$[E]_{\text{stoich.}}$ was determined by a fit of initial velocities to eqns. (1) and (2). Variable amounts of excess ACE were preincubated with ramiprilat in the standard assay buffer containing 0.300 M-NaCl. Hydrolysis of 50 μM -Fa-FGG at 25 °C was monitored at 328 nm. $[E]_i$ was determined by amino acid analysis. The concentration ranges used were: $[E]_i$, 0.03–0.4 nM (batch I), and $[I]_i$, 0.0–0.8 nM, or $[E]_i$, 0.05–0.5 nM (batch II), and $[I]_i$, 0.0–0.3 nM. n indicates the number of observations.

ACE batch	n	$[E]_{\text{stoich.}}$ from eqn. (1) (μM)	$[E]_{\text{stoich.}}$ from eqn. (2) (μM)	$[E]_{\text{stoich.}}/[E]_i$ ratio
I	64	3.6 ± 0.2	—	1.1 ± 0.2
II	19	1.3 ± 0.2	1.59 ± 0.05	$0.7 \pm 0.2^*$

* $[E]_i$ is not corrected for 88 % purity in batch II.

Fig. 2. Titration of enalaprilat and ramiprilat with ACE

Substrate hydrolysis was initiated by the addition of Fa-FGG to a final concentration of 50 μM in the standard assay buffer containing 0.300 M-NaCl at 25 °C and ACE (batch II) preincubated with (a) enalaprilat and (b) ramiprilat: $\square$, 0.0 nM; $\blacksquare$, 0.1 nM; $\triangle$, 0.2 nM; $\blacktriangle$, 0.3 nM. The continuous lines are fits by regression analysis of all initial rates, v , to eqn. (1). The broken lines are the asymptotes to eqn. (1), at high enzyme concentrations, at the corresponding $[I]_i$ (eqn. 2). Other assay and preincubation conditions were as described in the Materials and methods section.

Inhibition constants

The values for $k_{\text{obs.}}$ and $K_i^{\text{app.}}$ determined from the fit to eqn. (1) are presented in Table 2 parts (a) and (b). For the calculation

Fig. 3. Effect of temperature on ACE activity

Substrate hydrolysis was initiated by the addition of Fa-FGG to a final concentration of 50 μM in the standard assay buffer containing 0.120 M-NaCl ($\diamond$) or 0.300 M-NaCl ($\blacklozenge$) with 0.14 nM-ACE (batch I). The continuous line is a fit of all initial rates, v , to a straight line by regression analysis. Other assay and preincubation conditions were as described in the Materials and methods section.

Table 2. Effects of chloride and temperature on the steady-state inhibition of ACE by tight-binding inhibitors

The parameters were calculated from a fit of initial rates to eqn. (1). Hydrolysis was initiated by adding a final concentration of 50 μM -Fa-FGG to preincubated enzyme and inhibitor. Other standard assay conditions as described in the Materials and methods section. The variation in the assay temperature at 25 °C and 37 °C was less than ± 0.3 °C, but at 34 °C approx. ± 0.5 °C. n indicates the number of observations. Part (a): initial rates were taken from a titration of ACE (batch I) with inhibitor, with the use in each series a fixed enzyme concentration (0.1–0.3 nM). The $[I]_i$ range used was 0.0–0.8 nM. Part (b): initial rates were taken from a titration of inhibitor with ACE. Several enzyme concentrations (batch II) were used for each series. Almost all points were sampled with excess enzyme relative to inhibitor. The concentration ranges used were $[E]_{\text{stoich.}}$, 0.03–0.4 nM, and $[I]_i$, 0.0–0.3 nM.

Inhibitor	[NaCl] (M)	Temperature (°C)	$k_{\text{obs.}}$ (s^{-1})	$K_i^{\text{app.}}$ (pM)	n
Part (a)					
Enalaprilat	0.120	25	126 ± 5	170 ± 20	14
	0.120	34	130 ± 7	74 ± 8	11
	0.120	37	126 ± 3	54 ± 5	11
	0.300	25	69 ± 6	70 ± 10	10
	0.300	34	175 ± 7	45 ± 3	11
	0.300	37	114 ± 7	44 ± 6	12
Ramiprilat	0.120	25	73 ± 2	20 ± 1	13
	0.120	34	102 ± 6	16 ± 3	9
	0.120	37	112 ± 4	11 ± 1	11
	0.300	25	86 ± 8	16 ± 1	11
	0.300	34	156 ± 1	8.7 ± 0.7	13
	0.300	37	107 ± 5	6 ± 1	12
Part (b)					
Enalaprilat	0.300	25	70 ± 3	60 ± 10	29
	0.300	37	131 ± 3	29 ± 3	33
Ramiprilat	0.300	25	81 ± 5	6 ± 2	19
	0.300	37	112 ± 4	7 ± 2	34

of $K_i^{\text{app.}}$ and $k_{\text{obs.}}$ we used $[E]_{\text{stoich.}} = 3.6 \mu\text{M}$ and $1.6 \mu\text{M}$ for enzyme batches I and II respectively. Similar results, for the same inhibitor, temperature and chloride concentration, were obtained if the enzyme was titrated with the inhibitors (Table 2 part a) or if the inhibitors were titrated with the enzyme (Table 2 part b). Ramiprilat has consistently about 5–10-fold higher affinity for

Fig. 4. Inhibition of ACE by enalaprilat and ramiprilat

Substrate hydrolysis was initiated by the addition of Fa-FGG to a final concentration of $50 \mu\text{M}$ in the standard assay buffer containing 0.120 M-NaCl ($\square$ and $\triangle$) or 0.300 M-NaCl ($\blacksquare$ and $\blacktriangle$) at 37°C with 0.15 nM-ACE (batch I) preincubated with enalaprilat ($\square$ and $\blacksquare$) and ramiprilat ($\triangle$ and $\blacktriangle$). The continuous lines are fits of the initial rates, v , to eqn. (1) by regression analysis. The initial rate at $[I]_i = 0$ was approx. 20 nM/s for all experimental series. Other assay and preincubation conditions were as described in the Materials and methods section.

ACE than has enalaprilat (Fig. 4 and Table 2 parts *a* and *b*). The values for K_i^{app} of both inhibitors decreased on the average to approximately half in the temperature range $25\text{--}37^\circ\text{C}$ (Table 2), compared with an increase in ACE activity by 2-fold (Fig. 3). In this temperature range K_i^{app} values for enalaprilat and ramiprilat were roughly doubled by a decrease in the chloride concentration from 0.300 M- to 0.120 M-NaCl (Table 2 part *a*).

DISCUSSION

The study of the stoichiometry in the binding of specific inhibitors to ACE is of interest because the internal similarity of sequence of the two domains in the enzyme protein suggests the presence of two possible binding sites [2,3]. Moreover, kinetics of the hydrolysis of a synthetic substrate indicate that ACE has both a high-affinity substrate-binding site and a low-affinity substrate-binding site [21]. However, the presence in ACE of only one Zn^{2+} ion seems to be sufficient for optimal activity [6]. We used the inhibition of ACE by the very closely related tight-binding inhibitors ramiprilat and enalaprilat to characterize further the number of inhibitor-binding sites under the generally used assay conditions and also under more physiological conditions with respect to temperature and chloride concentration. The satisfactory fit of eqn. (1) for kinetic titrations, with both enalaprilat and ramiprilat, indicated that ACE has a single high-affinity binding site for these tight-binding inhibitors. In the reaction scheme under steady-state conditions we have thus not taken into account slow binding of these inhibitors to ACE or the conversion between the inactive *cis* and the active *trans* inhibitor forms [22], as is needed under pre-steady-state conditions.

The values for K_i^{app} that we have found by regression analysis to eqn. (1) under the generally used conditions at 25°C in the standard assay buffer containing 0.300 M-NaCl are comparable with those, reported for enalaprilat [18] and ramiprilat [9] obtained from Henderson plots [23], measured under these conditions. We have not used Henderson plots, a linear transformation of eqn. (1), to evaluate inhibition constants, since the use of these plots is associated with several statistical problems [14,24–26]. Used diagnostically, our data for enalaprilat and ramiprilat presented as Henderson plots (not shown) resulted in fairly straight lines (slope = K_i^{app}). Our K_i^{app} value for enalaprilat

is also similar to the value obtained from a fit to a modified eqn. (1), under the generally used assay conditions [8].

No other studies have reported values for K_i^{app} for these inhibitors with purified ACE under physiological conditions. However, the 3-fold higher affinity of enalaprilat at 0.300 M- compared with 0.120 M-NaCl (Table 2 part *a*) is consistent with the 7-fold increase between 0.300 M- and 0.020 M-NaCl at 25°C [27]. However, no increase in the rate of hydrolysis of Fa-FGG in the absence of inhibitor (Fig. 3) was found when the chloride concentration was raised from 0.12 M- to 0.30 M-NaCl , in contrast with ref. [20], where a 20% increase in the activity was reported. Our results confirm that the inhibition of these tight-binding inhibitors is enhanced by chloride [27]. The values for K_i^{app} with either enalaprilat or ramiprilat were decreased to approximately the same extent in the standard assay buffer with 0.300 M-NaCl compared with 0.120 M-NaCl over the temperature range $25\text{--}37^\circ\text{C}$.

In our diagnostic Henderson plots an ordinate intercept (= $[E]_{\text{stoich.}}$) was found that was considerably lower (about half) than the protein concentration determined from u.v. absorbance. However, it was approximately equal to $[E]_{\text{stoich.}}$ and to the protein concentration from amino acid analysis. This raises the question whether it is suitable to determine the concentration of ACE from the u.v. absorbance with the published ϵ_{280} values.

The determination of the protein concentration based on the published ϵ_{280} may overestimate the ACE concentration. The reported ϵ_{280} values were based on total protein concentration determined by the Lowry method [28] with BSA as standard. However, this method is not suitable for glycoproteins such as ACE [29]. A recalculation of ϵ_{280} , assuming identical amino acid composition for rabbit lung ACE [5,30,31] and mouse kidney ACE [3], using the reported number of amino acid residues (1274–1277) and M_r (147000) [2,3], gives an ϵ_{280} of $4.1 \times 10^5\text{--}4.4 \times 10^5 \text{ M}^{-1}\cdot\text{cm}^{-1}$. This new value is approximately twice the old value [5,31]. With the new value for ϵ_{280} , the protein concentration in batch I will be $4.1\text{--}4.4 \mu\text{M}$, which is more consistent with $[E]_{\text{stoich.}}$ and the protein concentration determined from amino acid analysis (Table 1).

The preferred method for $[E]_i$ determination, i.e. titration with a specific inhibitor [32], requires a powerful inhibitor and an independent method for enzyme protein determination, to determine enzyme/inhibitor stoichiometry. To fulfil these requirements we used enalaprilat and ramiprilat, two of the most powerful inhibitors so far reported [33], in inhibitor-binding-site titrations. Moreover, we determined the enzyme protein concentration by amino acid analysis. In general, eqn. (1) gives a useful description of the two different titration methods under the conditions used and appears to provide a correct description of the steady-state kinetics for these tight-binding inhibitors. Regression analysis using both eqns. (1) and (2) for the titration with ramiprilat provides almost the same $[E]_{\text{stoich.}}$ (Table 1). Our results, exemplified in Figs. 2(a) and 2(b), may suggest that $[E]_{\text{stoich.}}$ depends on the inhibitor used or the function used for regression analysis. However, erroneous conclusions can be drawn if only eqn. (2) is used. With enalaprilat (Fig. 2a), a fit to eqn. (2) will result in an apparent $[E]_{\text{stoich.}}$ larger than that obtained with ramiprilat, a far more powerful inhibitor (Table 2 parts *a* and *b*). The fit to eqn. (1) provided almost the same $[E]_{\text{stoich.}}$ with enalaprilat as with ramiprilat. Moreover, simulations inserting $[E]_i$ equal to twice the calculated $[E]_{\text{stoich.}}$ gave a poor fit.

A calculated average value for $k_{\text{obs.}}$ of $80 \pm 20 \text{ s}^{-1}$ ($n = 18$) in the standard assay buffer containing 0.300 M-NaCl at 25°C measured from titrations both with enalaprilat and with ramiprilat is approximately twice the previously reported value of 45 s^{-1} (0.30 M-NaCl buffer and 25°C) [12], but consistent with the recently reported 99 s^{-1} [2]. From preliminary K_m and k_0

determinations, in the absence of inhibitor, we found a k_{obs} of $100 \pm 20 \text{ s}^{-1}$ in the standard assay buffer containing 0.300 M-NaCl at 25 °C. A relatively large scatter was found in the calculated k_{obs} values obtained from the enzyme-inhibitor titrations at different temperatures (Table 2 part a) compared with the inhibitor-enzyme titration. This is probably due to a small number of data points with high reaction rates.

On the basis of the protein concentration, determined from amino acid analysis and the kinetic titrations, we conclude that the ACE-enalaprilat and ACE-ramiprilat binding stoichiometry is 1:1. This is consistent with the assumption of a single inhibitor-binding site. The stoichiometry is apparently consistent with the 1.1 $\pm$ 0.4:1 ratio between ACE and de-esterifiedtrandolapril [(2S-{1[R(R)],2 α ,3 α ,7 α })-1-(2-{[1-(ethoxycarbonyl)-3-phenylpropyl]amino}-1-oxopropyl)octahydro-1H-indole-2-carboxylic acid] [34] and the 1:1 ratio with enalaprilat [27] measured by equilibrium dialysis, and a 0.88:1 ratio with lisinopril {N-[1(S)-carboxy-3-phenylpropyl]-L-Lys-L-Pro} from enzyme kinetics [19], although the latter two in principle based their enzyme concentration on u.v. absorbance.

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