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THE ROLE OF ENZYME AND SUBSTRATE CONCENTRATION IN THE EVALUATION OF SERUM ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITION BY ENALAPRILAT *IN VITRO**

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Abstract—The relationship between serum angiotensin converting enzyme (ACE) activity and concentration of the ACE inhibitor enalaprilat was determined *in vitro* in the presence of different concentrations (S = 4–200 mM) of the substrate Hip-Gly-Gly. From Henderson plots, a competitive tight-binding relationship between enalaprilat and serum ACE was found yielding a value of ≈5 nM for serum ACE concentration (E_t) and an inhibition constant (K_i) for enalaprilat of ≈0.1 nM. A plot of reaction velocity (V_i) versus total inhibitor concentration (I_t) exhibited a non-parallel shift of the inhibition curve to the right with increasing S. This was reflected by apparent Hill coefficients > 1 when the commonly used inhibitory sigmoid concentration–effect model (E_{max} model) was applied to the data. Slopes > 1 were obviously due to discrepancies between the free inhibitor concentration (I_f) present in the assay and I_t plotted on the abscissa and could, therefore, be indicators of tight-binding conditions. Thus, the sigmoid E_{max} model leads to an overestimation of K_i. Therefore, a modification of the inhibitory sigmoid E_{max} model (called "E_{max} tight model") was applied, which accounts for the depletion of I_f by binding, refers to I_t and allows estimation of the parameters E_t and IC_{50f} (free concentration of inhibitor when 50% inhibition occurs) using non-linear regression analysis. This model could describe the non-symmetrical shape of the inhibition curves and the results for K_i and E_t correlated very well with those derived from the Henderson plots. The latter findings confirm that the degree of ACE inhibition measured *in vitro* is, in fact, dependent on the concentration of substrate and enzyme present in the assay. This is of importance not only for the correct evaluation of K_i but also for the interpretation of the time course of serum ACE inhibition measured *ex vivo*. The non-linear model has some advantages over the linear Henderson equation: it is directly applicable without conversion of the data and avoids the stochastic dependency of the variables, allowing non-linear regression of all data points contributing with the same weight.

Measurement of angiotensin converting enzyme (ACE) activity in serum/plasma samples has been carried out extensively during the last few years in order to assess the extent of the blockade of the renin angiotensin system (RAS) after administration of ACE inhibitors. However, the interpretation of serum ACE inhibition measurements remains difficult not only because of the clear discrepancy between the time course of inhibition of circulating ACE and the antihypertensive effect of these drugs [1] but also for several methodological reasons. Recently, Nussberger *et al.* [2] have shown that the use of two different methods for measuring ACE activity leads to different degrees of ACE inhibition in identical samples. This appears to be plausible since the various assays employ not only different synthetic substrates, e.g. Hip-Gly-Gly, Hip-His-Leu and Fa-Phe-Gly-Gly, but also different concentrations of substrate [3–5]. Regarding the competitive mechanism postulated for most of these inhibitors, the degree of inhibition should depend on the ratio of substrate concentration (S) to its K_m value (Michaelis constant) (S/K_m) used in the assay. Furthermore, the various methods differ in the kind of buffer and ions added, the temperature and pH of incubation, and the dilution of the sample before assaying. As pointed out by Burnier *et al.* [6], it is impossible to compare results unless the same methodology is used. Thus, it is not surprising that for enalaprilat the published *in vitro* IC_{50} values (concentration of inhibitor at which 50% inhibition occurs) for various sources of ACE vary in the range of 0.1 to 6 nM [7–9]. A comparison is impossible unless the S/K_m used is taken into account and inhibition constants (K_i) are calculated. Another problem occurs when characterizing the concentration–inhibition relationship of ACE inhibitors: because of their high affinity for the enzyme and the relatively large concentration of enzyme, so-called "tight-binding" inhibition (depletion of free inhibitor) occurs requiring a special evaluation of the measured data [10, 11].

The aim of the present investigations was to evaluate the *in vitro* relationship between enalaprilat

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concentration and serum ACE inhibition when the substrate concentration was varied within one method. Furthermore, we applied a non-linear model for the correct evaluation of the IC_{50} and K_i values of tight-binding inhibitors and compared the results with those derived from Henderson plots.

MATERIALS AND METHODS

Measurement of serum ACE activity

ACE activity in human serum (pooled sera from blood bank donors) was determined according to the method of Neels *et al.* [12] with slight modifications. The substrate–buffer solutions (S = 4–200 mM; pH 8.15 at 25°, pH 8.0 at 37°) were prepared as follows: 1613 mg of the substrate L-hippuryl-L-glycyl-L-glycine (Hip-Gly-Gly, purchased from Bachem Feinchemikalien, Heidelberg, F.R.G.) were dissolved in 25 mL of Hepes buffer (50 mM Hepes, 300 mM NaCl, 400 mM Na_2SO_4; pH 8.15 at 25°, pH 8.0 at 37°). (Since high concentrations of Hip-Gly-Gly change the pH, for complete dissolution intermediate pH adjustment with NaOH was necessary.) Using this solution a final assay concentration of 200 mM Hip-Gly-Gly was reached. Further dilutions with Hepes buffer were made to reach final assay concentrations of 150, 100, 50, 20, 10, 8 and 4 mM Hip-Gly-Gly.

Serum (10 μL) was incubated with 100 μL of the respective substrate solution at 37°. Incubation time (30–90 min) was adjusted so that substrate depletion was always below 10%. Incubation was terminated by consecutively adding 100 μL of 10% (w/v) sodium tungstate (Sigma GmbH, Munich, F.R.G.) and 100 μL of 0.33 M H_2SO_4, and mixing. After adding 1 mL of distilled water and mixing, the tubes were centrifuged for 4 min at 10,470 *g* (Hettich Mikroliter centrifuge, Tuttlingen, F.R.G.). Supernatant (750 μL) was transferred into microcuvettes and 1 mL of borate buffer ($Na_2B_4O_7 \cdot 10\ H_2O$, 100 mM, pH 9.6) and 50 μL of TNBS solution (406 mg of 2,4,6-trinitrobenzene sulfonic acid, purchased from Fluka Feinchemikalien GmbH, Neu-Ulm, F.R.G., dissolved in 20 mL of absolute alcohol) were added simultaneously. The cuvettes were allowed to stand for 30 min at room temperature and then extinction (ε) was read against air at a wavelength of 420 nm (UVIKON 810 spectrophotometer, Kontron AG, Zürich, Switzerland). Blanks were prepared by adding the deproteinizing reagents (sodium tungstate and H_2SO_4) to the substrate solution before the serum. ACE activity expressed as units per liter (units/L) was calculated as:

$$\frac{(\varepsilon_{\text{sample}} - \varepsilon_{\text{blank}} \times 3.144 \times 10^8}{\varepsilon_{420} \times \text{time of incubation (min)}}$$

where ε_{420} is the molar extinction coefficient of TNBS-Gly-Gly (=15,650 L/mol/cm. One unit per liter represents 1 μmol of hippuric acid or Gly-Gly, released per minute per liter of serum.

Standards were prepared by adding 10 μL of a defined solution of Gly-Gly (Boehringer Mannheim, F.R.G.) in distilled water to a blank incubation mixture. Using standard solutions corresponding to 266, 532, 798 and 1064 units/L, an accuracy of 92.3, 102.5, 103.4 and 99.2%, respectively, was obtained (N = 3). Over the whole range of standard dilutions, extinction was linear up to $\varepsilon = 2.0$. The intra-assay (N = 5) and inter-assay (N = 4) precision of a normal serum sample (240 units/L) were 2.2 and 1.6%, respectively. The lower detection limit was defined as the corresponding activity of the 3-fold standard deviation of the mean blank value and was calculated to be 9.4 units/L when 10 μL of serum was incubated for 90 min. Serum ACE activity was stable at 4° for at least two weeks. For the measurement of *in vitro* concentration–response curves for serum ACE inhibition by enalaprilat, dilutions of enalaprilat were freshly prepared in distilled water and 10 μL of each dilution were added to 1 mL of serum so that the concentration of enalaprilat in the spiked samples reached from 10^{-11} to 10^{-6} M. Spiked samples were allowed to preincubate for at least 6 hr at room temperature before starting the assay. Because of the dilution factor 11 in the assay, final concentrations were expected to be 9.09×10^{-13} to 9.09×10^{-8} M. Using the seven different substrate solutions described above, a series of inhibition curves was generated. All measurements were done in triplicate and data are presented in the figures as mean values ± SEM.

Data evaluation

Theory. The steady state rate equation for the reaction of one substrate with one enzyme is the well-known relationship derived by Michaelis and Menten [13]:

$$V_0 = \frac{V_{\max} \times S}{S + K_m} \tag{1}$$

where V_0 = velocity in the absence of inhibitor, $V_{\max}$ = maximum velocity, K_m = Michaelis constant and S = substrate concentration. The following equations are based on the assumption made by Michaelis and Menten that $K_m \approx K_s$ [K_s = dissociation constant of the enzyme–substrate complex (ES)].

In the presence of a reversible competitive inhibitor the velocity follows the equation [13]:

$$V_i = \frac{V_{\max} \times S}{S + K_m(1 + I/K_i)} \tag{2}$$

where V_i = velocity in the presence of inhibitor, I = inhibitor concentration and K_i = dissociation constant of the enzyme–inhibitor complex (EI).

In order to describe the velocity in the presence of different inhibitor concentrations in relation to the velocity in the absence of inhibitor (V_i/V_0), at one defined substrate concentration, Eqns 1 and 2 can be combined as:

$$V_i/V_0 = \frac{S + K_m}{S + K_m(1 + I/K_i)}. \tag{3}$$

This can be rearranged as:

$$V_i/V_0 = \frac{1 + S/K_m}{1 + S/K_m + I/K_i}. \tag{4}$$

The use of Eqns 1–4 is based on the following assumptions made by Michaelis and Menten: (1) equilibrium is rapidly reached between substrate, inhibitor and enzyme so that steady state velocities

are measured; (2) the reaction between inhibitor and enzyme follows a simple reversible competitive mechanism; (3) both substrate and inhibitor concentrations are much greater than the enzyme concentration (total enzyme concentration = E_t) so that S_{free} (S_f) $\approx S_{total}(S_t) \approx S$ and $I_{free}(I_f) \approx I_{total}(I_t) \approx I$, i.e. the bound fractions of S and I are negligibly small. The ratio E_t/K_i (E_t/K_m, respectively) should be 0.01 or less for a Michaelis–Menten analysis to be valid [14]. Otherwise, the substrate or inhibitor is called "tight-binding" and the above assumption (3) does not hold. Thus, one must bear in mind that Eqn 4 is a simplification and the correct general form must be written as:

$$V_i/V_0 = \frac{1 + S_f/K_m}{1 + S_{fi}/K_m + I_{fs}/K_i} \times \frac{S_{fi}}{S_f} \qquad (5)$$

where S_f and S_{fi} represent the free concentration of substrate in the absence (cf. Eqn 1) and presence (cf. Eqn 2) of inhibitor, respectively. I_{fs} represents the free concentration of inhibitor in the presence of substrate.

The concentration of ACE in serum is reported to be in the range of 4 to 10 nM [11, 15, 16]. Thus in our case, with respect to the dilution factor 11, E_t in the assay was assumed to be about 0.1–1 nM. The K_m of Hip-Gly-Gly and, hence, the substrate concentrations used were in the millimolar range. We can, therefore, assume that the bound fraction of substrate (=ES) was negligibly small, i.e. that $S_f \approx S_t \approx S$. However, as Francis *et al.* [11] and Bünning [10] noted, the ratio of E_t (serum)/K_i for most of the known ACE inhibitors is not <0.01 so that I_t used in the assay is not always equal to I_f. Thus, Eqn 5 simplifies to:

$$V_i/V_0 = \frac{1 + S/K_m}{1 + S/K_m + I_{fs}/K_i}. \qquad (6)$$

From this equation (6 or 4) Cheng and Prusoff [17] derived the relationship between K_i and the concentration of inhibitor (IC_{50}) at which the velocity is 50% of the uninhibited reaction ($V_i/V_0 = 1/2$). The term is here called IC_{50f} to bear in mind that this holds true only for the free concentration of inhibitor:

$$IC_{50f} = K_i(1 + S/K_m). \qquad (7)$$

From Eqns 6 and 7 one obtains:

$$V_i = \frac{V_0}{1 + (I_{fs}/IC_{50f})^n}. \qquad (8)$$

This function is equivalent to the inhibitory sigmoid E_{max} model [18] (in the following called "E_{max} model") where V_i represents the effect (E) and V_0 the maximum effect (E_{max}) at a defined substrate concentration. n is an additional parameter influencing the slope of the curve. It is called the Hill coefficient and was originally added to indicate positive or negative cooperativity.

A concentration–response plot is commonly based on total drug concentrations used in the assay (e.g. V_i vs I_t). Thus, in the case of a tight-binding inhibitor it is not possible to determine IC_{50f} directly from an inhibition curve. The IC_{50} values which are commonly evaluated from such inhibition curves are total inhibitor concentrations (IC_{50t}) and, as mentioned by Francis *et al.* [11] for the ACE inhibitor cilazaprilat, such inhibition curves become very steep and also asymmetrical around the midpoint.

For the detection of tight-binding conditions and the correct analysis of the K_i of such inhibitors, a linear steady state rate equation derived by Henderson [14] is often used which accounts for the depletion of free inhibitor by binding to the enzyme and refers to the total drug concentration I_t:

$$\frac{I_t}{1 - V_i/V_0} = E_t + K_i(1 + S/K_m) \times V_0/V_i. \qquad (9)$$

This is the form of the general equation for the special case of competitive inhibition.

A plot of $I_t/(1 - V_i/V_0)$ versus V_0/V_i permits calculation of the enzyme concentration E_t from the y-intercept of the regression line. The slope is $K_i(1 + S/K_m)$ and a replot of the slope versus S (using different concentrations of substrate) yields a linear relationship with the y-intercept = K_i and the slope = K_i/K_m. These plots at different substrate concentrations also allow detection of mechanisms of inhibition other than competitive.

Under conditions of first order enzyme kinetics ($S \ll K_m$), Eqn 9 simplifies to:

$$\frac{I_t}{1 - V_i/V_0} = E_t + K_i \times V_0/V_i \qquad (9a)$$

and K_i can then be computed directly from the slope of the plot.

However, the use of the Henderson equation has some disadvantages: it has been noted by Henderson [14] that the calculation of the best slope and intercept of the linear plots of Eqn 9 (9a) is very complex because of the stochastic dependence of the variables and the requirement of extrapolation from the data points to estimate the y-intercept, since V_0/V_i has a minimum value of 1. He recommends that calculations should be weighted in favour of the points in the range of V_0/V_i = 1.6–2.5 and that numerous replicate measurements should be done for a good approximation of the true mean. Thus, the mid-part of the inhibition curve (V_i/V_0 = 60–40%) is the most reliable for the calculation and other data are subject to greater error.

From Eqn 9, a relationship between IC_{50t} (the midpoint of each inhibition curve) and K_i can be derived as:

$$IC_{50t} = K_i(1 + S/K_m) + 0.5E_t \qquad (10)$$

which is an extended version of the relationship of Cheng and Prussof [17] (Eqn 7), to accommodate tight-binding inhibitors [19]. It is obvious that the difference between IC_{50t} and IC_{50f} is the additional term $0.5E_t$, which accounts for the depletion of free inhibitor by binding.

In order to avoid linearization (Henderson analysis) we modified the non-linear E_{max} model (Eqn 8) to make it applicable for tight-binding inhibition. For this purpose the term I_{fs} in Eqn 8 is replaced by $I_t - EI_s$, since $I_t = I_{fs} + EI_s$. This leads to:

$$V_i = \frac{V_0}{1 + (I_t - EI_s)/IC_{50f}} = \frac{V_0}{1 + I_t/IC_{50f} - EI_s/IC_{50f}} \quad (11)$$

where the Hill coefficient is eliminated since the term EI_s now accounts for the slope of the curve.

The term EI_s can be derived from the law of mass action, according to:

$$K_i = \frac{E_f \times I_{fs}}{EI_s} \quad \text{and} \quad K_m \approx K_s = \frac{E_f \times S_f}{ES} \quad (12)$$

for the interaction between enzyme and inhibitor or substrate, respectively. Under the assumption that $S \approx S_f \approx S_t$ (binding of substrate is negligible) and that $I_{fs} = I_t - EI_s$ (binding of inhibitor is not negligible), Eqn 12 becomes:

$$K_i = \frac{(E_t - EI_s - ES)(I_t - EI_s)}{EI_s} \quad (13)$$

and

$$K_m = \frac{(E_t - EI_s - ES) \times S}{ES}, \quad (14)$$

respectively. Solving Eqn 14 for ES one obtains:

$$ES = \frac{E_t \times S/K_m - EI_s \times S/K_m}{1 + S/K_m}. \quad (15)$$

Insertion of the above expression for ES into Eqn 13 yields:

$$K_i \times EI_s = \left[E_t - EI_s - \left(\frac{E_t \times S/K_m - EI_s \times S/K_m}{1 + S/K_m}\right)\right] \times (I_t - EI_s). \quad (16)$$

By rearrangement one obtains the quadratic equation:

$$EI_s^2 + [-I_t - E_t - K_i(1 + S/K_m)] \times EI_s + I_t \times E_t = 0 \quad (17)$$

according to $x^2 + bx + c = 0$ which can be resolved by the general formula [20]:

$$x_{1,2} = -\tfrac{1}{2}b \pm \sqrt{[(\tfrac{1}{2}b)^2 - c]}. \quad (18)$$

The appropriate solution is:

$$EI_s = \tfrac{1}{2}[I_t + E_t + K_i(1 + S/K_m)] - \sqrt{(\{\tfrac{1}{2}[I_t + E_t + K_i(1 + S/K_m)]\}^2 - I_t \times E_t)}. \quad (19)$$

Since $K_i(1 + S/K_m)$ is equal to IC_{50f} (Eqn 7), Eqn 19 can be written as:

$$EI_s = \tfrac{1}{2}(I_t + E_t + IC_{50f}) - \sqrt{\{[\tfrac{1}{2}(I_t + E_t + IC_{50f})]^2 - I_t \times E_t\}}. \quad (20)$$

Rearrangement yields:

$$EI_s = \tfrac{1}{2}\{I_t + E_t + IC_{50f} - \sqrt{[(I_t + E_t + IC_{50f})^2 - 4I_t \times E_t]}\}. \quad (21)$$

This expression for EI_s is inserted into Eqn 11 yielding:

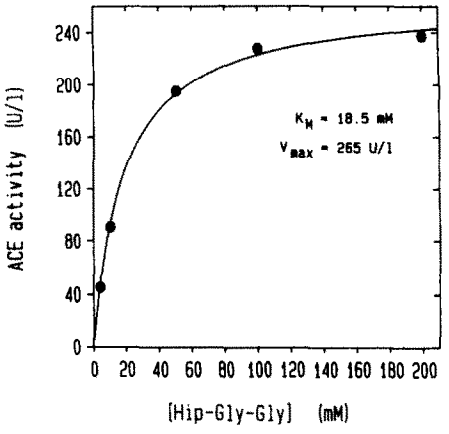

Fig. 1. Serum ACE activity as a function of the concentration of the substrate Hip-Gly-Gly. Curve-fitting was done using the saturation equation (Eqn 1) yielding K_m and V_{max} as depicted.

$$V_i = V_0/[1 + I_t/IC_{50f} - (\tfrac{1}{2}\{I_t + E_t + IC_{50f} - \sqrt{[(I_t + E_t + IC_{50f})^2 - 4I_t \times E_t]}\})/IC_{50f}]. \quad (22)$$

This equation (called "E_{max} tight model") can be applied to a plot of V_i versus I_t. Non-linear regression of this function to the inhibition curve yields the parameters V_0 as the maximum activity with the substrate concentration used, IC_{50f} as the free concentration of inhibitor in the case of 50% inhibition (which is smaller than the concentration at the midpoint of the competition curve!) and E_t as the enzyme concentration in the assay in terms of molar equivalents of inhibitor.

A plot of IC_{50f} values versus different S should yield a straight line which intersects the y-axis at $IC_{50f} = K_i$ and has a slope of K_i/K_m according to Eqn 7.

Under conditions of first order enzyme kinetics ($S \ll K_m$), Eqn 22 simplifies to:

$$V_i = V_0/[1 + I_t/K_i - (\tfrac{1}{2}\{I_t + E_t + K_i - \sqrt{[(I_t + E_t + K_i)^2 - 4I_t \times E_t]}\})/K_i]. \quad (22a)$$

Parameter estimation. Eqns 1 (=saturation isotherm of S), 8 (=E_{max} model) and 22 (=E_{max} tight model) were applied to the data using a program package of non-linear least squares model fitting (GIP, Gießener Iterationsprogramm, developed by D. H. Brockmeier and H. M. v. Hattingberg).

Linear regression analysis was performed using the same program.

RESULTS

Figure 1 shows the dependency of ACE activity on substrate concentration, covering the range

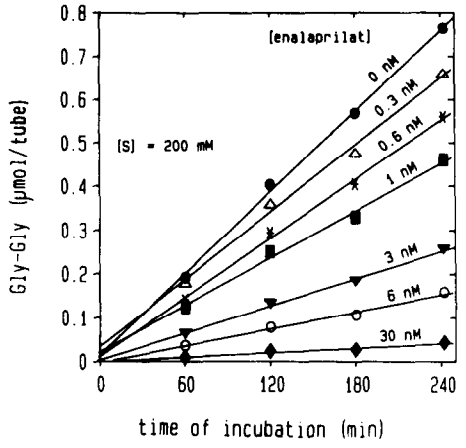

Fig. 2. Time-dependency of formation of the product Gly-Gly from ACE activity measurements using a final concentration of 200 mM of the substrate Hip-Gly-Gly. Serum samples were preincubated (6 hr) with different concentrations of enalaprilat (final concentrations depicted) and incubations with substrate were terminated at the time points indicated. For each enalaprilat concentration, the resulting regression line is depicted.

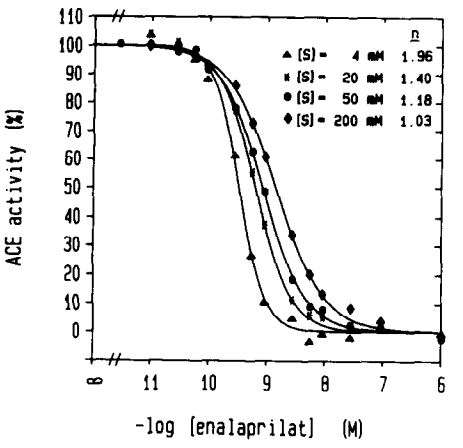

Fig. 3. Concentration-dependent inhibition of ACE activity by enalaprilat in the presence of different substrate concentrations (S). Data were fitted using the E_{max} model (Eqn 8) and the resulting curves are shown. The corresponding Hill coefficients (n) are given in the figure. Calculated IC_{50f} values (nM) were: 0.33 (▲), 0.63 (*), 0.83 (●), 1.40 (◆).

of concentrations used also in the inhibition experiments. Non-linear regression of Eqn 1 yielded K_m and V_{max} as depicted in the figure. Mean (±SD) values of eight saturation experiments were 19.3 ± 11.3 mM for K_m and 251 ± 61 units/L for V_{max}.

A prerequisite for the correct evaluation of inhibition data was that the velocities measured in the presence of enalaprilat were steady state velocities. Since the method was non-continuous, the incubation was terminated at different time points to make sure that release of the product was linear with time. The result for the case of 200 mM Hip-Gly-Gly in the presence of various enalaprilat concentrations is shown in Fig. 2. Since linearity was observed even for this high substrate concentration, one-point measurements for calculating V_i appear to be acceptable. From Figs 1 and 2 it becomes obvious that, for concentrations of up to 200 mM Hip-Gly-Gly, no substrate and/or product inhibition occurred.

A family of concentration–inhibition curves of enalaprilat obtained using different substrate concentrations is shown in Fig. 3. Application of the E_{max} model (Eqn 8) to these inhibition data (ignoring

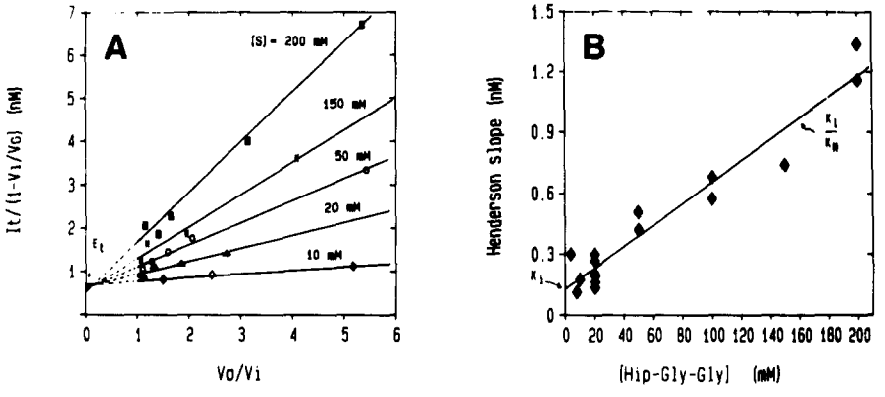

Fig. 4. (A) A representative Henderson plot (cf. Eqn 9) for the inhibition of serum ACE activity by enalaprilat when using the indicated substrate concentrations. Linear regression analysis yielded the following values for IC_{50f} (slope) and E_i (y-intercept) (nM): 0.18 and 0.68 (◇), 0.30 and 0.60 (▲), 0.51 and 0.59 (○), 0.74 and 0.55 (*), 1.2 and 0.52 (■), respectively. (B) Replot of the slopes (=IC_{50f}) obtained from Henderson plots (cf. Panel A) of n = 16 experiments against the different substrate concentrations used. Linear regression analysis (r = 0.968) yielded values of 0.13 nM for K_i (=y-intercept) of enalaprilat and 24.7 mM for K_m (=K_i/slope) of the substrate Hip-Gly-Gly, according to Eqn 7.

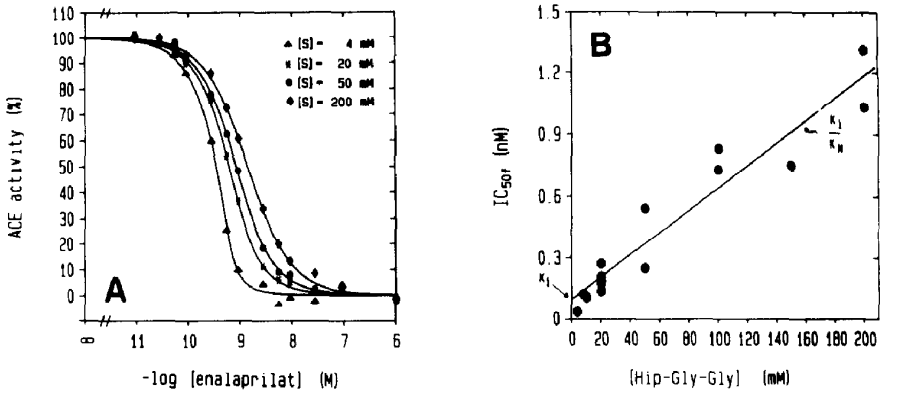

Fig. 5. (A) Concentration-dependent inhibition of serum ACE activity by enalaprilat in the presence of different substrate concentrations (S) as indicated. Data were fitted using the E_{max} tight model (Eqn 22) and the resulting curves are shown. Estimation of the parameters IC_{50f} and E_t gave the following values (nM): 0.033 and 0.63 (▲), 0.27 and 0.69 (*), 0.54 and 0.59 (●), 1.31 and 0.18 (◆), respectively. (B) Linear dependency of IC_{50f} (derived from non-linear regression analysis of N = 16 experiments, cf. Panel A) on the substrate concentration. Linear regression analysis ($r = 0.957$) yielded values of 0.1 nM for K_i (=y-intercept) of enalaprilat and 18.1 mM for K_m (=K_i slope) of the substrate Hip-Gly-Gly, according to Eqn 7.

the fact that I_t is probably not equal to I_f) yielded the curves depicted in the figure. As can be seen from the curves and from the Hill coefficients (n) increasing from 1 to approximately 2, the curves became steeper with reduced substrate. The calculated values for IC_{50} (= IC_{50t}) ranged from 0.33 nM (S = 4 mM) to 1.40 nM (S = 200 mM).

A representative example of a Henderson plot for such inhibition data is shown in Fig. 4A. The plot reveals linear dependencies with increasing slopes when S increases, as is expected for a competitive mechanism [14]. Linear regression analysis for all experiments (N = 16; N = 5 are depicted) yielded a mean (±SD) value for E_t of 0.49 ± 0.20 nM which corresponds to ≈5 nM for E_t in the undiluted serum. The replot of the slopes of all Henderson plots [representing the term $K_i(1 + S/K_m)$] versus S is shown in Fig. 4B. The regression line yielded K_i = 0.13 nM (y-intercept) and K_m = 24.7 mM.

Application of the E_{max} tight model (Eqn 22) to such inhibition data yields regression curves as shown in Fig. 5A. For comparison, the same data are plotted as in Fig. 3. The E_{max} tight model allows the curves to have a non-symmetrical shape in contrast to the E_{max} model which forces symmetry around the midpoint. This difference becomes most obvious in the range of low inhibitor concentrations. Non-linear regression yielded the parameter E_t = 0.48 ± 0.23 nM (mean ± SD; N = 16), which agrees very well with the result of the Henderson analysis. The calculated values of the parameter IC_{50f} for all of the experiments are plotted versus the corresponding S in Fig. 5B. As in the replot of Henderson (Fig. 4B), K_i and K_m were calculated from the regression line leading to very similar results, with K_i = 0.10 nM and K_m = 18.1 mM. Both K_m values (24.7 and 18.1 mM) agree fairly well with that derived independently from the saturation experiments with Hip-Gly-Gly (cf. Fig. 1).

The similarity of the results from the Henderson

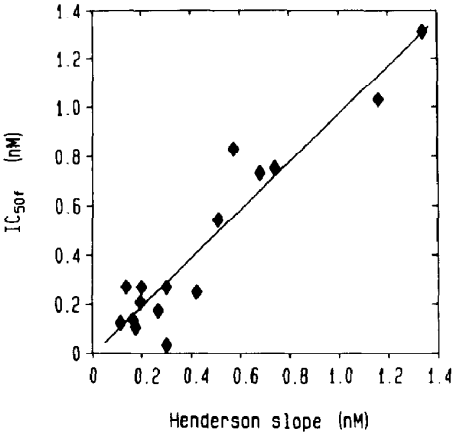

Fig. 6. Correlation between IC_{50f} (derived from the E_{max} tight model, cf. Fig. 5) and the slope from the Henderson plot (cf. Fig. 4). Each point represents the respective value obtained from a complete serum ACE inhibition curve of enalaprilat using different substrate concentrations *in vitro* (N = 16). The regression line ($y = 0.98x - 0.051$, r = 0.948) is depicted.

analysis and the parameter estimation using the E_{max} tight model is confirmed in Fig. 6, where the IC_{50f} and the corresponding Henderson slopes of each inhibition experiment are compared. The regression line was $y = 0.98x - 0.051$ ($r = 0.948$), indicating the close correlation between the two evaluation methods.

A comparison between the IC_{50t} (E_{max} model) and IC_{50f} (E_{max} tight model) values from all of the experiments is demonstrated in Fig. 7. The regression line ($y = 0.93x + 0.27$) confirms the relationship $IC_{50t} = IC_{50f} + 0.5E_t$ (cf. Eqn 10), since it parallels the line of identity with a positive intercept of

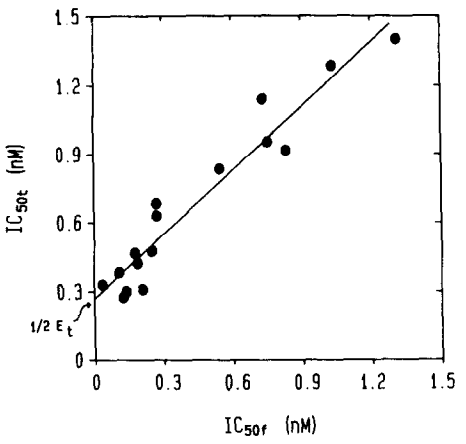

Fig. 7. Correlation between IC_{50t} (derived from the E_{max} model, cf. Fig. 3) and IC_{50f} (derived from the E_{max} tight model, cf. Fig. 5). Each point represents the respective value obtained from a complete serum ACE inhibition curve of enalaprilat using different substrate concentrations *in vitro* (N = 16). According to Eqn 10, the term $0.5E_t$ can be read from the y-intercept of the regression line ($y = 0.93x + 0.27$, $r = 0.950$).

0.27 nM. This value should represent the term $0.5E_t$. This is consistent with the results for E_t obtained directly from the Henderson plots and from the E_{max} tight model (0.49 and 0.48 nM, respectively).

DISCUSSION

The value of 251 units/L for the V_{max} of ACE in a pool of human sera determined with Hip-Gly-Gly, is consistent with that derived by Neels *et al.* [12] (281 units/L) using the same method. Our mean K_m was 4-fold higher than the literature values of 2.6–5 mM [4, 21], probably due to the different buffer systems used by these authors. The high precision of the ACE activity measurement reported by Neels *et al.* [12] is confirmed by the small coefficients of variance of our slightly modified method. The highest S used by these authors was 60 mM. Our results confirm the absence of substrate and/or product inhibition (at low fractional substrate utilizations) postulated by these authors and extend their observations up to a concentration of 200 mM Hip-Gly-Gly.

Enalaprilat concentration-dependently reduced serum ACE activity at every substrate concentration used. According to the postulated competitive mechanism [22, 23], a shift to the right of the enalaprilat inhibition curve with increasing substrate concentration was observed. However, the curve was shifted in a non-parallel manner as was reflected by the different Hill coefficients obtained when the E_{max} model (Eqn 8) was applied to the data. After transformation of the data according to Henderson [14], a competitive but in addition a "tight-binding" relationship between enalaprilat and serum ACE became evident. This is consistent with the observations of Shapiro and Riordan [23] and Bull *et al.* [24] with enalaprilat and purified ACE from rabbit lung. A significant enzyme concentration (E_t) of about 0.5 nM in the diluted serum could be read directly from the Henderson plot (Fig. 4A). Taking the dilution factor 11 into consideration, a serum ACE concentration of ≈5 nM is obtained; a value in agreement with the mean value of ≈4 nM (mean ± SD: 627 ± 205 ng/mL, MW ≈140,000) for ACE concentration in human serum reported by Lanzillo and Fanburg [15] using an independent immunological method. This finding confirms that E_t is remarkably high in comparison to the effective concentrations of most commercially available ACE inhibitors. Therefore, a significant portion of inhibitor will be bound to the enzyme, particularly at lower concentrations of inhibitor. Thus, we conclude that the increasing Hill coefficients obtained from the E_{max} model are due to the discrepancy between I_f present in the assay and I_t plotted on the abscissa. As noted by Holford and Sheiner [18], the Hill coefficient is useful for describing concentration–effect relationships but non-integer values of n deviating from 1 may provide no physical interpretation of receptor (or enzyme) binding sites, and one should be cautious in interpreting the meaning of n. Our results suggest that whenever $n > 1$ occurs a tight-binding situation has to be considered.

Our modified E_{max} model (E_{max} tight model) is in accordance with the general formula given by Morrison [25]. The E_{max} tight model not only substitutes the Hill coefficient with the more relevant parameter of enzyme concentration (E_t, in molar equivalents of I) but also reflects the non-symmetrical shape of the inhibition curves, appearing predominantly at low inhibitor concentrations. The parameter IC_{50f} obtained using this approach gives the correct value which can be used directly in the Cheng and Prusoff [17] correction (Eqn 7). It has to be considered that reading the midpoint only of such curves (IC_{50t}) leads to an overestimation of K_i unless E_t is taken into account. As can be read from Eqn 10, this error in the estimation of K_i increases with lower S and higher E_t. Under our assay conditions, if $S \ll K_m$ was used, K_i would be overestimated by about 4-fold (cf. Fig. 7). However, it must be noted that this factor becomes even higher when methods are used where the dilution factor of the serum samples is smaller (increase of E_t *in vitro*). Based on estimates of IC_{50t} solely, a correct value of K_i for tight-binding inhibitors can only be obtained if the enzyme concentration is known or if IC_{50t} is determined at different concentrations of E_t (method of Myers and Cha [26]).

Since the E_{max} tight model accounts for the possible difference between IC_{50t} and IC_{50f}, we suggest that it is a very useful model for the quantitative analysis of competitive inhibition when the free concentration of inhibitor is not known precisely. In the case of non-tight-binding, i.e. if $E_t/K_i \ll 0.01$, the approximation for E_t with the E_{max} tight model will be infinitely small and the calculated IC_{50f} will equal the midpoint of the curve (IC_{50t}). In this case a curve-fitting using the E_{max} model will yield $n \approx 1$ and would be sufficient for describing the data.

The results for E_t and IC_{50f} derived from the E_{max} tight model agree very well with those obtained from

the Henderson plots, confirming the identical mathematical extension in both equations. However, as a nonlinear model it has some advantages over the linear Henderson equation [26, 27]: it can be applied directly without conversion of the data and can be integrated into any iteration program which allows non-linear regression analysis. It avoids the stochastic dependency of the variables and all measured data contribute with the same weight (or with a weight according to their experimental precision) to the parameter estimation, in contrast to the Henderson plot. Finally, the equation (Eqn 22) can be used to estimate (predict) the fractional enzyme activity (V_i/V_0) for any given total concentrations of enzyme, inhibitor and substrate (e.g. to simulate the ACE activity in undiluted samples), when K_i and K_m are known.

The K_i value of $\approx$0.1 nM for enalaprilat obtained from both calculations is in accordance with the values derived by Shapiro and Riordan [23] and Natoff *et al.* [9] with purified ACE from rabbit lung, also considering tight-binding inhibition (0.05 and 0.15 nM, respectively). Bull *et al.* [24] obtained a similar value of 0.09 nM for K_i from equilibrium dialysis with [^{3}H]enalaprilat.

Concerning the different IC_{50} values for the interaction of enalaprilat with ACE as published in the literature (cf. introduction), we suggest that these differences can be explained, in part, by the assumptions made above about the evaluation of IC_{50t}, IC_{50f} and K_i. However, another conflicting situation arises from the fact that the dilution steps employed in the various assays are often ignored. In our case the samples, and hence I_t and E_t, were diluted by a factor of 11 during the assay procedure. This factor must be taken into account when calculating I_t on the abscissa for the reason that equilibrium is obviously reached between E_t, I_t and S_t during the incubation period as can be seen from our kinetic measurements (cf. Fig. 2). Additionally, if dissociation of EI into E_f and I_f to yield a new equilibrium had not occurred after dilution of the preincubated serum, then a shift to the right of the inhibition curve due to increasing S would not have been observed.

Finally, in the context of serum ACE activity as a possible correlate of the effect of ACE inhibitors *in vivo*, our results are relevant to the interpretation of *ex vivo* measurements after administration of an ACE inhibitor. The degree of inhibition measured is variable and will be dependent on S/K_m used in the assay and on the enzyme and inhibitor concentration in the (diluted!) sample. This has to be taken into account when one tries to interprete the time course of inhibition or to correlate I_t (measured chemically) with the inhibition effect.

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REFERENCES

1. Waeber B, Nussberger J, Juillerat L and Brunner HR, Angiotensin converting enzyme inhibition: discrepancy between antihypertensive effect and suppression of enzyme activity. *J Cardiovasc Pharmacol* **14**: S53–S59, 1989.
2. Nussberger J, Juillerat L, Perret F, Waeber B, Bellet M, Brunner HR and Menard J, Need for plasma angiotensin measurements to investigate converting-enzyme inhibition in humans. *Am Heart J* **117**: 717–722, 1989.
3. Cushman DW and Cheung HS, Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem Pharmacol* **20**: 1637–1648, 1971.
4. Ryan JW, Chung A, Ammons C and Carlton ML, A simple radioassay for angiotensin-converting enzyme. *Biochem J* **167**: 501–504, 1977.
5. Holmquist B, Bünning P and Riordan JF, A continuous spectrophotometric assay for angiotensin converting enzyme. *Anal Biochem* **95**: 540–548, 1979.
6. Burnier M, Waeber B, Nussberger J and Brunner HR, Pharmacokinetics of angiotensin converting enzyme inhibitors. *Br J Clin Pharmacol* **28**: 133S–140S, 1989.
7. Swanson BN, Hichens M, Mojaverian P, Ferguson RK, Vlasses PH and Dudash M, Angiotensin converting enzyme activity in human serum: relationship to enzyme inhibitor *in vivo* and *in vitro*. *Res Commun Chem Pathol Pharmacol* **33**: 525–536, 1981.
8. Reynolds CH, Kinetics of inhibition of angiotensin converting enzyme by captopril and by enalapril diacid. *Biochem Pharmacol* **33**: 1273–1276, 1984.
9. Natoff IL, Nixon JS, Francis RJ, Klevans LR, Brewster M, Budd J, Patel AT, Wenger J and Worth E, Biological properties of the angiotensin-converting enzyme inhibitor cilazapril. *J Cardiovasc Pharmacol* **7**: 569–580, 1985.
10. Bünning P, Inhibition of angiotensin converting enzyme by 2-(*N*-((*S*)-1-carboxy-3-phenylpropyl)-L-alanyl)-(1*S*,3*S*,5*S*)-2-azabicyclo(3.3.0)octane-3-carboxylic acid (Hoe 498 diacid). *Drug Res* **34**: 1406–1410, 1984.
11. Francis RJ, Brown AN, Kler L, Fasanella d'Amore T, Nussberger J, Waeber B and Brunner HR, Pharmacokinetics of the converting enzyme inhibitor cilazapril in normal volunteers and the relationship to enzyme inhibition: development of a mathematical model. *J Cardiovasc Pharmacol* **9**: 32–38, 1987.
12. Neels HM, van Sande ME and Scharpe SL, Sensitive colorimetric assay for angiotensin converting enzyme in serum. *Clin Chem* **29**: 1399–1403, 1983.
13. Fersht A, *Enzyme Structure and Mechanism*. W. H. Freeman and Company, New York, 1985.
14. Henderson PJF, A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors. *Biochem J* **127**: 321–333, 1972.
15. Lanzillo JJ and Fanburg BL, Development of competitive enzyme immunoassays for human serum angiotensin-1-converting enzyme: a comparison of four assay configurations. *Anal Biochem* **126**: 156–164, 1982.
16. Ryan JW, Assay of peptidase and protease enzymes *in vivo*. *Biochem Pharmacol* **32**: 2127–2137, 1983.
17. Cheng YC and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
18. Holford NHG and Sheiner LB, Understanding the dose–effect relationship: clinical application of pharmacokinetic–pharmacodynamic models. *Clin Pharmacokinet* **6**: 429–453, 1981.
19. Cha S, Tight-binding inhibitors—I: Kinetic behavior. *Biochem Pharmacol* **24**: 2177–2185, 1975.
20. *Wissenschaftliche Tabellen Geigy, Teilband Statistik*. CIBA-GEIGY AG, Basel, 1980.

21. Dorer FE, Kahn JR, Lentz KE, Levine M and Skeggs LT, Kinetic properties of pulmonary angiotensin-converting enzyme. Hydrolysis of hippurylglycylglycine. *Biochim Biophys Acta* **429**: 220–228, 1976.
22. Mendelsohn FAO, Csicsmann J and Hutchinson JS, Complex competitive and non-competitive inhibition of rat lung angiotensin-converting enzyme by inhibitors containing thiol groups: captopril and SA 446. *Clin Sci* **61**: 277s–280s, 1981.
23. Shapiro R and Riordan JF, Inhibition of angiotensin converting enzyme: mechanism and substrate dependence. *Biochemistry* **23**: 5225–5233, 1984.
24. Bull HG, Thornberry NA, Cordes MHJ, Patchett AA and Cordes EH, Inhibition of rabbit lung angiotensin-converting enzyme by N^{α}-((*S*)-1-carboxy-3-phenylpropyl)L-alanyl-L-proline and N^{α}-((*S*)-1-carboxy-3-phenylpropyl)L-lysyl-L-proline. *J Biol Chem* **260**: 2952–2962, 1985.
25. Morrison JF, Kinetics of the reversible inhibition of enzyme catalyzed reactions by tight-binding inhibitors. *Biochim Biophys Acta* **185**: 269–286, 1969.
26. Greco WR and Hakala MT, Evaluation of methods for estimating the dissociation constant of tight binding enzyme inhibitors. *J Biol Chem* **254**: 12104–12109, 1979.
27. Motulsky HJ and Ransnas LA, Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J* **1**: 365–374, 1987.