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Kinetics of the Inhibition of Human Renin by an Inhibitor Containing a Hydroxyethylene Dipeptide Isostere

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ABSTRACT: We have studied the inhibition of both human and hog renins by compound **1** [Boc-Pro-Phe-N^{α}-MeHis-Leuψ(CHOHCH$_2$)Val-Ile-(aminomethyl)pyridine] using kinetics. The inhibition of human renin was shown to be time dependent and followed a minimal two-step mechanism. A loosely bound EI complex was formed rapidly with a dissociation constant, K_I, of 12 nM. A second EI complex was slowly formed and was found to be 64-fold more strongly bound with an overall K_I* of 0.19 nM. The inhibition of human renin was shown to be competitive by both initial and final steady-state velocities. Compound **1** was also shown to be a competitive inhibitor of hog renin with a K_I of 12 nM, but no evidence for time-dependent inhibition was detected. The differences in overall K_I and inhibition kinetics may be a consequence of the similarities in structure between **1** and human angiotensinogen.

The aspartyl protease renin (EC 3.4.99.19) is secreted by specialized cells in the kidney into the bloodstream where it cleaves an N-terminal fragment from its sole substrate angiotensinogen to form the decapeptide angiotensin I. A C-terminal dipeptide is removed from angiotensin I by angiotensin-converting enzyme to generate the biologically active octapeptide angiotensin II. Angiotensin II causes vasoconstriction by binding to its receptor on the arterial walls, but it also stimulates the release of aldosterone from the adrenal glands. High aldosterone levels induce sodium and water retention, leading to an increase in blood pressure by a volume mechanism as well (Peach, 1977). The renin–angiotensin cascade has been shown to participate in both the maintenance of normal blood pressure (MacGregor et al., 1981) and certain forms of hypertension (Laragh, 1981).

The inhibition of renin is an attractive site for control of the cascade because renin has no other known physiological role. Potent renin inhibitors have been described that incorporate either a reduced peptide bond ψ(CH$_2$NH),[1] a hydroxyethylene bond ψ(CHOHCH$_2$), or the unusual amino acid statine[2] into peptides that contain portions of the angiotensinogen substrate sequence (Szelke et al., 1982, 1983; Boger et al., 1983). However, detailed investigations of the kinetics of inhibition for these compounds were not reported. Such

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[1] The abbreviation ψ[x], indicating that x replaces the amide –CONH– unit, has been defined by the IUPAC–IUB Joint Commission on Biochemical Nomenclature (1984).

[2] Abbreviations: statine, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid; PMSF, phenylmethanesulfonyl fluoride; pNPGB, *p*-nitrophenyl *p*-guanidinobenzoate; RIA, radioimmunoassay; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Boc, *tert*-butyloxycarbonyl; Amp, 2-(aminomethyl)pyridine; Na$_2$EDTA, ethylenediaminetetraacetic acid disodium salt; BSA, bovine serum albumin; AI, angiotensin I; EI, enzyme–inhibitor complex.

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work seemed to be warranted, considering that the inhibition of the related aspartyl protease pepsin by the statine-containing peptide pepstatin was unusually slow and tight binding (Rich & Sun, 1980). We now report the kinetics of inhibition of both human and hog renin by compound **1**, a hydroxyethylene-containing analogue of human angiotensinogen. Both enzymes rapidly form an initial EI complex with K_I of 12 nM, but a second, more strongly bound, EI complex is formed slowly with the human enzyme. This process is not seen for the hog EI complex. The differences in inhibitory mechanisms are discussed on the basis of the structure of **1** and several explanations for the slow binding of inhibitors to enzymes. Compound **1** has potential therapeutic usefulness because, in addition to being a very potent human renin inhibitor in vitro, it also possesses high protease specificity (Thaisrivongs et al., 1986) and long-lasting oral efficacy (Pals et al., 1986).

Materials and Methods

Materials

Human renin was provided by Dr. W. Hsueh, University of Southern California, and was purified according to Do et al. (1987) to a specific activity of 1100 units/mg. Hog renin (9.1 units/mg) was obtained from Dr. E. Haas, Mt. Sinai Hospital, Cleveland, OH. We are indebted to Dr. D. Tewksbury, Marshfield Medical Foundation, for providing us with purified human angiotensinogen. Hog angiotensinogen, PMSF, pNPGB, and MES were purchased from Sigma. Lyophilized human plasma, concentrated maleate buffer solution, [^{125}I]iodoangiotensin I and anti-angiotensin I coated tubes were obtained from Clinical Assays-Travenol Labs.

Methods

Boc-Pro-Phe-N^{α}-MeHis-Leuψ(CHOHCH$_2$)Val-Ile-Amp inhibitor **1** was synthesized as previously described (Thaisrivongs et al., 1986).

RIA. The quantitative determination of angiotensin I generated in the incubation mixtures was performed by using a modification of the vendor's procedure. Briefly, 100-μL aliquots were removed from the incubation mixtures at specified intervals and placed into prechilled antibody-coated tubes. Immediately thereafter a 1.0-mL aliquot of chilled [^{125}I]iodoangiotensin I tracer was added. The RIA tubes were allowed to equilibrate overnight at 4 °C, after which time the supernatants were decanted and the empty tubes counted in a gamma counter. The results from the RIA were calculated by a computer program (Rodbard et al., 1974).

Angiotensinogen Determination. The angiotensinogen content of the hog preparation was determined by reacting 5.2 μg of the preparation with excess hog renin (5.4 nM) in a total volume of 2.0 mL of 0.15 M sodium phosphate, 0.16 M NaCl and 3 mM Na$_2$EDTA at pH 6.0 buffer containing 1 mg/mL BSA. Aliquots were removed at 30-min intervals and assayed by the RIA procedure. The angiotensinogen content was calculated from an extrapolation of the plateau region of a plot of nanograms of angiotensin I vs time. The angiotensinogen content in human plasma and in the purified human angiotensinogen preparation was determined in a similar manner except that excess human renin (1.1 nM) was used to form the angiotensin I product.

Endogenous Plasma Renin Concentration. A series of incubation mixtures was prepared that contained 0.5 mL of reconstituted plasma, 5 μL of 0.3 M PMSF, 50 μL of maleate buffer, and a variable, but known, amount of human renin. Incubations lasted for 90 min followed by RIA for the angiotensin I product. A plot of product vs exogenous renin concentration was prepared with the endogenous renin concentration determined by linear regression. Units of enzyme activity were converted to moles by using a specific activity of 1200 units/mg (Slater & Strout, 1981) and a molecular weight of 37 200 (Imai et al., 1983) for pure human renin.

Time-Dependent Inhibition of Human Renin in Plasma. Lyophilized human plasma was reconstituted with sterile water and contained both renin and angiotensinogen. Incubation mixtures contained 2.1 mL of plasma, 21 μL of 0.3 M PMSF, and 0.21 mL of maleate buffer, pH 6.0. The mixture was equilibrated for at least 5 min in a 37 °C bath, and then the assay was begun by the addition of 20 μL of an appropriate dilution of inhibitor **1** in 1 mM citric acid. Aliquots were removed at time zero and periodically thereafter and assayed for angiotensin I product by the RIA procedure. Progress curves were fitted to the equation $P = P_0 + v_s t + (v_0 - v_s)[1 - \exp(-k't)]/k'$ by using the nonlinear regression program of Duggleby (1984). The parameters P_0 and P represent product concentrations at time zero and t, respectively, and v_0 and v_s represent velocities at time zero and steady state. The parameter k' represents a pseudo-first-order rate constant for the decrease in reaction velocity. The individual rate and inhibition constants were calculated as described under Results.

A similar experiment was performed with plasma spiked with purified angiotensinogen. The spiking was accomplished by the substitution of the purified human angiotensinogen in 0.05 M Tris-HCl and 0.10 M NaCl, pH 7.0, buffer for 7% of the volume of water normally used to reconstitute the plasma.

Initial Velocity Measurements for the Inhibition of Human Renin. Reactions were performed in 0.15 M MES and 0.07 M NaCl, pH 6.0, buffer containing 1.8 mM PMSF and 0.4 mM pNPGB. The stock human renin buffer was 0.10 M Tris–acetate, pH 7.4, containing 1 mg/mL gelatin. Reaction mixtures containing purified angiotensinogen and inhibitor were equilibrated in a 37 °C water bath for at least 5 min. The assay was begun upon addition of 20 μL of human renin (1.0 nM). Aliquots were removed every 20 s and placed into glass tubes containing 0.05 M Tris-HCl and 0.10 M NaCl buffer in a boiling water bath to quench the reactions. After boiling for at least 5 min, the quench tubes were allowed to cool and were then centrifuged at 2000g for 20 min. Aliquots of the supernatant were removed and assayed for angiotensin I by the RIA procedure. Initial velocities were calculated by using a least-squares regression program from the linear portion of the curves. Constants resulting from double-reciprocal plots were replotted as a function of inhibitor concentration to obtain the dissociation constant K_I (Segel, 1975).

Hog Renin Inhibition. All reactions were performed in 0.05 M sodium phosphate and 0.10 M NaCl buffer, pH 7.5, containing 3 μM PMSF. Hog angiotensinogen (1.05 μM) and inhibitor **1** were equilibrated in a 37 °C water bath for at least 5 min prior to starting the reaction with hog renin (24 pM). Aliquots were removed at time zero and periodically thereafter and assayed by the RIA procedure. Initial velocities were calculated from a least-squares regression analysis of plots of product vs time. The dissociation constant was calculated according to the method of Dixon (1953).

Results

In the absence of inhibitor the hydrolysis of human angiotensinogen by human renin reaches its steady-state velocity almost immediately (Figure 1). However, in the presence of inhibitor, there is a progressive decrease in reaction rate that varies with inhibitor concentration until the linear steady-state velocity is reached. This behavior has been termed slow-binding inhibition by Morrison (1982). Cha (1975, 1976),

Table I: Relationship of k' to Inhibitory Mechanism

mechanism	k'	inhibition constant
A	$k_2[1 + [I]/[K_I(1 + [S]/K_M)]]$	$K_I = k_2/k_1$
B	$k_4[[1 + [I]/K_I^*(1 + [S]/K_M)]/[1 + [I]/K_I(1 + [S]/K_M)]]$	$K_I = k_2/k_1$; $K_I^* = K_I[k_4/(k_3 + k_4)]$
C	$[k_2 + k_1/(1 + [S]/K_M)]\{[1 + [I]/K_I[1 + (k_2/k_1)(1 + [S]/K_M)]]/[1 + [I]/K_I]\}$	$K_I = k_4/k_3$

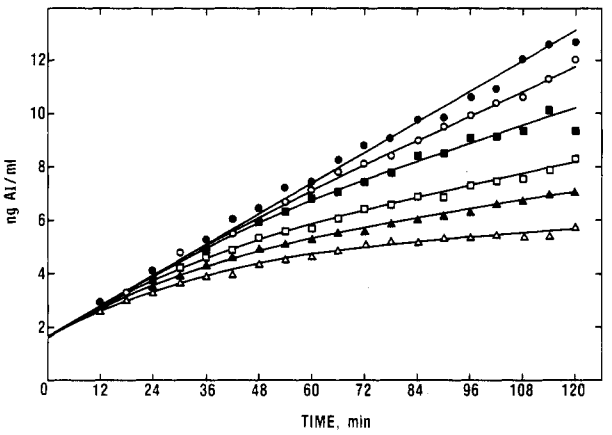

FIGURE 1: Reaction progress curves for the hydrolysis of human angiotensinogen by human renin in plasma at 37 °C and pH 6.0. Assays were initiated by the addition of 20 μL of 1 mM citric acid containing an appropriate dilution of inhibitor **1** to 2.231 mL of a preincubated solution of human plasma in pH 6.0 maleate buffer. Aliquots (100 μL) were removed at the intervals indicated and assayed for angiotensin I by an RIA procedure as described under Materials and Methods. Final concentrations of **1** were 0 (●), 0.10 (○), 0.33 (■), 0.67 (□), 1.00 (▲), and 1.33 (△) nM.

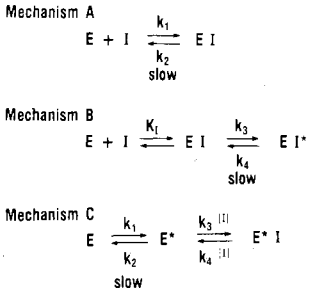

FIGURE 2: Possible mechanisms for slow-binding inhibition.

Duggleby (1982), and Morrison and Stone (1985) have shown that the progress curves can be described by

$$P = P_0 + v_s t + (v_0 - v_s)[1 - \exp(-k't)]/k' \qquad (1)$$

Fitting of progress curve data to this equation yields values for k', P_0, v_0 and v_s directly. However, the physical significance of k' depends on some knowledge of the slow-binding mechanism (Figure 2 and Table I).

In mechanism A, the enzyme–inhibitor equilibrium is established slowly either because the inhibitor concentration is very low or due to nonproductive binding of inhibitor with enzyme. In mechanism B, there is an immediate establishment of the enzyme–inhibitor complex (EI) that then slowly changes to form a new enzyme–inhibitor complex (EI*) in which the inhibitor is bound more strongly. Mechanism C postulates a slow isomerization between unbound enzyme forms prior to formation of the enzyme–inhibitor complex (E*I). Duggleby et al. (1982) have described how plots of k' vs inhibitor concentration can distinguish among mechanisms A–C. Briefly, mechanism A predicts a linear relationship with an ordinate intercept equal to k_2. Mechanisms B and C predict hyperbolic relationships but differ in that the hyperbola is concave down for mechanism B and concave up for mechanism C. The values for k' obtained from the data in Figure 1 were plotted against inhibitor concentration (data not shown). A linear

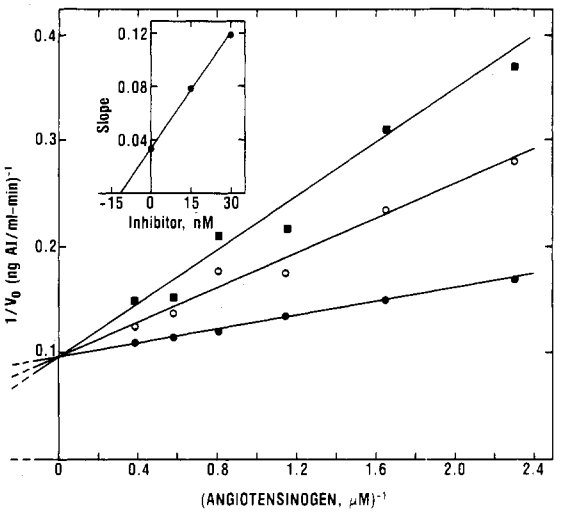

FIGURE 3: Effect of inhibitor concentration on initial velocity. Reactions were initiated by the addition of 20 μL of human renin to 0.98 mL of a preincubated solution of 0.15 M MES and 0.17 M NaCl, pH 6.0, buffer containing purified human angiotensinogen and inhibitor **1** at 37 °C. Final human angiotensinogen and renin concentrations were 0.87 μM and 1.0 nM, respectively. Final concentrations of **1** were 0 (●), 15 (○), and 30 (■) nM. (Inset) Replot of the slopes from Figure 3 against inhibitor **1** concentration to obtain the $-K_I$ from the abscissa intercept (Segel, 1975).

dependence of k' on inhibitor concentration was obtained which was characteristic of mechanism A. Linear regression gave a slope of 1.3×10^7 M^{-1} min^{-1} and an intercept of 1.7×10^{-2} min^{-1}. A value for k_2 of 2.8×10^{-4} s^{-1} was calculated directly from the intercept. The slope of the plot of k' vs inhibitor concentration is equal to $k_1(1 + S/K_M)$. The concentration of angiotensinogen in the reaction mixture was found to be 0.42 μM (data not shown). Combining this value with the K_M for angiotensinogen in human plasma of 0.800 μM (Favre & Valloton, 1973), we get $k_1 = 3.2 \times 10^5$ M^{-1} s^{-1}.

This value is somewhat less than the 10^7–10^8 M^{-1} s^{-1} expected for a diffusion-controlled, bimolecular associative process for molecules of this size (Gutfreund, 1974). It would appear that mechanism A adequately describes the slow-binding behavior of **1** with human renin. However, Morrison (1982) has noted that mechanism B can degenerate into mechanism A if K_I is much greater than K_I^* and the slow-binding investigation is conducted with inhibitor concentrations in the region of K_I^*. This can be verified by inspection of the relationships for k' in Table I. When $K_I \gg K_I^*$, k' for mechanism B has the same form as mechanism A.

An investigation of the relationship between initial velocities and inhibitor concentration can further distinguish mechanism A from B. If mechanism A is applicable, then v_0 should be independent of inhibitor concentration (Morrison & Stone, 1985). Mechanism B would show a decrease in v_0 with increasing inhibitor concentration due to instantaneous formation of the EI complex. Figure 3 shows a double-reciprocal plot which indicates that initial velocities decrease with increasing inhibitor concentration. Moreover, Figure 3 shows that **1** is a competitive inhibitor of human renin with an initial dissociation constant (K_I) of 12 nM. Thus, the mechanism of slow-binding inhibition is best explained by mechanism B. It should be noted that this experiment required, relative to the

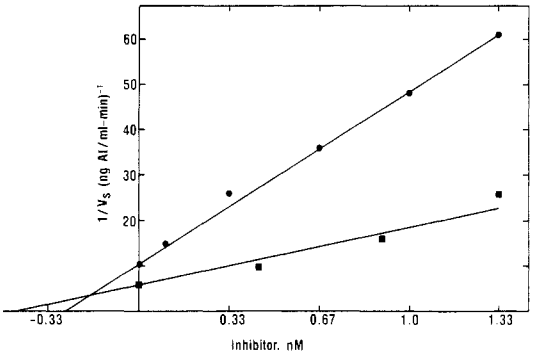

FIGURE 4: Effect of human angiotensinogen and inhibitor **1** concentration on final steady-state velocities. Nonlinear regression analysis of progress curves similar to Figure 1 yielded steady-state velocities that were plotted according to the method of Dixon (1953). Human angiotensinogen concentrations were 0.42 (●) and 1.52 (■) μM. The point of intersection of the two lines yields the overall $-K_I^*$.

experiment in Figure 1, much higher inhibitor concentrations in order to detect K_I and much higher renin concentrations to accurately measure the initial velocities.

Steady-state velocities from progress curves similar to those in Figure 1 at two different human angiotensinogen concentrations were plotted as a function of inhibitor concentration, resulting in a Dixon plot (Figure 4). Figure 4 shows that the overall K_I^* is equal to 0.19 nM and that competitive inhibition occurs at steady state as well. It should be noted that the overall K_I^* is about 64-fold smaller than the initial K_I. In this case a plot of k' vs inhibitor concentration would appear to be straight, leading to the erroneous conclusion that mechanism A is obeyed.

The value for the first-order rate constant for the reverse reaction of the slow step (designated k_2 for mechanism A or k_4 for mechanism B) was determined to be 2.8×10^{-4} s^{-1} as discussed earlier. A value for $k_3 = 1.7 \times 10^{-2}$ s^{-1} was determined from (Morrison, 1982)

$$k_3/k_4 = K_I/K_I^* - 1 \qquad (2)$$

Analysis of the k_3 and k_4 values shows that the majority of the time-dependent inhibition results from the slow rate of dissociation of the EI* complex ($t_{1/2}$ = 42 min).

Many slow-binding inhibitors also show tight-binding inhibition (Morrison, 1982). A reversible, tight-binding inhibitor exerts its inhibitory influence at concentrations comparable to that of the enzyme. Binding of inhibitor to the enzyme no longer has a negligible effect on the free inhibitor concentration. In this situation eq 1 should not be used for data analysis because the assumption that the inhibitor concentration changes by a negligible amount upon binding to the enzyme made in the derivation of eq 1 is no longer valid. In order to determine if compound **1** was a tight-binding inhibitor of human renin, we used a standard addition method and determined that the concentration of renin in the plasma was 2.0×10^{-11} M (data not shown). This value is consistent with the results from other investigations on normal human plasma (Boger et al., 1985). Inhibitor **1** was present in 5–66-fold excess during the slow-binding investigations. Thus, tight-binding should not greatly affect the estimates of the kinetic parameters obtained from the use of eq 1.

Inhibition of Hog Renin. We have previously reported (Pals et al., 1986) that preincubation of **1** with hog renin for 90 min followed by addition of hog angiotensinogen resulted in linear time courses. In that paper a double-reciprocal plot of steady-state velocity vs hog angiotensinogen showed that **1** was a competitive inhibitor of hog renin with a K_I of 12 nM. In light of the slow-binding inhibition of human renin by **1**, we

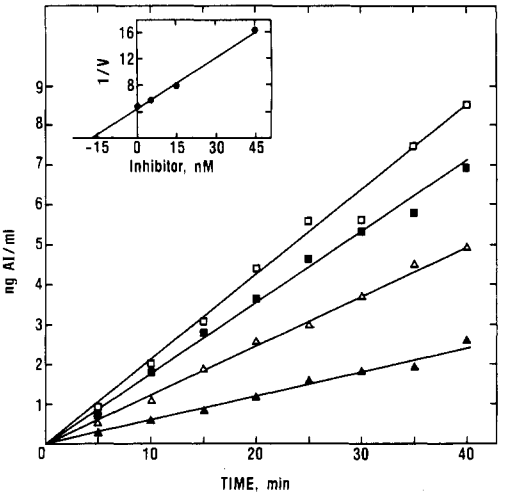

FIGURE 5: Inhibition of hog renin by **1**. Reaction progress curves obtained by the addition of 20 μL of hog renin to 0.98 mL of a preincubated solution containing 0.05 M sodium phosphate, 0.10 M NaCl, and inhibitor **1** at 37 °C and pH 7.5. Final hog angiotensinogen and hog renin concentrations were 1.05 μM and 24 pM, respectively. Concentrations of inhibitor **1** were 0 (□), 5 (■), 15 (△), and 45 (▲) nM. Aliquots (100 μL) were removed at the intervals indicated and assayed for angiotensin I by an RIA as described under Materials and Methods. (Inset) Initial velocities were calculated from the slopes of the lines in Figure 5 and plotted according to Dixon (1953). The dissociation constant, K_I, was calculated from the abscissa intercept by using a value of 3.7 μM for K_M (Pals et al., 1986).

investigated the possibility that **1** was a slow-binding inhibitor of hog renin as well. The time courses shown in Figure 5 indicate no decrease in reaction velocity with times up to 40 min. The Dixon replot of initial velocities vs inhibitor concentration (inset) resulted in a K_I of 13 nM, which is not significantly different from the K_I of 12 nM calculated when the enzyme was preincubated with inhibitor. These data suggest that the initial and steady-state velocities were equivalent. We also investigated the possibility that the slow binding might have occurred within the first 5 min of the reaction. Progress curves resulting from the assay of aliquots removed at 1-min intervals were linear as well and agreed with the velocities shown in Figure 5 (data not shown). Therefore, we found no evidence for time-dependent inhibition of hog renin by compound **1**.

DISCUSSION

The inhibition of human renin by **1** was shown to be consistent with a two-step reaction (mechanism B). This mechanism involves establishment of a rapid equilibrium for an initial EI complex followed by a slow isomerization step to form the more strongly bound EI* complex. The extent of the isomerization reaction is calculated from the k_3/k_4 value. In this case human renin binds **1** in the EI* complex 64-fold stronger than in the initially formed EI complex. In contrast, only the rapidly formed EI complex occurs with hog renin. The K_I values for the EI complexes are identical for the two enzymes (12 nM).

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \qquad \text{human renin}$$

$$E + I \overset{K_I}{\rightleftharpoons} EI \qquad \text{hog renin}$$

While the exact nature of these observations must await further study, it is possible that the differences in overall K_I and inhibition kinetics may be a consequence of the similarities in structure between **1** and human angiotensinogen (Table II).

While human renin can efficiently hydrolyze the peptide bond between positions 10 and 11 of human angiotensinogen,

Table II: N-Terminal Sequences of Human and Hog Angiotensinogen and Structure of Compound **1**

	Position												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Hog Angiotensinogen[a]	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr
Human Angiotensinogen[b]	———									Leu	Val	Ile	His
Compound 1						Boc	Pro	Phe	N-methyl His	Leuψ(CHOHCH$_2$)Val		Ile	Amp

[a] Lee and Wilson (1971). [b] Tewksbury et al. (1981).

renins from nonprimates cannot (Braun-Menendez et al., 1946). It appears that sequence differences between human and hog angiotensinogens at the C-terminal side of the renin cleavage site may partially contribute to this observation. Recent work with synthetic substrates has shown that substitution of the human angiotensinogen sequence for the hog at positions 11–13 markedly decreased substrate hydrolysis by nonprimate renins (Poe et al., 1984; Quinn & Burton, 1981). One possible explanation for these results is that some of the substrate residues in positions 11–13 interact with the renins primarily in the transition state. Perhaps the transition-state structure of human substrate hydrolysis provides interactions that, relative to human renin, are either weaker or fewer in number with nonprimate renins, thereby resulting in a decreased rate of hydrolysis. Transition-state-analogue theory (Wolfenden, 1976) would also predict that the binding of an analogue of the transition state for human substrate hydrolysis would yield greater interactions with human renin than with hog renin. Compound **1** was designed, in part, to be a transition-state analogue of human substrate hydrolysis. The Leu-Val hydroxyethylene isostere is thought to be a mimic of the high-energy tetrahedral intermediate formed in human substrate hydrolysis. If it is accepted that **1** is a transition-state analogue, then it is not surprising that **1** binds to human renin more tightly than with hog renin.

It is also significant that slow-binding inhibition was only observed with human renin. The exact nature of slow-binding inhibition for those situations that follow a two-step mechanism is unknown. Slow enzyme conformational changes, nonproductive encounters between enzyme and inhibitor, or slow extrusion of an active-site water molecule are all possible explanations.

Experimental evidence from kinetic studies (Fruton, 1976) and fluorescence measurements (Fruton, 1980) with pepsin have indicated that conformational flexibility of active-site groups plays an important role in the catalytic mechanism of aspartyl proteases. Conformational flexibility is also documented in the binding of inhibitors by aspartyl proteases. X-ray crystallographic data showed that a major conformational change occurred in penicillopepsin as a result of binding a statine-containing inhibitor (James, et al., 1982). In addition, smaller changes in conformation of amino acids at the active site have also been detected (James et al., 1983). Significantly, many of the amino acids involved in these conformational changes make contact with the inhibitor. Thus, it appears that the aspartyl proteases can exist in at least two different conformations: a ground-state conformation that allows the substrate to diffuse in and a transition-state conformation that makes a large number of contacts with the altered substrate or transition-state-analogue inhibitor. It is likely that all enzymes share this property; otherwise, they would not be efficient catalysts (Wolfenden, 1974).

Why might the binding of a transition-state analogue be slow (e.g., $k_3 = 0.017\ s^{-1}$) if the induced enzyme conformational changes that occur during its binding are similar to the rapid changes found in catalysis of substrates? Frick et al. (1986) have suggested that enzymes may have minimized the kinetic obstacles for this conformational change during the binding of substrates but that binding an analogue of the transition state is something for which the enzyme is unprepared. For the aspartyl proteases Fruton (1980) proposed that the portion of the substrate responsible for the primary specificity of the protease (e.g., Phe-Phe for pepsin or Leu-Val for human renin substrates) is bound rapidly at the active site of the enzyme. The rest of the substrate is drawn into the active site at a rate that depends on the flexibility of the active site. Considering the specific interaction of **1** with human renin, we propose that perhaps rapid binding of the Leu-Val hydroxyethylene isostere of **1** to the ground-state enzyme conformation is so favorable (e.g., K_I is approximately 1000-fold greater than the K_M for a substrate) that the enzyme becomes constrained in the attainment of its transition-state conformation. Overcoming these limitations on the active-site flexibility may occur slowly, although the transition-state conformation of the enzyme is probably reached in due course. We propose that additional binding interactions are possible between **1** and the transition-state conformation of human renin, resulting in the observed two-step binding interaction. The lack of slow-binding inhibition of **1** with hog renin might be rationalized on the basis of the proposal that **1** would make fewer contacts with hog renin than with human renin. Perhaps all the contacts are made with **1** by hog renin before it reaches its transition-state conformation. The conformational flexibility of hog renin may still be constrained upon the binding of **1**, but attainment of the complete transition-state conformation provides no additional contacts with the inhibitor. Thus, slow-binding inhibition would not be observed in this case. In a more general sense, it is possible that similar constraints on enzyme conformational flexibility produced during the binding of transition-state analogues to their respective enzymes may be occurring in those instances where the inhibition is shown to follow mechanism B.

While the above discussion has assumed that the inhibition of human renin follows mechanism B, an alternative mechanism is also possible. The mechanism involves formation of EI and EI* directly from free E and I.

$$\begin{array}{ccc} E + I & \overset{\text{slow}}{\rightleftharpoons} & EI^* \\ \downarrow\uparrow & & \\ EI & & \end{array} \qquad (3)$$

The slow "on" rate for the EI* complex could be explained by assuming that many enzyme–inhibitor encounters are nonproductive, perhaps due to the fact that the conformation of the inhibitor may be considerably different from that of the normal substrate. Indeed, one would expect that compound **1**, with a tetrahedral carbon in the center of the molecule, could adopt many more conformations than a peptide substrate. The loosely formed EI complex might arise when the enzyme binds only a few of the inhibitor's binding determinants (e.g., the N-terminal region). It is possible that compound **1** may form EI complexes with both hog and human renin, but only human renin can take advantage of the C-terminal structural features of **1** to form the tight complex EI*. Unfortunately, it is difficult if not impossible to experimentally distinguish mechanism B from the mechanism shown in eq 3.

A third explanation for the slow step is that, in addition to being an analogue of the tetrahedral intermediate, the hydroxyl group of the hydroxyethylene isostere may slowly displace a strongly bound water molecule from renin's active site. This argument was originally proposed by Rich and co-workers (Rich, 1985, Rich et al., 1985) to account for the slow-binding inhibition of pepsin by pepstatin. In theory, the favorable increase in entropy gained from returning a strongly bound active-site water molecule to bulk solvent could produce 3–5 kcal/mol of energy, which could stabilize the EI* complex by as much as 4000-fold. Although this explanation is theoretically possible, more experimental evidence is required to support it. It remains to be determined if hydroxyethylene isostere containing inhibitors displace the reactive water molecule from the enzyme's active site. If such a process were occurring during the binding of **1** to human renin, then perhaps the structure of the C-terminal portion of **1** would not allow the identical positioning of **1** in the active site of hog renin such that the bound water molecule would not be displaced. We anticipate that the X-ray crystal structures of human renin and of the human renin/**1** complex will eventually be solved and provide more information about the slow-binding phenomenon as well as contribute to a rational guide for the design of future renin inhibitors.

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