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Comparison of Angiotensinogen and Tetradecapeptide as Substrates for Human Renin

Substrate Dependence of the Mode of Inhibition of Renin by a Statine-Containing Hexapeptide

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The kinetic properties of two different substrates for human renin, a synthetic tetradecapeptide and the natural substrate human angiotensinogen, have been compared. While the V_{max} was similar for the two substrates, the K_m values differed by a factor of 10, i.e., 11.7 ± 0.7 μM (tetradecapeptide) and 1.0 ± 0.1 μM (angiotensinogen). The mode of inhibition of renin by a statine (Sta)-containing hexapeptide, BW897C, that is a close structural analog of residues 8-13 of human angiotensinogen (Phe-His-Sta-Val-Ile-His-OMe), was determined for the two substrates. Competitive inhibition was observed when tetradecapeptide was the substrate (K_i = 2.0 ± 0.2 μM), but a more complex mixed inhibition mode (K_i = 1.7 ± 0.1 μM, K_i' = 3.0 ± 0.23 μM) was found with angiotensinogen as substrate. This mixed inhibition probably results from the formation of an enzyme-inhibitor-substrate or enzyme-inhibitor-product complex and reflects the more extensive interactions that the protein angiotensinogen, as opposed to the small tetradecapeptide substrate, can make with renin. We conclude that the mixed inhibition observed when angiotensinogen is used as renin substrate could be important in the clinical application of renin inhibitors because it is less readily reversed by increased concentrations of substrate than is simple competitive inhibition.

The renin-angiotensin-aldosterone system has an important role in the regulation of blood pressure (1). Angiotensinogen, a plasma protein, is first cleaved by renin (EC 3.4.23.15), to give the decapeptide angiotensin I, which subsequently has the C-terminal dipeptide removed by angiotensin converting enzyme (EC 3.4.15.1), to give the potent vasoconstrictor angiotensin II.

Inhibitors of components of the system have been sought as a means of treating hypertension. To date, the most successful strategy has involved the inhibition of angiotensin converting enzyme. Thus drugs such as captopril and enalapril are clinically useful in treating hypertension and congestive heart failure (2).

Attention is now turning increasingly toward the development of renin inhibitors (3). These, as well as being of possible therapeutic benefit, may also help in defining more exactly the role of the renin-angiotensin system in the control of blood pressure. This is because renin is known to cleave only angiotensinogen *in vivo*, whereas angiotensin converting enzyme has a wider substrate specificity and will cleave, in addition to angiotensin I, other vasoactive peptides such as bradykinin.

Renin is a 40-kDa aspartyl protease synthesized in the juxtaglomerular cells of the kidney. It is released into the circulation both as an active form and as an inactive precursor (4). Angiotensinogen is synthesised in the liver; the human protein has a molecular mass of approx 64 kDa and con-

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tains 452 amino acids and 14% carbohydrate (5, 6). In addition to being a renin substrate, it is also a substrate for a granulocyte cathepsin G (7).

Human angiotensinogen has proved difficult to purify (9) and thus many test systems for renin inhibitors *in vitro* make use of small synthetic substrates (10, 11), commonly a tetradecapeptide based on the N-terminal sequence of equine angiotensinogen (12). The first 14 amino acid residues of human angiotensinogen differ from this in the four positions at the C-terminal side of the scissile bond, i.e., Val^{11}-Ile^{12}-His^{13}-Asn^{14} instead of Leu^{11}-Val^{12}-Tyr^{13}-Ser^{14} with a putative glycosylation site on Asn^{14} (6).

The question arises as to whether the synthetic substrate represents in all respects the natural substrate, and in particular how closely an assay system using the synthetic substrate *in vitro* relates to renin inhibition *in vivo*. Different substrate K_m values and different modes of inhibition of renin could result in variations of renin inhibition observed *in vitro* and *in vivo*. Literature data show large variations in measured K_m values for both angiotensinogen and tetradecapeptide; these range at physiological pH from 5.7 to 0.4 μM for human angiotensinogen (13–18) and 32 to 3.1 μM for TDP[2] (19, 17, 20). Most of these studies have used relatively crude renin preparations which probably contain other protease activities. The angiotensinogen used has also mainly been of low purity. The presence of possible endogenous renin inhibitors (15, 21) could affect measurements of K_m. Many of these studies have derived kinetic parameters from unweighted double-reciprocal plots. Of the methods of analyzing kinetic data this has been shown to be the least sound statistically, and can give inaccurate K_m and V_{max} values (22). We describe here experiments using highly purified human kidney renin which compare equine TDP with both partially and fully purified human angiotensinogen as renin substrates. Nonlinear least-squares and median-estimate methods have been used to analyze the data in preference to linear plots.

Potent renin inhibitors have been developed by the incorporation of a nonhydrolyzable group at the scissile bond of a substrate fragment. For example the incorporation of statine (11), or other modified peptides (23, 25) into part of the angiotensinogen sequence has been reported. Although there have been some reports of the mode of inhibition of renin by various inhibitors (10, 31, 32), it has yet to be established rigorously whether the same mechanism of inhibition of renin occurs with both synthetic and natural substrates. Accordingly we have synthesized a statine-containing hexapeptide based on part of the human angiotensinogen sequence (residues 8–13) and have investigated how its inhibition of human renin varies with different substrates.

MATERIALS AND METHODS

Human kidney renin was prepared by the method of McIntyre *et al.* (24) with the modification described previously (25).

Partially purified human angiotensinogen was prepared from fresh human plasma using anion-exchange chromatography on DEAE-Sepharose equilibrated with 0.1 M Tris-HCl, 30 mM NaCl, pH 6.6, followed by albumin removal on Con A-Sepharose (26). The angiotensinogen had a specific angiotensin I content of 0.9 μg/mg protein.

Highly purified human angiotensinogen was obtained using three additional column steps: hydrophobic interaction chromatography on phenyl-Sepharose (27), adsorption chromatography on hydroxyl apatite-Ultrogel equilibrated in 10 mM phosphate, pH 6.4, followed by gradient elution with 0.1 M potassium phosphate, pH 6.2. The final step was gel permeation chromatography on Sephacryl S-200 equilibrated in 0.1 M potassium phosphate, 0.02% sodium azide, pH 7.4. Angiotensinogen was obtained in 5% yield with a specific angiotensin I content of 20.8 μg/mg protein, close to the theoretical maximum of 21 μg/mg protein. Sequence analysis of the first 10 residues gave the known N-terminal sequence of human angiotensinogen (27).

BW897C was synthesised by standard sequential coupling to the N-terminus to give Boc-Sta-Val-Ile-His-OMe. After deprotection further N-terminal elaboration was performed by coupling Boc-Phe-$His-N_3$ under standard Rudinger coupling conditions (28).

[2] Abbreviations used: TDP, equine tetradecapeptide; AI, angiotensin I; Boc, *tert*-butyloxycarbonyl; Sta, statine; OMe, methyl ester; PMSF, phenylmethylsulfonyl fluoride.

Purification was carried out by reverse-phase HPLC and deprotection by standard acidolytic methods. The structure was confirmed by amino acid analysis, ^1H NMR, mass spectrometry, and elemental analysis.

Equine tetradecapeptide was obtained from Beckman. Other chemicals were from Sigma and BDH.

The radioimmunoassay for angiotensin I was carried out using assay kits obtained from Travenol-Genentech. Samples were counted to constant standard deviation in an LKB Wallac 80000 gamma counter interfaced to an Apple IIe computer to provide automated data collection.

K_m determination and inhibition studies. The reaction was carried out at pH 7.4. Substrates, inhibitors, and enzyme were equilibrated in 0.1 M potassium phosphate buffer containing 0.01% (w/v) Tween 20 and 1 mM PMSF. The reaction was started by addition of renin and samples were incubated at 37°C for 60 min. The reaction was stopped by cooling the samples to 0°C and dilution for the radioimmunoassay of angiotensin I.

Initial estimates of K_m were made using a wide range of substrate concentrations. More accurate K_m and V_{max} values were determined using five substrate concentrations in the range of $[S]/K_m$ from 0.2 to 5 separated by equal intervals on a log scale. Five replicate measurements were made at each substrate concentration. A corresponding set of renin free blanks was set up simultaneously. Each experiment for tetradecapeptide and partially-purified angiotensinogen was carried out on three separate occasions. A single experiment using highly purified angiotensinogen was performed because of the limited supply of material.

For the inhibition studies both substrate and inhibitor concentrations were varied according to a four by four matrix up to a maximum $[S]/K_m$ of between 4 and 5, the value of $[I]/K_i$ being varied from 0 to 7. Three replicate measurements were made at each [I], [S] value and also a corresponding set of renin free blanks. Each experiment was repeated on three separate occasions with a single experiment with highly purified angiotensinogen.

Studies of the time course of both inhibited and uninhibited reactions were made with different substrate concentrations by removing samples at 10 min intervals up to 1 h.

Analysis of kinetic data. The mean values of net initial rate measurements were fitted to the appropriate rate equations using a nonlinear least-squares minimization program written for the Apple II by Dr. R. Wootton. The program was modified to incorporate a number of inhibition models including the four most commonly considered types of inhibition: competitive, uncompetitive, mixed (competitive/uncompetitive), and pure noncompetitive. Both proportional weighted and unweighted fits were used. The model that fitted the inhibition data best was determined by comparison of the residual sum of squares for different inhibition models obtained after refinement. Generally the inhibition model that had the smallest sum of squares also gave a more even run of signs of the residuals and values of K_m and V_{max} closer to those previously determined in experiments without inhibitors.

The data were also analyzed by the median estimate method of Cornish-Bowden and coworkers (29). A program for this was written for the Apple II (D. K. Stammers, unpublished work). The program calculates K_m (app) and V_{max} (app) together with 68% confidence limits. K_i and K'_i values for inhibition data are calculated from the median regression lines for K_m (app)/V_{max} (app) against [I] and $^1/V_{max}$ (app) against [I]. The inhibition mode could be deduced from the relative values of K_i, K'_i and the maximum concentration of inhibitor used, together with the "spread" of values for the inhibitor constants observed in the secondary plots.

RESULTS

Studies of the time course of the reaction, both with and without inhibitor, showed that this was essentially linear over the time period used for the measurement of initial rates. The depletion of substrate for the uninhibited reaction with angiotensinogen was 1.9, 3.4, 5.6, 7.9, and 10.4% for the five different substrate concentrations (5.26, 2.38, 1.05, 0.47, and 0.21 μM, respectively). With TDP as substrate depletion was 1% or less in all cases. These data indicate that the substrates and inhibitors are in rapid equilibrium with the enzyme in relation to the time of the experimental measurements, and that there is not enough depletion of substrate or buildup of product to cause significant slowing of the reaction; this indicates that equilibrium and initial rate assumptions made in deriving the kinetic equations used to analyze the data hold in this instance.

The K_m and V_{max} values obtained for the two substrates are shown in Table I. The different methods of data analysis, i.e., least squares and median estimate, gave values for K_m and V_{max} that agreed within 5%. Proportional-weighted least squares gave values closer to the median method than did unitary weighting and hence was used for analysis of subsequent data. Plots of initial rate as a function of substrate concentration are shown in Fig 1.

TABLE I

K_m AND V_{max} VALUES FOR DIFFERENT RENIN SUBSTRATES FROM NONLINEAR LEAST SQUARES

Substrate	K_m (μM) Means (±SE, n = 3)	K_m (μM) Range	V_{max} (ng AI ml^{-1} hr^{-1}) Means (±SE, n = 3)	V_{max} (ng AI ml^{-1} hr^{-1}) Range
1. Tetradecapeptide	11.7 ± 0.7	10.7-13.1	197 ± 15	179-227
2. Angiotensinogen				
a. Partially purified	1.0 ± 0.1	0.84-1.2	163 ± 9	153-181
b. Highly purified	0.9 ± 0.08[a]	—	143 ± 7[a]	—
Overall mean	1.0 ± 0.08		158 ± 8	

[a] Value from a single experiment, standard error from least squares.

For the inhibition of renin by BW897C a comparison of different inhibition models in terms of the residual sum of squares is shown in Table II. This shows clearly that with tetradecapeptide as renin substrate, the inhibition observed by BW897C fits better to competitive inhibition than uncompetitive or pure noncompetitive inhibition. The mixed inhibition model reverts to competitive inhibition since, in this case, K_i' tends to infinity and the remaining parameters then become very similar to the competitive model. In contrast, for angiotensinogen as substrate, mixed inhibition is a much better fit to the data than competitive or uncompetitive inhibition. A small but consistent preference is shown for mixed inhibition when compared with pure noncompetitive inhibition. The same result was observed with both partially and highly purified angiotensinogen. The inhibitor constants obtained for BW897C from least squares fitting are shown in Table IIIA. Table IIIB shows the K_i and K_i' values together with confidence limits obtained using the median estimate method to analyze the inhibition data. It can be seen that for TDP as substrate the K_i value is well defined. By contrast K_i' is much larger with a wide spread of values (including one negative) giving rise to a large standard error that is in fact larger than

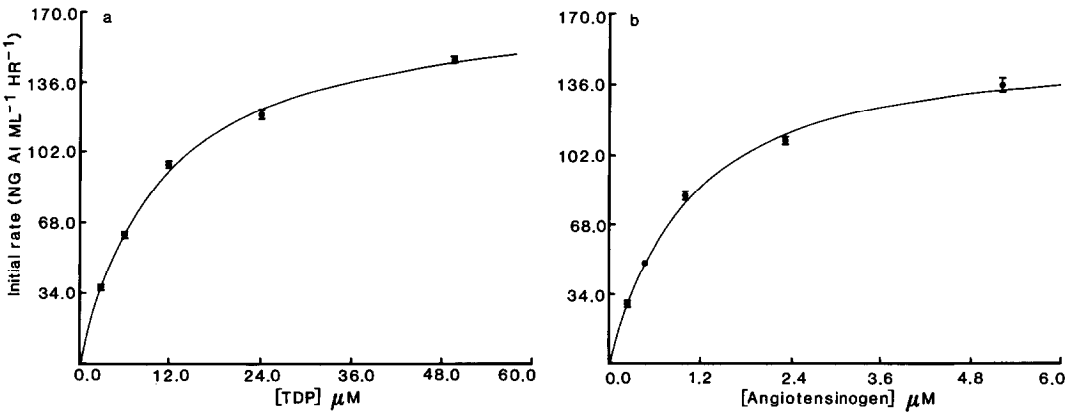

FIG. 1. Initial velocity of the human renin reaction as a function of (a) tetradecapeptide and (b) angiotensinogen concentration. Observed points are shown together with error bars (omitted when less than the symbol size). Lines are calculated from the parameters obtained by least squares minimization.

TABLE II

SUBSTRATE-DEPENDENT INHIBITION OF RENIN BY BW897C: COMPARISON OF INHIBITION MODELS FROM LEAST SQUARES FITTING USING PROPORTIONAL WEIGHTING

Substrate	Experiment no.	Weighted sum of squares / Degrees of freedom $\left(\Sigma \frac{(v_{calc}-v_{obs})^2}{v_{calc}+v_{obs}} \Big/ (n-p)^a\right)\times 10^{-4}$			
		Competitive	Uncompetitive	Mixed	Pure noncompetitive
TDP	1	16	569	—[b]	117
	2	11	510	—[b]	61
	3	20	587	—[b]	120
Angiotensinogen					
a. Partially purified					
	1	249	277	16	22
	2	307	378	22	26
	3	249	398	11	19
b. Highly purified					
	1	259	341	16	23

[a] v = initial rate, n = number of observations, p = number of parameters.
[b] Reverts to competitive inhibition.

the K_i' value itself. This indicates that the K_i' parameter is undefined and hence the inhibition mode is competitive. With angiotensinogen as substrate both K_i and K_i' are well defined, consistent with mixed inhibition.

Plots of initial rate as a function of substrate concentration at different concentration of inhibitor are shown in Fig 2.

DISCUSSION

The K_m value for angiotensinogen determined here of 1.0 ± 0.1 μM can be compared with values previously reported at physiological pH, viz 5.7 μM (13) 4.1–2.8 μM (14), 1.4 μM (15), 0.8 μM (16), 0.5 μM (17), and 0.4 μM (18).

The similar K_m values obtained for both partially purified and fully purified angiotensinogen indicate there are no endogenous inhibitors present in the partially purified material and hence that in this respect the latter is suitable for kinetic studies with renin.

With equine tetradecapeptide the value of K_m is 11.7 ± 0.7 μM. Other values determined at physiological pH are 32 μM (19), 25 μM (17), and 3.1 μM (20).

By contrast the V_{max} is similar for both substrates, this being consistent with there being the same rate-limiting step in both cases.

The *in vitro* assay system described by us previously for assessing renin inhibitors (25), will give IC_{50} values that are close approximations to K_i values for competitive inhibitors because it uses a low concentration of TDP (0.5 μM) compared to its K_m (11.7 μM).

The difference in K_m values might be largely explained by a difference in affinity of the two substrates for the enzyme. The sequence differences present at positions 11–14 of the substrate might give rise to this apparent difference in affinity; alternatively this difference might be due to extra interactions made by angiotensinogen with renin sites away from the active site region. The fact that the K_m and V_{max} values for equine tetradecapeptide and human tridecapeptide are very similar (19) would indicate that the latter possibility is more likely.

From a hypothetical model of renin, based on the amino acid sequence of human renin fitted to the known three-dimensional structure of the related aspartyl

TABLE III

INHIBITOR CONSTANTS DETERMINED FOR BW897C WITH RENIN USING DIFFERENT RENIN SUBSTRATES AND DIFFERENT METHODS OF DATA ANALYSIS

	A. Nonlinear least squares				B. Median estimate			
	K_i (μM)	Range	K'_i (μM)	Range	K_i (μM)	Range	K'_i (μM)	Range
Substrate								
1. Tetradecapeptide	2.0 ± 0.2	1.7 to 2.3	—[a]	—	1.7 ± 0.2	1.2 to 2.0	29.4 ± 58.0	−73 to 126
2. Angiotensinogen								
a. Partially purified	1.7 ± 0.2	1.5 to 2.0	2.9 ± 0.4	2.4 to 3.6	1.5 ± 0.3	1.0 to 2.1	3.8 (±0.4)	3.4 to 4.5
b. Highly purified	1.6 ± 0.2[b]	—	3.0 ± 0.4[b]	—	1.8 (+0.1,-0.6)[c]	—	4.7 (+1.7,−1.0)[c]	
Overall mean (2a and 2b)	1.7 ± 0.1 (n = 4)		3.0 ± 0.3		1.6 ± 0.3 (n = 4)		4.0 (±0.3)	

Note. K_i and K'_i values are all means (±SE, n = 3) unless otherwise stated.

[a] Value tends to infinity.

[b] Value from a single experiment, standard error from least squares.

[c] Median value from a single experiment, nonparametric 68% confidence limits.

protease, endothiapepsin (30), Blundell and co-workers have identified at least two regions outside the active site that might interact with angiotensinogen. These include a basic region of Lys^{240}, Lys^{241}, and Arg^{242}, as well as a conformationally rigid proline rich region (residues 293-298).

The renin inhibitor BW897C described here, is a close structural analog of the cleaved region of human angiotensionogen (residues 8 to 13). It thus would be expected to be a simple competitive inhibitor of renin. Although this was observed to be the case with tetradecapeptide as substrate a more complex mixed inhibition pattern was found when angiotensinogen was substrate. The reproducibility of this effect over different experiments together with the consistent results when using either nonlinear least-squares or median-estimate methods gives us confidence that this substrate-dependent mode of inhibition of renin is real. We have also observed that the mixed inhibition mode is not dependent on the presence of Tween 20, as mixed inhibition was also found in experiments in which Tween was replaced by 0.05% lysozyme.

The observed mixed inhibition is consistent with a number of possible mechanisms that involve the binding of inhibitor to more than one form of the enzyme. These could include the formation of an enzyme-inhibitor-substrate complex (EIS) complex, the simultaneous binding of two inhibitor molecules to the enzyme, or inhibitor binding to an enzyme-product complex. In each case these ternary complexes are formed in addition to an enzyme-inhibitor (EI) complex.

The explanation of mixed inhibition in terms of an EIS complex as well as an EI complex seems feasible in the case of renin and angiotensinogen, since, as described above, there is evidence of interactions between the two proteins occurring outside the immediate active site region. Thus, if the N-terminal region of angiotensinogen could move out of position, the inhibitor might be accommodated together with the substrate. In contrast, this would not be possible with the tetradecapeptide because this cannot make the same interactions as

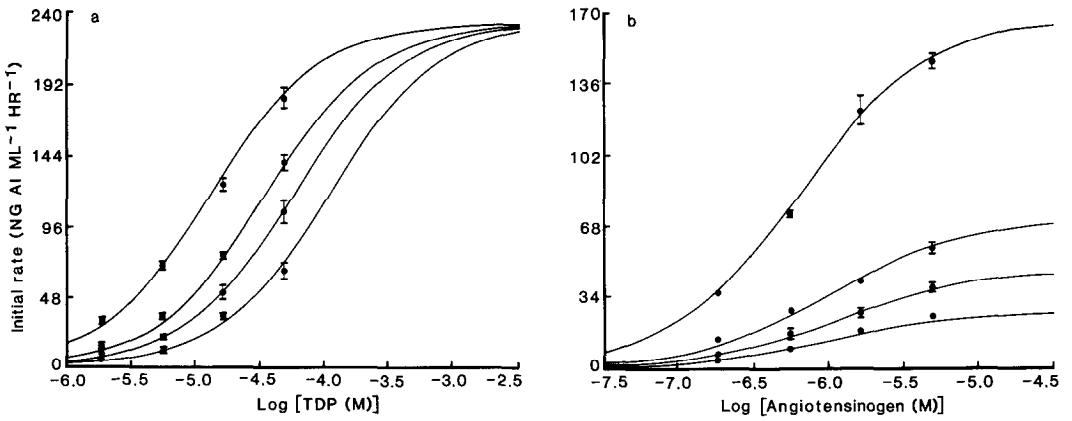

FIG. 2. Pattern of inhibition of human renin by BW697C with (a) tetradecapeptide and (b) angiotensinogen as substrate. Conditions of substrate and inhibitor concentrations are shown in the text. Observed points are shown together with error bars (omitted when less than the symbol size). Lines are calculated from the parameters obtained by least squares minimization.

angiotensinogen away from the active site of renin. An alternative mechanism would be if the substrate binds to renin as normal but the inhibitor then attaches to this renin-angiotensinogen complex away from the active site causing a reduction in catalytic turnover rate. In this case the EIS complex could also break down to product but at a reduced rate when compared to the ES complex. When the data were fitted to such a partially mixed inhibition model, however, the rate constant for conversion of EIS to product tended to zero. It was not possible to investigate the stoichiometry of inhibitor binding to renin since only a few micrograms of enzyme were available.

There is evidence that des-angiotensin I-angiotensinogen, the by-product of the renin-catalyzed cleavage of angiotensinogen might be a potent inhibitor of human renin (15), although this has not been observed in other studies (8, 18). If BW897C is able to form a renin-inhibitor-[des-angiotensin I-angiotensinogen] (EIP) complex this could explain the observed mixed inhibition. It seems likely that the inhibitor could be accommodated more easily in this EIP complex than an EIS complex since the AI portion of the substrate has been removed in the former.

It is not known if this substrate-dependent mode of inhibition is a general phenomenon for all renin inhibitors. Preliminary results with tetrapeptide and octapeptide inhibitors related to BW897C show the same substrate dependent modes of inhibition. Similar K_i/K_i' ratios were observed for these as for BW897C when angiotensinogen was used as renin substrate. It will be of interest to see if this K_i/K_i' ratio alters in different series of renin inhibitors.

The occurrence of mixed inhibition of renin *in vivo*, i.e., with angiotensinogen as substrate, could be important in the therapeutic application of renin inhibitors. This is because a mixed inhibitor, with $K_i \simeq K_i'$, is more effective than a simple competitive inhibitor when the substrate concentration is at or above the K_m. Normal plasma levels of angiotensinogen are $\simeq 1$ μM (13), a value comparable with the K_m determined in this work. There is also evidence that, in certain hypertensive states, the level of angiotensinogen is significantly increased (1). With a mixed inhibitor of the type described here, the degree of inhibition of renin will be virtually independent of substrate level, whereas, by contrast, a competitive inhibitor of comparable affinity for the enzyme will be two to three times less effective under these conditions.

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