Biochimica et Biophysica Acta 952 (1988) 8–12
Elsevier

BBA 33020

The amino-acid residues on the C-terminal side of the cleavage site of angiotensinogen influence the species specificity of reaction with renin

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(Received 21 July 1987)

Key words: Renin; Angiotensinogen; Tetradecapeptide; Enzyme kinetics; Species specificity

The N-terminal sequences of human and canine angiotensinogen and two hybrid sequences were synthesized and used to determine whether the species specificity of renin is influenced by amino-acid residues adjacent to the cleavage site. k_{cat}/K_m for the generation of angiotensin I from the N-terminal tridecapeptide of human angiotensinogen by canine renin is 0.37% of that observed when the N-terminal tetradecapeptide from canine angiotensinogen is used as a substrate. Replacement of the valine residue at P_1' in the human tridecapeptide with the leucine residue from the canine sequence triples k_{cat} and improves K_m 4-fold. Replacement of isoleucine residue at P_2' with the valine residue from the canine sequence enhances K_m 8-fold. Substitution of the histidine residue at P_3' with the tyrosine serine sequence of canine angiotensinogen increases k_{cat} an order of magnitude. Results obtained with the synthetic substrate are similar to those observed with the protein substrates. Canine renin does not cleave human angiotensinogen. Also, k_{cat}/K_m of canine renin for its homologous substrate is about 6-times greater than the k_{cat}/K_m value for human renin acting on human angiotensinogen.

Introduction

The only known naturally occurring substrate for renin (EC 3.4.23.25, formerly EC 3.4.99.19) is angiotensinogen [1]. Angiotensinogen isolated from most species is cleaved by human renin. Human angiotensinogen, however, is not hydrolyzed by non-primate renin [2]. This is termed species specificity.

Skeggs et al. [3] showed that synthetic peptides containing the N-terminal amino-acid sequence of angiotensinogen were renin substrates. The amino-acid sequences on the N-terminal side of the cleavage site of angiotensinogen from most species are identical, and changes in this portion of the molecule cannot explain species specificity (Fig. 1).

The recent publication of the amino-acid sequence of canine angiotensinogen [4] allowed synthesis of peptides which were used to determine how changes in specific amino-acid residues on the C-terminal side of the cleavage site affected the cleavage rates by human and canine renin.

A preliminary account of this work which contains some incorrect values was presented at the Seventh American Peptide Symposium [7].

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Experimental

Peptide synthesis

Procedures used for peptide synthesis and purification have been described [5].

The N-terminal tetradecapeptide sequence from canine angiotensinogen [4] and [Ile^5]angiotensin I were purchased from Peninsula (San Mateo, CA) and had the expected amino-acid composition.

*Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Val–Ile–His (Human angiotensinogen(1–13)tridecapeptide); **1***

Boc-His(Dnp) was attached to the support (22.4 μmol/g) and the peptide chain elongated by standard techniques [5]. [^{3}H]Proline was incorporated at position 7. On completion of synthesis, 3.05 g peptidyl polymer were treated with 1 M sodium thiophenoxide in dimethylformamide for 1 h at room temperature to remove the 2,4-dinitrophenyl (Dnp) group [6]. Peptide was then cleaved from the polymer with HF/10% anisole for 1 h at 0 °C. After evaporation, the reaction product was transferred to a sintered glass funnel and sequentially extracted with ethyl acetate, 1, 5, 10 and 25% (v/v) acetic acid solutions. 95% of the radioactivity was in the 1% acetic acid extract which was lyophilized to yield 51.2 mg crude **1**. This was gel filtered on Sephadex G-50 (2.5 × 100 cm) in 10% acetic acid solution and fractions eluting between 420–500 ml pooled and lyophilized. The product (22%) was purified to homogeneity by semi-preparative HPLC (ODS, 1 × 25 cm) at a flow rate of 2.0 ml/min using a linear gradient between 20 and 50% of CH_3CN/0.1% CF_3COOH, and water/0.1% CF_3COOH, for 26 min.

D, 1.06; P, 1.09; V, 1.77; I, 1.61; L, 1.09; Y, 0.91; H, 2.05; R, 1.04.

R_F: T2, 0.30; T3, 0.56; T4, 0.07. Spec. radioactivity 0.437 Ci/mol. $[M]_{589}$, −2349°. ε_{280}, 1946.

*Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Ile–His; **2***

2 was prepared with the Boc-His(Dnp)-polymer used to synthesize **1**. On completion of synthesis and removal of the Dnp group, 2.75 g peptidyl polymer were treated with HF/anisole. Sequential extraction and lyophilization of the 5% extract yielded 72.3 mg crude **2** (71%). This was gel filtered on Sephadex G-50 (25 × 100 cm) in 5% acetic acid solution, and fractions eluting at 500–540 ml were pooled and lyophilized to yield 60 μmol peptide. This was purified to homogeneity using the semi-preparative HPLC system described for **1**, for 18 min.

D, 1.07; P, 1.19; V, 0.96; I, 1.82; L, 2.17; Y, 0.96; F, 1.02; H, 2.90; R, 1.28.

R_F: T2, 0.31; T3, 0.57; T4, 0.09. Spec. radioactivity 0.0852 Ci/mol. $[M]_{589}$, −1977°. ε_{280}, 1802.

*Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–His; **3***

3 was prepared with the Boc-His(Dnp)-polymer used to synthesize **1**. On completion of the synthesis and removal of the Dnp group, 2.79 g peptidyl polymer treated with HF/anisole for 1 h at 0 °C. Lyophilization of the 1% extract from cleavage yielded 164 mg crude **3**. Of this material, 101 mg were gel filtered on Sephadex G-50 as described for **1** and the fractions eluting between 510–540 ml were lyophilized. This material was then purified to homogeneity by semi-preparative HPLC using the system described for **1**, for 16 min.

D, 1.05; P, 1.01; V, 2.02; I, 0.97; Leu, 2.28; Tyr, 0.79; Phe, 0.95; H, 2.98; R, 1.04.

R_F: T2, 0.27; T3, 0.56; T4, 0.10. Spec. radioactivity 0.0892 Ci/mol. $[M]_{549}$, −2416°. ε_{280}, 2217.

Enzyme kinetics

Cleavage of both the angiotensinogens and the synthetic angiotensin analogs was carried out in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5% bovine serum albumin as described previously [5]. The concentration of angiotensin I was determined by RIA (New England Nuclear, Boston, MA). The appropriate controls for cross-reactivity were run in all cases. Data from the RIA were fitted to a double-reciprocal plot using an unweighted linear regression which was solved for K_m and V_{max}. The velocity of the reaction was expressed as mol angiotensin I/mol renin per min which gives identical values for V_{max} and turnover number. For all equations, r^2 was greater than 0.98.

Homogeneous human kidney renin was obtained from E.E. Slater and contained no proteinases capable of degrading either [^{14}C]methe-

moglobin (New England Nuclear) or [Ile^5]angiotensin I as measured by RIA and HPLC. Standardization against human renin obtained from the Medical Research Council (London, U.K.) showed that the specific activity of the human renin preparation was $3.6 \cdot 10^{10}$ GU/mol (Goldblatt units/mol).

Partially purified canine renin and angiotensinogen were obtained from V.J. Dzau. The renin preparation contained proteinases capable of degrading both angiotensin I and [^{14}C]methemoglobin. A previous report from this laboratory [7] was not corrected for destruction of angiotensin I and the kinetic constants published for canine renin are in error. Addition of 2,3-dimercaprol, phenylmethanesulfonyl fluoride and EDTA to the reaction mixture prevents proteolysis of both the methemoglobin and angiotensin I. The specific activity of homogeneous canine renin was calculated to be $1.6 \cdot 10^{11}$ GU/mol [8].

Concentration of synthetic substrates was determined by amino-acid analysis. Concentration of angiotensinogen was determined by complete proteolysis with renin and measurement of angiotensin I formed.

Results

Cleavage of human and canine angiotensinogen and the synthetic substrates by renin from both species exhibited Michaelis-Menten kinetics. Data were fitted by an unweighted linear regression and had correlation coefficients (r^2) greater than 0.98. K_m and k_{cat} values are given in Table I. K_m and k_{cat} values for cleavage of the synthetic substrates are given in Table II.

Discussion

The rate at which renin generates angiotensin I from angiotensinogen depends on the species from which the substrate and enzyme are derived. This is termed species specificity [2], and can result from the substrate, proteinase or both. Canine

TABLE I

KINETIC PARAMETERS FOR CLEAVAGE OF ANGIOTENSINOGENS BY RENIN

k_{cat}, mol angiotensin I/mol renin per min; k_{cat}/K_m, 10^6/mol per min. The reactions are performed in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5% albumin at 37 °C.

Angiotensinogen	Human renin			Canine renin		
	K_m (μM)	k_{cat}	k_{cat}/K_m	K_m (μM)	k_{cat}	k_{cat}/K_m
Human	2.5	2.8	1.1	not cleaved		
Canine	0.23	1.4	6.3	0.38	14	38

TABLE II

KINETIC PARAMETERS FOR CLEAVAGE OF ANGIOTENSINOGENS BY RENIN

k_{cat}, mol angiotensin (ANG) I/mol renin per min; k_{cat}/K_m, 10^6/mol per min. The reactions are performed in 0.1 M Tris-HCl buffer (pH 7.5) at 37 °. TDP, tetradecapeptide.

	Substrate	Human renin			Canine renin		
		K_m (μM)	k_{cat}	k_{cat}/K_m	K_m (μM)	k_{cat}	k_{cat}/K_m
1	ANG I-Val-Ile-His	24	12	0.50	170	10	0.06
2	ANG I-Leu-Ile-His	15	11	0.73	41	28	0.69
3	ANG I-Leu-Val-His	16	13	0.80	5.5	15	2.9
TDP	ANG I-Leu-Val-Tyr-Ser	33	13	0.37	7.0	112	16

angiotensinogen is cleaved by both human and canine renin with similar K_m values (Table I). The k_{cat} value is an order of magnitude greater for the canine substrate. Morris and co-workers [9] obtained a K_m value of 1.8 μM for cleavage of canine angiotensinogen by canine renin. The difference between this value and that reported here could be due to the more acidic pH employed by Morris et al. for the reaction (pH 5.5). The K_m value of the human substrate for the human enzyme is about an order of magnitude larger than observed with canine angiotensinogen, while k_{cat} is 2-fold greater. Human angiotensinogen is not a substrate for canine renin, and its K_m is not available for comparison. Skinner et al. [10] reported that human angiotensinogen does not inhibit the cleavage of ovine angionensinogen by ovine renin, and thus does not bind to this species of non-primate renin. k_{cat}/K_m, and ultimately species specificity, appear to be controlled more by the substrate than by the enzyme.

K_m values for the cleavage of synthetic tetradecapeptide by human renin are in agreement with published values [5]. The k_{cat} for the cleavage of synthetic substrates by renin is rarely reported, although Poe and co-workers [11] found that human renin cleaved tetradecapeptide at 7.5/min and synthetic human angiotensinogen(1–14)tetradecapeptide at 51/min [11]. The 2.5-fold increased in k_{cat}/K_M is slightly greater than the 1.4-fold increase reported here. Mouse submaxillary renin does not hydrolyze the human homologue, but cleaves tetradecapeptide about 1000-times more rapidly than does human renin. Inagami and Murakami [12] obtained a value of about 280 per min for the cleavage of *N*-acetyltetradecapeptide by hog renin at pH 5.4. The differences between the various values for k_{cat} can probably be accounted for by differences in the substrate and reaction pH.

The human angiotensinogen analog is hydrolyzed much less efficiently by canine renin than the canine sequence. k_{cat}/K_m for hydrolysis of **1** is only 0.4% of that observed for tetradecapeptide.

Replacement of the valine at P_1' in the human sequence (**1**) with the leucine residue found in the canine sequence (Fig. 1) yields a tridecapeptide (**2**) which has a k_{cat}/K_m about an order of magnitude greater than **1**. This is due both to a decrease in K_m (41 vs. 168 μM) and a trebling of k_{cat}.

Replacement of the isoleucyl at P_2' (**3**) further decreases K_m without greatly changing k_{cat}.

Replacement of the histidine residue at P_3' with the ryrosylserine residues found in canine angiotensinogen improves k_{cat} an order of magnitude without changing K_m for reaction with canine renin. Skeggs and co-workers [3] previously reported that deletion of the C-terminal serine residue does not change kinetic parameters for cleavage of tetradecapeptide by equine renin; and, it is unlikely that this amino acid is responsible for the increase in k_{cat} observed with canine renin.

The kinetic parameters for cleavage of the synthetic substrates parallel these seen with intact

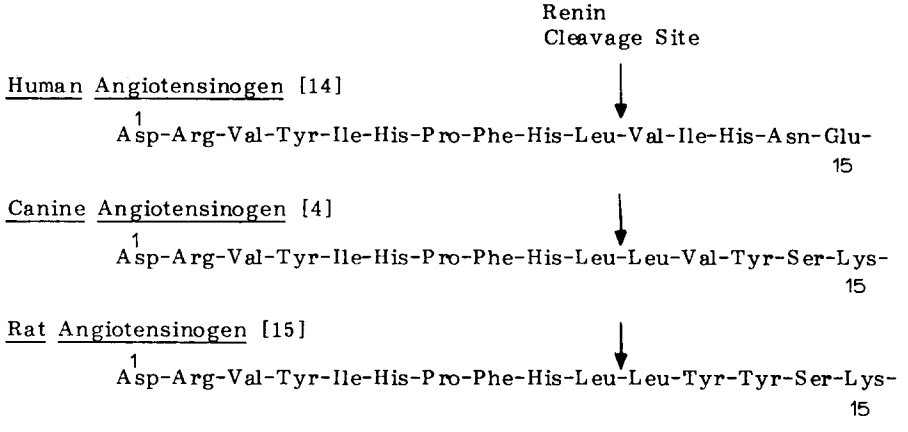

Fig. 1. N-terminal amino-acid sequences (1–15) of angiotensinogen from human, dog and rat.

angiotensinogen. This supports the hypothesis of Dzau et al. [8] that homogeneous canine renin has a greater specific activity (4200 GU/mg) than human renin (1000 GU/mg) [8].

A large part of species specificity appears to be due to changes in a relatively limited number of amino-acid residues in the substrate adjacent to the cleavage site. As postulated by Tewksbury et al. [13], the histidine residue at P_3' in the human sequence significantly influences k_{cat}/K_m. The seemingly homologous replacements at P_1' and P_2', however, appear to be even more important. Generally, replacements at P_1' and P_2' alter K_m, while the replacement at P_3' has its greatest effect on k_{cat}.

Acknowledgments

Research was supported by a grant from the Heart, Lung and Blood Institute (HL19517). Support from the trustees of the Evans Memorial Department for Clinical Research at University Hospital is also gratefully acknowledged. Dr. E.E. Salter of Merck (Rahway, NJ) provided purified human renin and Dr. V.J. Dzau of The Brigham and Women's Hospital (Boston, MA) supplied partly purified canine renin and canine angiotensinogen.

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