Granulocyte–Angiotensin System. Identification of Angiotensinogen as the Plasma Protein Substrate of Leukocyte Cathepsin G†

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ABSTRACT: Cathepsin G, a human lysosomal neutral protease, converts angiotensin I to angiotensin II and cleaves angiotensin II from a plasma protein substrate. Experiments were designed that identified and characterized cathepsin G substrate as human angiotensinogen. A total of 2, 5, and 10 μg of purified substrate, incubated with 2 μL of partially purified human renin (2 Goldblatt units/mg) for 60 min at 37 °C, generated 2, 9, and 22 pmol of angiotensin I. Cathepsin G substrate and renin substrate activities copurified during Affi-Gel Blue affinity chromatography, hydroxylapatite chromatography, phenyl-Sepharose chromatography, and S-200 gel filtration. Disc gel electrophoresis of 10 μg of purified protein gave a single band containing both activities. The amino-terminal sequence contained the covalent structure of angiotensin I and was Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-X-Glu-Ser-Thr-Cys-Glu-. Reduced and unreduced angiotensinogens were subjected to sodium dodecyl sulfate gel electrophoresis, and each gel showed two bands of M_r 65 000 and 62 000. The isoelectric point of the M_r 65 000 form was pH 4.5–4.3 and the M_r 62 000 form was pH 4.9. Functional, structural, and physiochemical evidence demonstrates that the substrate of cathepsin G is angiotensinogen. Thus, human neutrophils may utilize angiotensin I or angiotensinogen as substrate for angiotensin II generation. The granulocyte–angiotensin system does not require renin or converting enzyme and may function as a mobile effector pathway which modulates tissue blood flow and/or vascular permeability.

Two human angiotensin II generating pathways have been described. Renin cleaves angiotensin I from angiotensinogen (Skeggs et al., 1967), and angiotensin I is converted to angiotensin II by angiotensin-converting enzyme (Skeggs et al., 1954). Recently, a neutrophil-dependent angiotensin II generating system has been recognized (Wintroub et al., 1981a). The latter pathway was initially detected as a smooth muscle contractile activity formed during incubation of homogenized neutrophils with heated plasma (Wintroub et al., 1974). Subsequent experiments showed that cathepsin G, a neutral protease found in the neutrophil azurophil granule, converts angiotensin I to angiotensin II (Klickstein et al., 1982) and releases angiotensin II from a plasma protein substrate (Tonnesen et al., 1982). The substrate of cathepsin G has been purified to homogeneity (Wintroub et al., 1981b) and was assumed to be angiotensinogen on the basis of size, charge, and identification of the product as angiotensin II. Experiments described in this report present definitive functional, physicochemical, and structural evidence that the plasma protein substrate of cathepsin G is human angiotensinogen.

Experimental Procedures

Materials

The following materials were obtained as noted: phenyl-Sepharose CL-4B, Ficoll-Hypaque, Dextran T500, and Sephacryl S-200 (Pharmacia Fine Chemicals, Divison of Pharmacia, Inc., Piscataway, NJ); Affi-Gel Blue and hydroxylapatite (Bio-Gel HTP) (Bio-Rad Laboratories, Richmond, CA); *N*-succinyl-(L-Ala)$_3$-*p*-nitroanilide [suc-(Ala)$_3$-pNA] (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA); carboxymethylcellulose (Whatman Ltd., Kent, England); dimethyl sulfoxide (Me_2SO) (Mallinckrodt, St. Louis, MO); angiotensin I and II (Sigma Chemical Co., St. Louis, MO); specific antisera to angiotensin I and II, (tyrosyl-5-L-[^{125}I]iodoisoleucyl)angiotensin I and II, and iodo-[^{3}H]acetic acid (New England Nuclear, Corp., Cambridge, MA); dioxane, acetonitrile, and tetrahydrofuran (Burdick & Jackson Laboratories, Inc., Muskegon, MI); pyridine, trifluoroacetic acid, and 1,2-dichloroethane (reagent grade, Pierce Chemical Co., Chicago, IL); 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (BOC-ON) (Aldrich Chemical Co., Milwaukee, WI); phenyl isothiocyanate (Beckman Instruments, Inc., Palo Alto, CA).

Methods

Purification of Plasma Protein Substrate of Leukocyte Cathepsin G. Purification of the substrate of cathepsin G was carried out from human plasma by a six-step procedure (Wintroub et al., 1981b), and appropriate fractions were examined for cathepsin G substrate activity. Briefly, fresh human citrated plasma (240 mL) was brought to a final concentration of 45% ammonium sulfate (w/v) at 4 °C, and the supernatant was concentrated to 100 mL, dialyzed against 0.2 M phosphate buffer, pH 7.1/0.02% sodium azide, and applied to blue dextran–Sepharose column equilibrated in the same buffer. The effluent was concentrated to 120 mL, dialyzed against 1 mM phosphate buffer, pH 7.9, and applied to a hydroxylapatite column which was equilibrated in the same buffer and eluted with a 4-L gradient from 1 to 67 mM phosphate buffer, pH 7.9. Active fractions were pooled, concentrated to 70 mL, and dialyzed against 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)/6 mM phosphate buffer/0.6 M $(NH_4)_2SO_4$, pH 8.2/ 20% (v/v) ethylene glycol and applied to phenyl-Sepharose CL-4B at room temperature. The column was eluted by a 500-mL gradient from the starting buffer to 50 mM Tris, pH 10.5/50% (v/v) ethylene glycol. Column fractions were dialyzed against 10 mM Tris-HCl, pH 7.4/0.15 M NaCl prior to assay, and appropriate fractions were pooled, concentrated to 1.4 mL,

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filtered, and recycled through Sephacryl S-200 in 10 mM Tris-HCl, pH 7.4/0.15 M NaCl. Unless otherwise noted, procedures were carried out at 4 °C. Solutions were concentrated on an Amicon DC-2 (Amicon Corp., Lexington, MA) hollow fiber concentration apparatus or by means of positive pressure by using Amicon PM-10 membranes (Amicon Corp., Lexington, MA). Ten micrograms of purified cathepsin G plasma protein substrate gave a single stained protein band following disc gel electrophoresis (Maurer, 1968) and yielded a single arc following immunoelectrophoresis against rabbit antibody to human serum.

Measurement of Plasma Protein Substrate of Cathepsin G. Capthepsin G substrate was measured by quantitation of angiotensin II generated following incubation of a substrate source with appropriate amounts of cathepsin G. Substrate-containing samples were heated for 2 h at 61 °C to inactivate plasma inhibitors of cathepsin G and then incubated with 10 μg of purified cathepsin G for 30 min at 37 °C in 100 μL of 10 mM Tris, pH 7.4/0.15 M NaCl. The reaction was stopped by addition of 900 μL of 0.1 M HCl, and 5–50-μL aliquots were assayed for angiotensin II content by competitive-binding radioimmunoassay according to the manufacturer's method (New England Nuclear Corp., Boston, MA). Alternatively, reactions were stopped by placing samples on ice, and angiotensin II content was determined by an isocratic, reverse-phase, ion-pair high-performance liquid chromatography (HPLC) assay (Klickstein & Wintroub, 1982).

Measurement of Angiotensinogen. Angiotensinogen was measured by detection of angiotensin I produced after incubation with partially purified human renin. Five- to ten-microliter angiotensinogen-containing samples were incubated with 2 μL of partially purified human renin for 30 min at 37 °C in 100 μL of 0.1 M phosphate buffer, pH 6.0/0.15 M NaCl. The reaction was stopped by addition of 900 μL of 0.1 N HCl, and 5–50-μL aliquots were assayed for angiotensin I content by competitive-binding radioimmunoassay according to the methods of the manufacturer (New England Nuclear Corp., Boston, MA) or by HPLC assay (Klickstein & Wintroub, 1982).

Purification of Cathepsin G. Cathepsin G was purified from human peripheral blood neutrophil granules by a modification of the method of Baugh & Travis (1976) as described (Tonnesen et al., 1982). Five micrograms of purified cathepsin G yielded three stained protein bands of M_r 26 000, 27 000, and 29 000 when analyzed by reduced sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Ten micrograms of cathepsin G contained no detectable elastase activity as measured by hydrolysis of suc-$(Ala)_3$-pNA (Beith et al., 1974).

Purification of Renin. Human renin was partially purified from cadaver kidney through the fourth step of a previously published procedure (Slater et al., 1978; Slater & Strout, 1981) with the further addition of an α-casein–Sepharose affinity chromatography step to remove cathepsin D (Dzau, 1982). Purification was 1000-fold, and specific activity was 2 Goldblatt units/mg. Recovery of starting activity was 32%, and there was no detectable proteolytic activity as measured by neutral degradation of methyl[^{14}C]hemoglobin (Williams & Lin, 1971).

Procedures for Characterization of the Plasma Protein Substrate of Cathepsin G and Angiotensinogen. Polyacrylamide disc gel electrophoresis was performed at pH 9.4 in 7.5% gels in a Buchler apparatus (Buchler Instrument, Fort Lee, NJ) (Maurer, 1968). Gels were stained with Coomassie blue or sliced into 2-mm sections, and each slice was eluted overnight in 0.5 mL of 10 mM Tris, pH 7.4/10 mM NaCl. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described (Wintroub et al., 1981). Gradient polyacrylamide gel electrophoresis was carried out at pH 9.4 in preformed 4–30% acrylamide gels (Pharmacia Fine Chemicals, Piscataway, NJ) in a Pharmacia GL-4 apparatus according to the methods of the manufacturer.

Isoelectric focusing was carried out at 4 °C in a 1.25 × 15.3 cm tube containing 2% ampholytes (70% pH 3.5–10 and 30% pH 4–6) in a linear 19-mL 10–40% sucrose gradient. The pH gradient was established by application of 9 mA for 3.5 h, after which a 100-μL sample in 25% sucrose containing the 2% ampholyte mixture was inserted into the gradient via a 22-gauge spinal needle. A 500-V potential was applied for 3.5 h, at which time the current had remained stable for 1 h, and the gradient was fractionated into 19 1.0-mL samples. The pH of each fraction was determined before the buffer was adjusted for assay.

Procedures for Structural Analysis. The procedures used in the amino acid composition and N-terminal sequence determination have been described in a previous publication (Wintroub et al., 1981a). Briefly, amino acid analyses of reduced and carboxymethylated material were performed either on a Durrum D-500 (Dionex Inc., Sunnyvale, CA) or Beckman 121MB (Beckman Instrument Inc., Palo Alto, CA) amino acid analyzer. Angiotensinogen was analyzed after total acid hydrolysis for 24, 48, and 72 h (5.7 N HCl, 0.1% phenol, 108 °C). Tryptophan was determined after a 24-h acid hydrolysis with 5.7 N HCl in the presence of 10% mercaptoacetic acid. Sequential automated Edman degradations of protein were performed on a Beckman 890C (Beckman Instruments Inc., Palo Alto, CA), utilizing 0.1 M Quadrol program 121078 as supplied by the manufacturer. Identification of each amino acid residue was achieved by back-hydrolysis of the anilinothiazolinone derivative followed by amino acid analysis and by HPLC analyses of its phenylthiohydantoin derivative as described (Wintroub et al., 1981a). The identity of carboxymethylated cysteine at position 18 was further substantiated by recovery radioactivity after reducing and alkylating one sample with iodo[^{3}H]acetic acid prior to sequence analysis.

A second protein sample was deglycosylated by hydrogen fluoride treatment. An aliquot of 5 nmol of protein was dried in vacuo over P_2O_5 for 24 h and KOH for 3 days. Next, the sample was treated with 8–10 mL of anhydrous hydrogen fluoride in a Kel-F apparatus for 1 h at 0 °C with continuous stirring. After complete removal of hydrogen fluoride by nitrogen for 20 min, the residue was dissolved in 88% formic acid and applied on the sequencer for automatic Edman degradation.

Temporary reduction of background in sequence analyses was achieved by blocking all newly generated amino termini at the end of cycle 6 when proline was found to be the next amino terminus (Machleidt et al., 1982; Bhown et al., 1983; Shoemaker et al., 1983). Briefly, the method involved interruption of the sequences run at the end of residue 6 and treatment of the sample within the spinning cup with 500 μL of *o*-phathalaldehyde (OPA, 5 mg/mL in 0.1 M Quadrol and 20 μL of mercaptoethanol/mL) for 20 min at room temperature. The regular program was resumed automatically with sample drying, organic wash, acid cleavage, and extraction with *n*-chlorobutane. The normal Edman program was then allowed to continue for cycle 7 with an extra 10-min period of HFBA cleavage at the prolyl residue.

Results

Angiotensinogen Activity in Purified Cathepsin G Substrate.

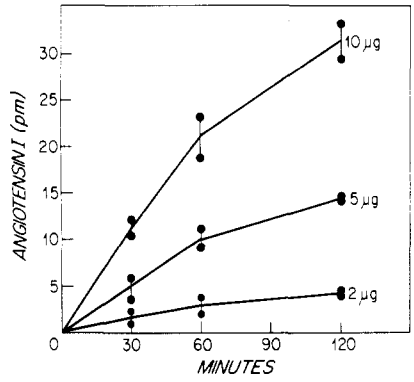

FIGURE 1: Renin-dependent angiotensin I generation from cathepsin G substrate. A total of 2, 5, and 10 μg of cathepsin G substrate was incubated with partially purified human renin as described in the text.

Table I: Digestion of Cathepsin G Substrate by Cathepsin G and Renin[a]

incubation time (h)	angiotensin II generated (pmol/sample)	angiotensin I generated (pmol/sample)
0	ND	636
1	92.5	564
4	73.5	462
6	42.5	498
24	46	336

[a] Incubation of renin with angiotensinogen alone or with angiotensinogen and soybean trypin inhibitor generated 714 and 738 pmol of angiotensin I, respectively.

Since the interaction of cathepsin G and its plasma protein substrate generated angiotensin II (Wintroub et al., 1981a), experiments were designed to confirm that the protein substrate of cathepsin G was human angiotensinogen. Various amounts of purified plasma protein substrate (2, 5, and 10 μg) were incubated with 2 μL of partially purified renin. At appropriate time intervals, 30 μL of each reaction mixture was removed, placed on ice, and assayed for angiotensin I content by radioimmunoassay (Figure 1). The generation of angiotensin I was time and substrate concentration dependent, indicating that the purified protein substrate of cathepsin G contained substrate activity for human renin.

To determine if cathepsin G utilized the angiotensinogen activity in a cathepsin G substrate preparation, 5 pmol of cathepsin G was incubated with 10^{-5} M purified protein substrate in 500 μL 0.01 M Tris, pH 7.4/0.15 M NaCl, and 100-μL samples were removed from the reaction mixture at 0, 1, 4, and 24 h. Each sample was divided into two fractions. One fraction was assayed for angiotensin II content by HPLC, and the second fraction of each sample was made 10^{-4} M in soybean trypsin inhibitor to inactivate cathepsin G, incubated with 2 μL of human renin for 15 min at 37 °C, and then assayed directly for angiotensin I content by HPLC. The results (Table I) indicate that under the conditions of this experiment, digestion of the cathepsin G substrate by cathepsin G diminished but did not exhaust the ability of cathepsin G substrate to support generation of angiotensin I by renin. For this reason, the following experiments were designed to definitively demonstrate that the substrate of cathepsin G is angiotensinogen.

Identification of the Substrate of Leukocyte Cathepsin G as Angiotensinogen. Cathepsin G substrate and renin substrate activities were followed during the purification procedure designed for isolation of the plasma protein substrate of cathepsin G. Two-hundred and forty milliliters of fresh human

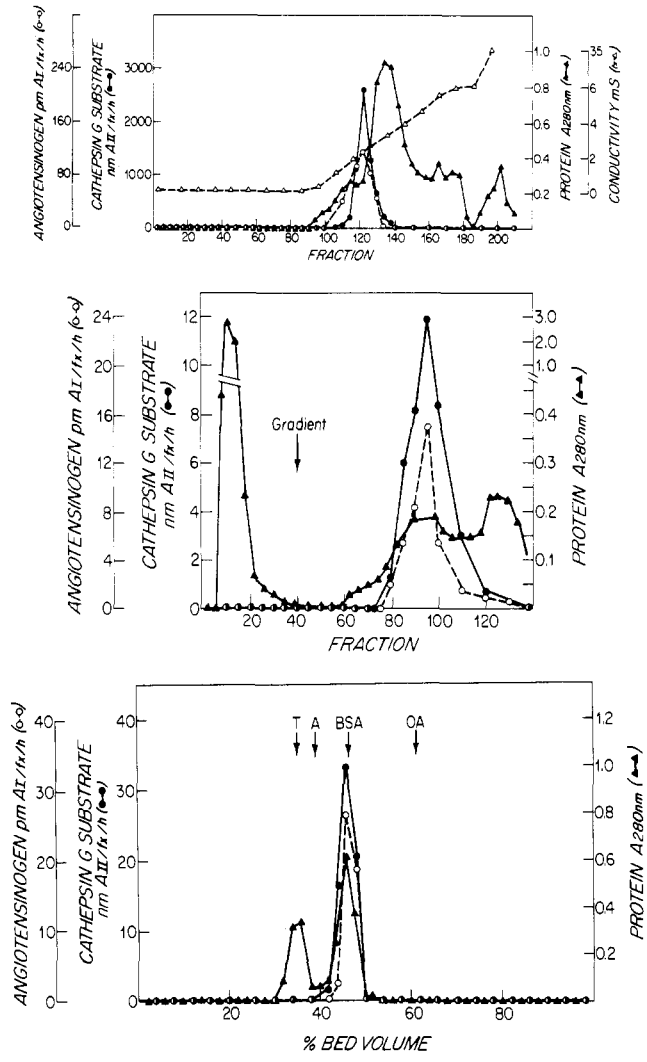

FIGURE 2: Purification of cathepsin G substrate activity and angiotensinogen. (Upper panel) Hydroxylapatite chromatography of the effluent of the blue dextran affinity column; (middle panel) phenyl-Sepharose hydrophobic chromatography of hydroxylapatite fractions 90–132; (lower panel) Sephacryl S-200 gel filtration of phenyl-Sepharose fractions 80–110.

plasma was subjected to 45% ammonium sulfate precipitation, and the supernatant which contained cathepsin G and renin substrate activities was carried through blue dextran affinity chromatography, to remove albumin. Both activities were detected only in the column effluent, and this material was further purified by hydroxylapatite chromatography, phenyl-Sepharose hydrophobic chromatography, and Sephacryl S-200 gel filtration (Figure 2). At each purification step, appropriate column fractions were assayed, and fractions that contained cathepsin G and renin substrate activities were pooled, concentrated, and subjected to further purification. At each purification step, cathepsin G- and renin substrate activities behaved identically (Figure 2).

To confirm that angiotensinogen and cathepsin G substrate were identical, the purified material was subjected to procedures designed to separate proteins on the basis of physicochemical characteristics. Ten micrograms of cathepsin G substrate was subjected to alkaline disc gel electrophoresis and stained with Coomassie blue. A second parallel gel, containing 25 μg of protein, was electrophoresed and sliced, and each slice was eluted prior to assay for cathepsin G substrate or angiotensinogen. A single-stained protein band, which corresponded to cathepsin G substrate and angiotensinogen activities, was detected (Figure 3).

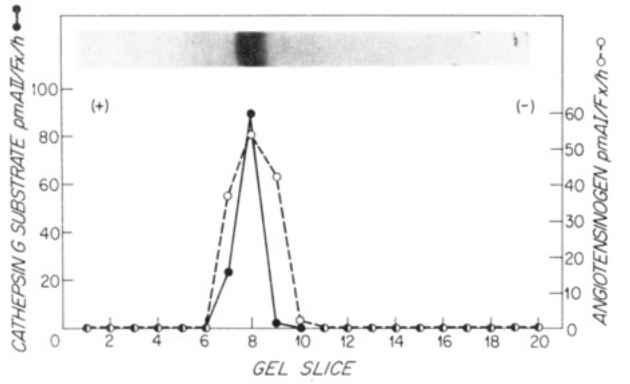

FIGURE 3: Acrylamide gel electrophoresis of purified cathepsin G substrate and angiotensinogen.

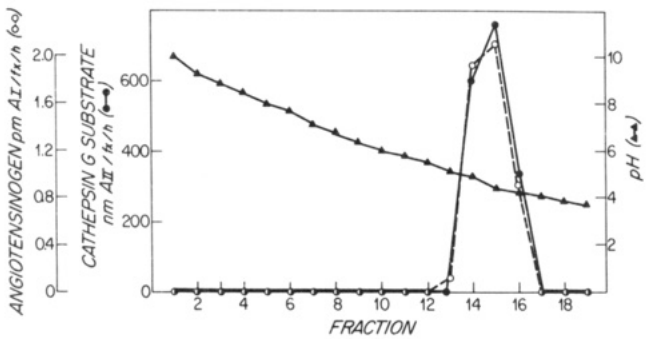

FIGURE 4: Isoelectric focusing of purified cathepsin G substrate and angiotensinogen.

Table II: N-Terminal Sequence of Human Angiotensinogen

residue	1	2	3	4	5	6	7	8	9	10
amino acid	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu
nmol	3.5	2.5	3.4	3.0	2.7	1.0	1.0	2.9	0.8	2.7
residue	11	12	13	14	15	16	17	18	19	20
amino acid	Val	Ile	His	X	Glu	Ser[a]	Thr[a]	Cys[b]	Glu	Gln
nmol	2.2	1.6	0.8		2.1	0.6	0.5	1.1	2.5	2.0
residue	21	22	23	24	25	26	27	28	29	30
amino acid	Leu	Ala	Lys	Ala	Asn	Ala	Gly	Lys	Pro	Lys
nmol	1.8	1.4	1.0	1.3	0.9	1.2	0.8	0.5	0.5	0.4

[a] Identified as dehydroserine or dehydrothreonine at 313 nm. [b] Identified as radioactive CM-Cys derivative on HPLC, scintillation counting, and back-hydrolysis.

Five hundred micrograms of purified protein was subjected to isoelectric focusing in a 10–40% sucrose gradient, and each fraction was assayed for cathepsin G substrate and angiotensinogen activities. The two activities behaved identically and were detected in fractions corresponding to pH 4.3–4.9 (Figure 4).

Four separate preparations that gave a single-stained protein band on disc gel electrophoresis were examined for amino-terminal sequence, and one of these was analyzed after alkylation with iodo[^{3}H]acetic acid. The amino-terminal sequence of each preparation was Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu, and the sequence of the first 30 amino-terminal residues is presented for 5 nmol of a single preparation with OPA treatment at step 7, the first prolyl residue (Table II). Every residue reported, except step 14, was unequivocally identified in four separate N-terminal analyses. Because the 14th residue was not identified, another 5 nmol of purified material was chemically deglycosylated and automatically sequenced. Again no amino acid was detected at position 14.

The amino acid composition of a similar angiotensinogen preparation is presented (Table III).

Table III: Amino Acid Composition of Human Serum Angiotensinogen[a]

	24-h residues/ 500[b]	48-h residues/ 500[b]	72-h residues/ 500[b]	average residues/ 500[b]
CM-Cys[f]	5.2	5.1	5.1	5.2 (5)
Asx	42.9	43.2	43.2	43.1 (43)
Thr	33.0	32.1	31.9	33.0[d] (33)
Ser	31.5	28.9	28.4	31.5[d] (32)
Glx	56.3	57.0	57.1	56.8 (57)
Pro	26.9	26.9	26.9	26.9 (27)
Gly	26.7	26.8	27.0	26.8 (27)
Ala	45.1	45.2	45.5	45.3 (45)
Val	41.3	43.6	43.7	43.7[e] (44)
Met	15.7	12.6	12.2	15.7[d] (16)
Ile	15.6	15.6	15.6	15.6 (16)
Leu	70.8	71.7	71.6	71.4 (71)
Tyr	9.0	9.3	9.1	9.1 (9)
Phe	24.1	24.9	25.0	24.7 (25)
Lys	20.3	20.6	21.0	20.6 (21)
His	14.7	15.1	15.2	15.0 (15)
Trp	5.4[c]			5.4 (5)
Arg	15.8	16.1	16.2	16.0 (16)

[a] The values given are the averages of duplicate runs of 24-, 48-, and 72-h acid hydrolysis. Each analysis represents approximately 250 pmol of protein. [b] Calculated on the basis of 500 residues. [c] Determined by acid hydrolysis for 24 h in the presence of 10% mercaptoacetic acid. [d] 24-h values were used. [e] 72-h values were used. [f] See footnote *b* of Table II.

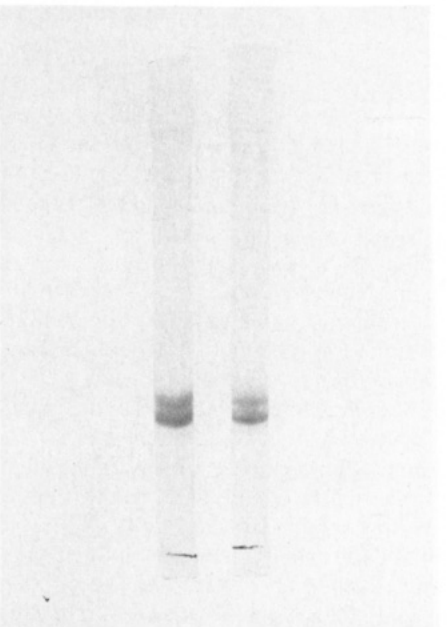

FIGURE 5: SDS gel electrophoresis of cathepsin G substrate and angiotensinogen. The gel on the left is reduced. The high molecular weight bands are attributed to trace contaminant.

Heterogeneity of Angiotensinogen. The molecular weight and subunit structure of material in which the amino-terminal sequence had been determined (Table I) was assessed by SDS–PAGE of 10 μg of unreduced and reduced protein. Two major stained bands of M_r 65 000 and 62 000 were observed (Figure 5) in unreduced and reduced material.

Next, 500 μg of this material was subjected to isoelectric focusing as described above. Activity was detected in three fractions corresponding to pH 4.9, 4.5, and 4.3. Twelve microliters of each angiotensinogen-containing fraction was subjected to gradient gel electrophoresis, and the gel was stained with Coomassie blue. Both forms of angiotensinogen were detected in active fractions; however, the predominant

form of angiotensinogen in the pH 4.9 fraction was of M_r 62000, and the predominant form in the pH 4.5 and 4.3 fractions was of M_r 65000.

Discussion

Cathepsin G, a human neutrophil lysosomal neutral serine protease, converts angiotensin I to angiotensin II (Klickstein et al., 1982; Reilly et al., 1982) and directly cleaves angiotensin II from a plasma protein substrate (Wintroub et al., 1981a). Although the plasma protein substrate of cathepsin G had been purified to homogeneity, its possible identification as human plasma angiotensinogen was primarily based on recognition of the reaction product as angiotensin II (Wintroub et al., 1981b). The plasma protein substrate of leukocyte cathepsin G has now been identified as human angiotensinogen on the basis of functional, physicochemical, and structural criteria. The purified protein served as substrate for generation of angiotensin I by partially purified human renal renin (Figure 1) (Table I), copurified with renin substrate activity throughout purification (Figure 2), and behaved identically with renin substrate activity during disc gel electrophoresis and isoelectric focusing (Figures 3 and 4). Finally, the amino-terminal sequence of the first 17 residues (Table II) was identical with that previously described for human angiotensinogen (Tewksbury et al., 1981).

Further studies with purified material are consistent with two forms of human plasma angiotensinogen (Figure 5) which differ in size and charge. One form is a single polypeptide chain of approximately M_r 65000 by SDS–PAGE (Figure 5) and gradient gel electrophoresis and has an isoelectric point of approximately 4.5. The second form is a single polypeptide chain of approximately M_r 62000 and has an approximate isoelectric point of pH 4.9. While the explanation for size and charge heterogeneity is not clear, the M_r 62000 material may represent the native form of angiotensinogen, or the low molecular weight form may arise by limited proteolysis during the purification procedure. Since the amino-terminal sequence of the two forms is identical, the observed size and charge heterogeneity must result from alterations at the carboxy terminus or from carbohydrate differences. That the two forms may represent native angiotensinogen is suugested by the observation that multiple forms of human plasma angiotensinogen have been noted by others (Printz et al., 1977). It is of interest that rat plasma angiotensinogen displays heterogeneity similar to that described for human angiotensinogen and is composed of an M_r 5500–5900 form with an isoelectric point of 4.70–4.75 and an M_r 52000–57000 form with a pH of 4.4–4.5 (Bouhnik et al., 1981; Hilgenfeldt et al., 1982; Voight et al., 1982).

The amino-terminal sequence of human angiotensinogen (Table II) was similar to but not identical with that previously reported by others (Tewksbury, et al., 1981). The sequence of the first 10 amino-terminal residues is identical with the structure of decapeptide angiotensin I; the 11th amino acid is valine rather than leucine as reported in rat (Skeggs et al., 1954, Ohkubo et al., 1983) angiotensinogen. However, amino acid identification by amino acid analyzer, HPLC, and detection of [^{3}H]CM-Cys indicate that the the 18th amino acid is cysteine rather than serine as previously reported for human angiotensinogen (Tewksbury et al., 1981). Recently, Ohkubo et al. (1983) reported the amino acid sequence of rat angiotensinogen as deduced from cloning and nucleotide sequence analysis of a nearly full-length cDNA coding for rat angiotensinogen precursor. Interestingly, the rat angiotensinogen molecule has a sequence of Leu-Tyr-Tyr-Ser-Lys-Ser-Thr-Cys from positions 11 to 18 as compared to the corresponding sequence of Val-Ile-His-X-Glu-Ser-Thr-Cys in human angiotensinogen, the last three residues being identical.

In our first three sequence attempts, background on HPLC appeared in significant amount at and after the sequence of His^6-Pro^7 and completely interferred with further identification beyond step 29. Furthermore, all three attempts failed to identify an amino acid at position 14. This prompted us to chemically block the background at the end of cycle 6 with OPA treatment in the fourth sequence run which utilized 5 nmol of protein. The significant reduction of background after the prolyl residue enabled us to corectly identify residue 24 as alanine and residue 30 as lysine. Residue 14 was still unidentified, suggesting a possible modified serine or threonine residue covalently linked with some polar groups such as phosphate or with an O-glycosidic linkage. Thus, another 5 nmol of human angiotensinogen was chemically deglycosylated and sequenced automatically. For the fifth time, no amino acid was detected for position 14. Although the reason for our inability to identify residue 14 is unclear, it is likely that the modified amino acid may be so hydrophilic that a more polar solvent is required to extract the anilinothiazolinone. In addition, the ready β elimination of such residues and possibly the aldehyde groups of released carbohydrate may contribute to our inability to identify this residue.

The biologic importance of the granulocyte–angiotensin pathway is unclear; however, this pathway is a potential mechanism which permits neutrophil-dependent tissue generation of angiotensin II. Recent data suggest that tissue generation of angiotensin II may modulate local vascular responses (Fantone et al., 1982) or granulomatous inflammation (Weinstock et al., 1981; Schrier et al., 1982). Captopril, an inhibitor of angiotensin-converting enzyme, inhibits rat cutaneous vascular permeability responses to a panel of vasodilators (Schrier et al., 1982) and reduces the size of granulomas in mice infected with BCG (Fantone et al., 1982) or *Schistosoma mansoni* eggs (Weinstock et al., 1981). Because neutrophil-dependent angiotensin generation is not inhibited by captopril (Klickstein et al., 1982), it is unlikely to account for these effects. Experimental evidence demonstrates that increased blood flow and enhanced vascular permeability associated with acute cutaneous, zymosan-induced, inflammation is leukocyte dependent (Wedmore & Williams, 1981) and is mediate by two leukocyte-generated factors. Increased blood flow results from local generation of prostaglandin PGE_2 (Wedmore & Williams, 1981) while the factor responsible for the permeability response is unknown. Angiotensin II is known to enhance permeability in large vessels (Harhult, 1971; Robertson & Khairallah, 1972) and induces widening of interendothelial cell space in aortic, coronary, mesenteric, and peripheral arteries (Robertson & Khairallah, 1972, 1973; Giacomelli et al., 1976; Wiener & Giacomelli, 1973). The leukocyte-dependent angiotensin-generating system may be a mechanism permitting tissue generation of a biologically active peptide through which the neutrophil may modulate vascular permeability.

Acknowledgments

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Registry No. Cathepsin G, 56645-49-9; angiotensinogen, 11002-13-4.

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Structural Changes in Membranes Produced by the Binding of Small Amphipathic Molecules†

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ABSTRACT: In their interactions with membranes, amphipathic small molecules exhibit detergent-like properties. At sufficiently high concentrations (above their critical micelle concentrations, if they form micelles), they substantially dissolve membranes. At lower concentrations, between maximally antihemolytic and lytic, we show here that the amphipaths significantly perturb membrane structure. Each of six small-molecule amphipaths was shown by hygroscopic desorption filtration to induce the extraction of small but significant amounts of membrane components, partly in the form of vesicular fragments, from red blood cell membranes. These extracts were enriched in the lipid to protein ratio as compared to the intact membrane, and the protein composition was highly unrepresentative. A similar set of extractions from sarcoplasmic reticulum membranes was induced by the six amphipaths. We conclude that small-molecule amphipaths, at concentrations lower than lytic, promote gross redistributions of components in the plane of a membrane that result in the observed extractions.

The interactions of amphipathic small molecules with membranes are of great interest in physiology and biochemistry. Such amphipaths include the bile salts, many anesthetics and tranquilizers, mitochondrial uncouplers, and many other drugs and metabolites. In addition, many of the molecular probes used to study membrane fluidity and other properties are amphipaths. These molecules have two distinct domains, one hydrophobic and the other hydrophilic. It is widely believed that over a broad range of concentrations most amphipaths dissolve in the fluid lipid regions of a membrane, intercalating their hydrophobic domains into the hydrophobic interior of the lipid bilayer, while their hydrophilic domains are positioned in the region of the lipid polar head groups. This is thought to be responsible for the fluidity changes that are generally

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