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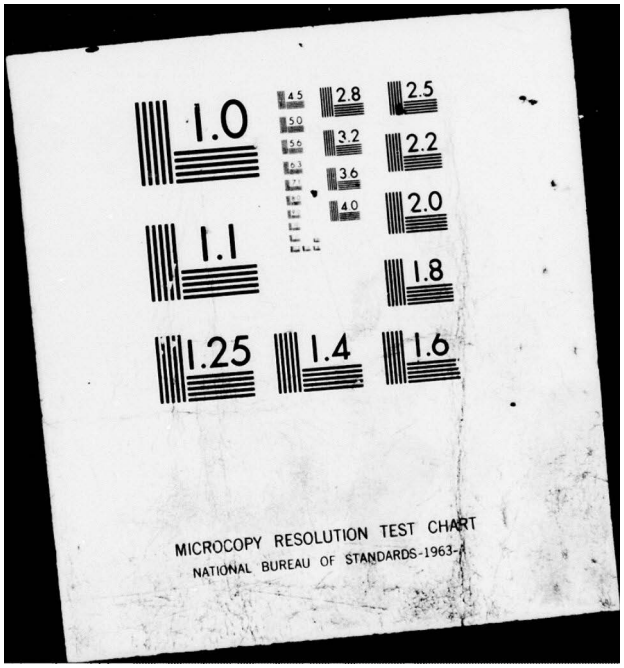
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Inhibition of Kininase II (Angiotensin I Converting Enzyme) by Human Serum Albumin and Its Fragments

Prepared by

Ervin G. Erdös

For Publication in
Symposium on Kinins

Departments of Pharmacology and Internal Medicine
University of Texas Health Science Center at Dallas
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(10) Ervin G. Erdos, Rainer J. Klauser
Carol J. G. Robinson

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Departments of Pharmacology and Internal Medicine
University of Texas Health Science Center at Dallas
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Because we have recently reported that blood contains inhibitors of angiotensin I converting enzyme (kininase II; CE), we investigated whether albumin and its fragments can inhibit CE, thus block the release of angiotensin II and the inactivation of bradykinin. Human albumin inhibited (K_i = 3 x 10^{-4} M) purified human pulmonary CE, assayed with hippuryl-glycyl-glycine, in a non-competitive manner. When albumin was cleaved with CNBr, the resulting isolated fragments inhibited more potently than albumin. One of them, fragment C (sequence 124-298; K_i = 1.7 x 10^{-5} M), was purified to homogeneity by

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gel filtration and ion exchange chromatography. When five of the six disulfide bridges in fragment C were reduced and carboxymethylated, as confirmed by amino acid analysis, a more potent inhibitor was obtained and the K_i dropped to 3×10^{-6} M. Reduction and carboxymethylation of the sixth disulfide bridge, however, yielded a less active inhibitor ($K_i = 7.5 \times 10^{-6}$ M). The inhibition by the fragments was not due to binding of the metal cofactor of CE by thio groups. Fragment C also inhibited the inactivation of bradykinin by CE on intact cultured human vascular endothelial cells. Thus the K_i values of the fragments suggest that these proteins inhibit CE at concentrations two orders of magnitude lower than the concentration of albumin in plasma. Transfusion of plasma protein preparations may cause severe hypotension and such preparations may contain bradykinin and a prekallikrein activator. Albumin fragments formed during preparation of protein fractions could potentiate the hypotensive effect of bradykinin by blocking its inactivation by CE. These fragments could interfere with the regulation of blood pressure by also inhibiting the release of angiotensin II by the same enzyme.

INHIBITION OF KININASE II (ANGIOTENSIN I CONVERTING ENZYME) BY HUMAN SERUM ALBUMIN AND ITS FRAGMENTS

Rainer J. Klauser, Carol J.G. Robinson and Ervin G. Erdös

Departments of Pharmacology and Internal Medicine

University of Texas Health Science Center @ Dallas, TX

Commercial 5% plasma protein preparations, and human serum albumin and its fragments inhibit in vitro purified peptidyl dipeptidase of human lung or hog kidney. Fragment C of albumin (sequence 124-298) is a more potent inhibitor (K_i = 1.7 x 10^{-5} M) than albumin itself. Reduction and carboxymethylation of five of the six S-S bridges in fragment C increase the inhibitory potency (K_i = 3 x 10^{-6} M) but reduction of the sixth bridge raises the K_i. This suggests the importance of the tertiary structure in fragment C for inhibition of the enzyme. Albumin and fragment C are not substrates of the enzyme. Fragment C and its derivative also inhibit the inactivation of bradykinin in vitro by the human enzyme as shown by bio-assay. Inhibition of peptidyl dipeptidase may contribute to the hypotension caused by infusion of plasma protein preparation, by potentiating the effects of bradykinin and blocking the release of angiotensin II.

Peptidyl dipeptidase (angiotensin I converting enzyme or kininase II, E.C. 3.4.15.1; CE) cleaves dipeptides from the C-terminal end of peptides such as angiotensin I, bradykinin (Yang et al., 1970, 1971) or enkephalins (Erdös et al., 1978). The enzyme is present in blood of man and animals (Skeggs et al., 1956; Yang and Erdös, 1967) probably only in partially active form because plasma contains one or more inhibitor(s) of CE (Yang et al., 1971; Oshima et al., 1974).

Because of the obvious biological importance of CE we have studied its inhibition by plasma proteins. Here we report on the inhibition of human CE by human albumin and its fragments.

Index: Kininase II inhibition, plasma proteins, infusion, albumin, hypotension.

MATERIALS AND METHODS

Human serum albumin essentially fatty acid free was obtained from Sigma Chemicals (St. Louis, Mo.). Five % plasma protein preparations (Plasmatein, Protenate and Plasmanate) were obtained from Abbott Laboratories, Hyland Laboratories and Cutter Laboratories respectively. CE was prepared from swine kidney by the method of Oshima et al. (1974). Human lung CE was prepared by gel filtration on a Sephadex G-200 column followed by chromatography on DEAE-Sephadex A-50 and hydroxyapatite columns (Klauser and Erdös, to be published). Human endothelial cells were cultured according to Johnson and Erdös (1977). Antibody to human lung CE and to human albumin was elicited in the goat.

CE was assayed using tert-butyloxycarbonyl-Phe(NO_2)-Phe-Gly (BPPG) or hippuryl-glycyl glycine (Hip-Gly-Gly) as substrate (Yang et al., 1971). One unit of enzyme (U) cleaved 1 μmole of substrate per min. When inhibitors were used they were preincubated for 10-15 min with the enzyme. The best lung CE preparation contained 8 U per mg and showed a single protein band in polyacrylamide gel electrophoresis.

The inactivation of bradykinin by CE was followed by measuring the decrease in activity on the isolated rat uterus (Yang et al., 1971; Oshima et al., 1974).

Amino acid analysis was performed in a Durrum Amino Acid Analyzer after hydrolyzing the peptides at 110° for 16 hr with 6 N HCl in sealed evacuated tubes. Values for residues of amino acids per mol fragment were calculated by using amino acid determinations of McMenamy et al. (1971) and amino acid sequence data of Meloun et al. (1975).

The concentration of protein in the solutions was determined either by the method of Lowry et al. (1951) or by measuring the absorption at 280 nm using fragment C as a standard.

Human serum albumin was cleaved to fragments A, B and C with CNBr and the fragments were separated and purified by the sequential use of gel filtration and column chromatography according to McMenamy et al. (1971).

The reduction and carboxymethylation of the disulfide bridges of the fragment was performed according to Crestfield et al. (1963) with slight modifications. Mercaptoethanol was added to fragment C under nitrogen in 25-fold excess with respect to the 12 possible SH-residues of fragment C. Iodoacetic acid was then added dropwise at pH 8.7 (Method 1).

Alternatively the reduction was carried out in 8 M urea (Method 2) or with only a 4-fold excess of mercaptoethanol with respect to the possible SH-groups (Method 3).

Immunodiffusion was carried out on Ouchterlony plates using antiserum to human albumin and to purified human lung CE.

RESULTS

Inhibition by Albumin

First we tested commercially prepared plasma protein preparations which contained over 83% albumin. Different preparations of three manufacturers inhibited purified CE of hog kidney. Thirty-five ul of the 5% protein solution inhibited the hydrolysis of BPPG by 50%.

Because commercial plasma protein preparations contain 4 x 10^{-3} M acetyltryptophan added as stabilizer we tested this compound for inhibition of CE. The I_{50} with Hip-Gly-Gly substrate and with either human or hog CE was 5 x 10^{-4} M.

Since, during purification of human CE (Oshima et al., 1974; Nishimura et al., 1977), crude preparations exhibit low enzymic activity, we examined human lung CE preparations during the various stages of purification for the presence of albumin. CE of homogenized human lung extracted with detergent and purified with gel filtration on Sephadex G-200 column still contained albumin as shown by immunodiffusion. On immunodiffusion plates the preparation of CE formed a precipitin band with antibody to human lung CE but also with antibody to human serum albumin. Albumin, which has a molecular weight of 66,500 eluted in gel filtration on a Sephadex G-200 column with CE which has a much higher molecular weight; thus CE probably forms a complex with albumin.

After additional steps of purification on ion exchange columns CE did not react with antibody to human albumin indicating that albumin was removed from the preparation during purification (Stewart, to be published). CE, in the preparation, however, still formed a precipitin band with antibody to the human lung enzyme.

Commercially prepared plasma protein preparations contain over 83% albumin (FDA, 1977) thus we studied the inhibition of CE by purified, fat free albumin. We found that purified CE of human lung and hog kidney are equally inhibited by albumin. The I_{50} values with Hip-Gly-Gly substrate were 2 x 10^{-4} M and 1.9 x 10^{-4} M. The inhibition of human lung enzyme was non-competitive. The K_i determined in the Dixon plot was 3 x 10^{-4} M.

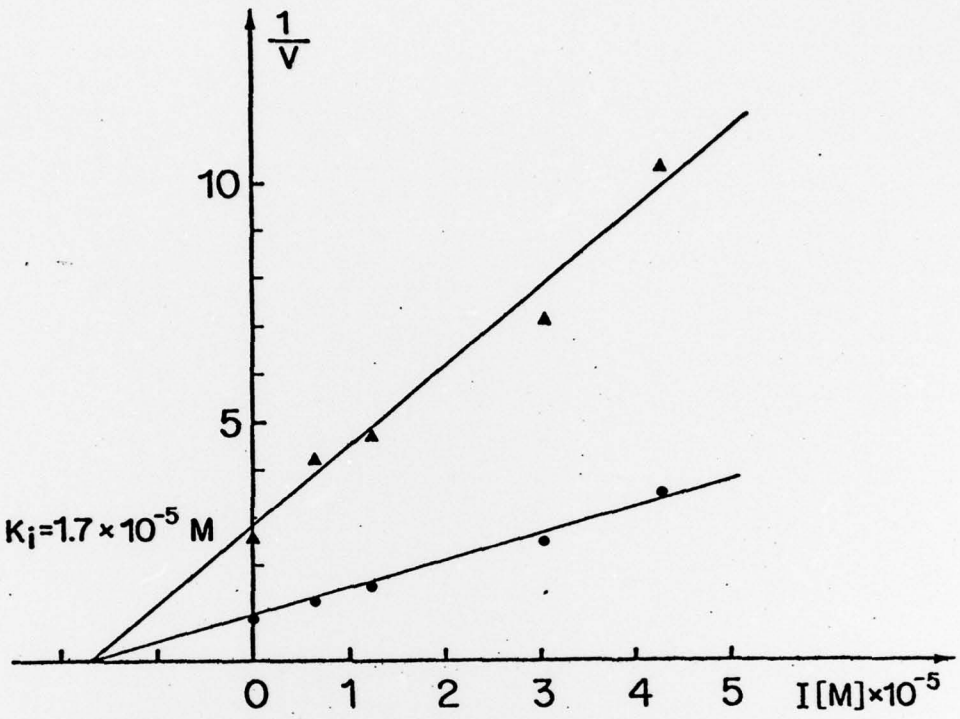

Fig. 1. Dixon plot of inhibition of human lung CE by fragment C. ▲——▲ 2×10^{-4} M Hip-Gly-Gly, ●——● 10^{-3} M Hip-Gly-Gly. $K_i = 1.7 \times 10^{-5}$ M.

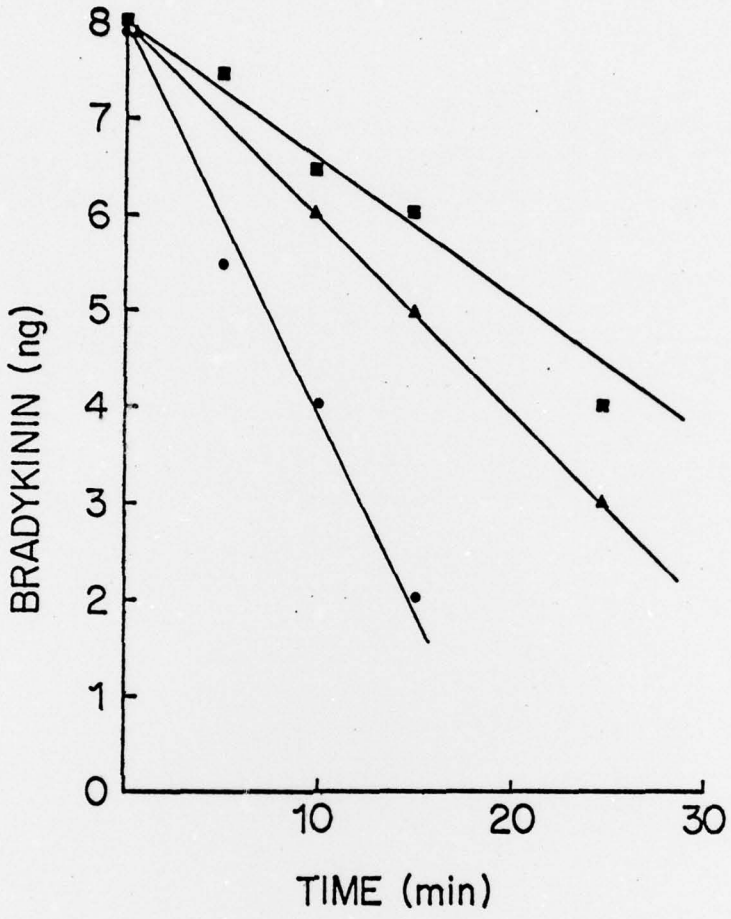

Fig. 2. Inhibition of the inactivation of bradykinin of human lung CE by fragment C and by its reduced and carboxymethylated derivative (CMFC 3). Bio-assay on the isolated rat uterus, ●——● inactivation of bradykinin, ▲——▲ inhibition by 7.5×10^{-5} M fragment C, ■——■ inhibition by 4.5×10^{-5} M CMFC 3.

Inhibition by Fragments

Since the commercial plasma protein solutions inhibited CE relatively more than accounted for by albumin content, we investigated whether fragments of human albumin may be more potent inhibitors than the native protein. Human albumin was cleaved to three fragments at the methionine residues with CNBr and the fragments were separated by column chromatography (McMenamy et al., 1971; Meloun et al., 1975). Following their nomenclature we called fragment B the residues 1-123 of albumin, fragment C residues 124-298 and fragment A residues 299-585. All three fragments inhibited CE. The I_{50} values were 2.2×10^{-5} M, 7.4×10^{-5} M, 2.5×10^{-5} M for fragments A, B and C respectively.

The inhibition of CE by homogeneous fragment C and its derivatives was studied further. In order to establish the structural features of fragment C important for inhibition of CE we reduced and carboxymethylated the disulfide bridges in fragment C formed by 12 cysteine residues (CM-cysteine) and denatured the peptide. Three different conditions were used for reduction. Method 1: mercaptoethanol was used in 25-fold excess over the theoretically available SH groups to obtain fragment CMFC 1. Method 2: mercaptoethanol was used in 25-fold excess with the addition of 8 M urea (CMFC 2). Method 3: mercaptoethanol was used in 4-fold excess (CMFC 3). Thus, with each method the conditions of denaturing fragment C differed. With methods 1 and 2 all disulfide bridges of fragment C were derivatized in CMFC 1 and CMFC 2. In addition, method 2 disrupted the secondary structure of the peptide. Method 3 left one of the 6 bridges intact in CMFC 3. Accordingly amino acid analysis of fragment C indicated 12.1 and 12.8 CM-cysteine residues obtained with methods 1 or 2 but only 9.6 with method 3, since 2 CM-cysteines are formed after reduction of each bridge. All three reduced fragments inhibited purified human CE non-competitively, but to a different degree. The calculated K_i values for non-competitive inhibition of CE by fragment C and CMFC 1, CMFC 2 and CMFC 3 are shown in Table I. Mild reduction which leaves one disulfide bridge intact (method 3) lowered the K_i of fragment C from 1.7×10^{-5} M to 3×10^{-6} M (Fig. 1). Carboxymethylation of all cysteine residues (method 1) decreased the inhibition, $K_i = 7.5 \times 10^{-6}$ M. When fragment C was completely denatured by urea and by reducing all the disulfide bridges (method 2) the resulting fragment inhibited less than fragment C, $K_i = 8 \times 10^{-5}$ M.

In separate experiments it was established that the inhibition by fragment C and CMFC 3 was not due to binding a metal cofactor and that neither albumin nor fragment C were substrates of the enzyme. Fragment C and CMFC 3 inhibited the inactivation of bradykinin by human lung CE as assayed on the isolated rat uterus (Fig. 2).

Table I

Inhibition of Human Lung CE by Fragment C and its Derivatives

Inhibitor	CM-Cysteine (mol/mol)	K_i (M)
Fragment C	---	1.7×10^{-5}
CM Fragment C (Method 1; CMFC 1)	12.1	7.5×10^{-6}
CM Fragment C (Method 2; CMFC 2)	12.8	8×10^{-5}
CM Fragment C (Method 3; CMFC 3)	9.6	3×10^{-6}

Fragment C also inhibited the inactivation of bradykinin by the enzyme on the surface of intact suspended cultured human endothelial cells. CMFC 3 was a better inhibitor of CE on the endothelial cells than fragment C was, because CMFC 3 inhibited 58% at 10^{-5} M concentration while fragment C (10^{-4} M) inhibited 49%.

DISCUSSION

Commercial plasma proteins are prepared by harsh methods which include alcohol precipitation and heating over ten hr at 60°C (Mulford et al., 1955; Hink et al., 1957). They contain over 83% albumin, plus acetyltryptophan and other additives. These protein preparations may have bradykinin (Izaka et al., 1974) and a prekallikrein activator, Hageman factor fragment, as contamination (Alving et al., 1978). Infusion of such proteins may cause severe hypotensive reactions in patients (Harrison et al., 1971; Bland et al., 1973; FDA, 1977; Alving et al., 1978). The reactions are particularly severe in patients with cardiopulmonary bypass.

In plasma bradykinin is inactivated mainly by kininase I or carboxypeptidase N Kininase II, CE, which is identical with the angiotensin I converting enzyme (Yang et al., 1970; 1971; Igic et al., 1972), has low activity in plasma; it is a more active kininase on the surface of endothelial (Bakhle and Vane, 1974) and epithelial cells (Erdös, 1977).

Inhibition of CE prolongs the hypotensive effects of kinins and abolishes the vasoconstrictor effect of renin by blocking the

release of angiotensin II (Erdös, 1977).

The low activity of CE in plasma may be due in part to its inhibition by albumin. Because the concentration of albumin is 5 to 8 x 10^{-4} M in plasma hypothetically it is sufficiently high enough to inhibit much of the activity of CE. Albumin may interfere with assaying of CE activity in plasma on in crude extracts. Although in gel filtration CE exhibits a molecular weight about three times as high as albumin, albumin was eluted in the peak containing CE, indicating binding. Albumin present in partially purified human CE preparations may account for the low CE activity of these preparations.

Our experiments have shown that albumin and its fragments inhibit CE noncompetitively, without being substrates of the enzyme. This inhibition may be enhanced by the presence of acetyltryptophan, an additive in commercial preparations. In general, fragments of albumin were more potent inhibitors than albumin itself. All three fragments obtained by cleaving human serum albumin with CNBr have lower K_i values than albumin. Fragment C which contains residues 124-298 of albumin is of particular interest because it has most of the ligand binding potency of albumin (Gambhir and McMenamy 1973).

Changes in the secondary and tertiary structure of fragment C can change its inhibitory action as shown by carboxymethylation and denaturation of this fragment. The K_i of CMFC 3, which has only 5 reduced and carboxymethylated bridges, is much lower than that of fragment C. In fragment CMFC 1 all the cysteine residues were derivatized (Method 1). Its K_i is half of that of fragment C. These experiments suggest that introduction of carboxyl groups increases the inhibition of CE by fragment C, especially when one intact disulfide bridge preserves the original conformation. When derivatization is carried out under denaturing conditions in urea, the fragment (CMFC 2) inhibits less than fragment C itself. Because the CM-cysteine content of fragments CMFC 1 and CMFC 2 is identical but CMFC 2 has a K_i ten times as high as CMFC 1, preservation of the secondary structure appears to affect the inhibition constant more than the incorporation of carboxyl groups.

In conclusion, inhibition of CE on vascular endothelial cells and in plasma by albumin and its fragments may enhance the hypotensive effects of kinins if they are present in or liberated by infused plasma protein preparations. Hypotension may be most marked when the lung is excluded from the circulation since the pulmonary vascular bed is very rich in CE (Bakhle and Vane, 1974). In addition the inhibition of CE can also block the release angiotensin II from angiotensin I by the same enzyme.

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