Inhibition of Human Peptidyl Dipeptidase (Angiotensin I Converting Enzyme: Kininase II) by Human Serum Albumin and its Fragments

RAINER J. KLAUSER, PH.D., CAROL J. G. ROBINSON, M.S., D. V. MARINKOVIC, PH.D., AND ERVIN G. ERDÖS, M.D.

SUMMARY Purified peptidyl dipeptidase (angiotensin I converting enzyme or kininase II) from human lung or hog kidney is inhibited by commercially prepared plasma protein preparations, by human serum albumin and by the additive albumin stabilizer, acetyltryptophan. After the initial steps of purification, albumin was detected by immunodiffusion as a component in human lung peptidyl dipeptidase preparation. Fragment C of albumin (sequence 124-298) is a more potent inhibitor than the parent molecule ($K_i = 1.7 \times 10^{-8} \text{ M}$). Reduction and carboxymethylation of five of the six S-S bridges in Fragment C yield the most potent noncompetitive inhibitor ($K_i = 3 \times 10^{-9} \text{ M}$). Reduction of the sixth bridge raises the $K_i$. This indicates that maintenance of the tertiary structure in Fragment C is of importance for the inhibition. Neither albumin nor Fragment C are substrates of the enzyme. Fragment C and its derivative also inhibit the inactivation of bradykinin by the purified human enzyme and by the peptidyl dipeptidase on the surface of intact cultured human endothelial cells. (Hypertension 1: 281-286, 1979)

KEY WORDS transfusion • shock • protein inhibitor • albumin • kininase inhibition • angiotensin I conversion • bradykinin • converting enzyme • peptidyl dipeptidase

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 or bradykinin. In addition converting enzyme (CE) hydrolyzes other biologically active peptides such as enkephalins. The enzyme is present in plasma of man and animals, but plasma also contains an inhibitor or inhibitors of CE.

Because of the obvious importance of CE in the metabolism of vasoactive peptides, we investigated the inhibition of the enzyme by protein preparations and by albumin and its fragments in vitro.

Methods and Materials

Human serum albumin, essentially free of fatty acid, was obtained from Sigma Chemicals (St. Louis, MO); cyanogen bromide (87%) and iodoacetic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Goat antiserum to human albumin was obtained from Miles Laboratories (Elkhart, IN). Hippuryl-glycyl-glycine (Hip-Gly-Gly) was from Vega-Fox Biochemicals (Tucson, AZ). Plasma protein preparations (Plasmatein, Protenate and Plasmanate) were obtained from Abbott Laboratories, Hyland Laboratories and Cutter Laboratories, respectively. Converting enzyme was prepared from swine kidney by the method of Oshima et al. Human lung CE was prepared by gel filtration of a Sephadex G-200 column followed by chromatography on DEAE-cellulose and hydroxyapatite columns. Cultured human endothelial cells were also used as a source of enzyme.

Assay of Converting Enzyme

We assayed CE using tert-butyloxycarbonyl-Phe (NO$_2$)-Phe-Gly (BPPG) or Hip-Gly-Gly as substrate. With the former substrate the hydrolysis was followed at 310 nm and with the latter, at 254 nm in a Cary Model 118 spectrophotometer. In typical experiments, 0.1 ml enzyme solution in 5 mM tris pH 7.4 was added to 0.8 ml of 0.2 M tris buffer, pH 7.4, containing 0.2 M NaCl and preincubated at 37°C for
Inhibition of purified hog kidney peptidyl dipeptidase by commercially prepared 5% plasma protein preparations. Four different batches of three manufacturers (see Methods section) were used. Substrate: t-Boc-Phe-p-\(\text{NO}_2\)-Phe-Gly. Abscissa: µl plasma fraction. Ordinate: changes in absorption at 310 nm.

Bioassay

The inactivation of bradykinin by CE was followed by measuring contractions of isolated rat uterus.\(^3\)\(^7\)

Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out in 7.5% polyacrylamide gels (120 × 6 mm) at pH 8.9 with a constant current of 3 mA per tube at 4°C for 3 hours. The sample was heated in 5% mercaptoethanol and 3% sodium dodecyl sulfate (SDS) for 1 hour at 60°C and the molecular weight was estimated by plotting log mobility against molecular weights of human serum albumin, ovalbumin, chymotrypsinogen and ribonuclease A protein standards.

Amino Acid Analysis

Amino acid analysis was performed in a Durrum Amino Acid Analyzer after hydrolyzing the peptides at 110°C for 16 hours with 6 N HCl in sealed evacuated tubes. Mean values of three determinations were used. Values for residues of amino acids per mol fragment were calculated with amino acid determinations of McMenamy et al.\(^11\) and amino acid sequence data of Meloun et al. for comparison.\(^12\) A Beckman 121 automatic amino acid analyzer was used when attempts were made to release dipeptides from albumin and Fragment C with CE.

Protein Determination

The concentration of protein in the solutions was either determined by the method of Lowry et al.\(^13\) or by measuring the absorption at 280 nm with Fragment C as a standard.

Preparation of Albumin Fragments

Human serum albumin was cleaved to Fragments A, B and C with cyanogen bromide and the fragments were separated and purified by the sequential use of gel filtration and column chromatography according to McMenamy et al.\(^11\)

The reduction and carboxymethylation of the disulfide bridges of the fragment were performed according to Crestfield et al.\(^14\) with slight modifications. Fragment C (55.5 mg) was dissolved in 5 ml of 0.5 M tris buffer, pH 8.6. The solution (6.2 × 10\(^{-4}\) M Fragment C) was stirred under nitrogen for 1 hour. The mercaptoethanol was added in a concentration sufficient to give a 25-fold excess over the 12 possible sulfhydryl (SH) residues of Fragment C. The reaction was allowed to proceed for 90 minutes at room temperature. Iodoacetic acid (1.73 g) was then added dropwise, and the pH was simultaneously adjusted to 8.7 with 0.2 N NaOH. After stirring for 1 hour, the solution was dialyzed for 48 hours against distilled water and then lyophilized (Method 1). Alternatively, the reduction was carried out in 8 M urea (Method 2) or with only a fourfold excess of mercaptoethanol over the possible SH-groups (Method 3).

Immunodiffusion

Immunodiffusion was carried out on Ouchterlony plates using antiserum to human albumin and to purified human lung CE. The latter was produced in the goat by injecting purified enzyme.

Results

Commercially prepared plasma protein preparations inhibited purified CE of hog kidney. Four different preparations of three manufacturers were tested. Thirty-five µl of the 5% protein solution inhibited the hydrolysis of BPPG by 50% (fig. 1).

Because commercial plasma protein preparations contain 4 × 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 × 10\(^{-4}\) M. The other additive, sodium caprylate, had no effect on the activity of the enzyme. Related compounds such as tryptophan, acetylhistidine and histidine were also inactive at 10\(^{-4}\) M concentration.

Presence of Albumin in CE

During purification of human CE\(^7\)\(^16\) crude preparations have low enzymic activity, so we examined human lung CE preparations during the various stages of purification for the presence of albumin. (The
details of purification of human CE will be published elsewhere.) We found that CE of homogenized human lung extracted with detergent and partially purified by gel filtration on Sephadex G-200 column still contained albumin in the active peak (fig. 2), as shown by immunodiffusion. Figure 2 indicates that the preparation of CE reacted with antibody to human lung CE and also with antibody to human serum albumin. Albumin, which has a molecular weight of 66,500, eluted in gel filtration on Sephadex G-200 column with CE, which has a much higher molecular weight. In gel filtration, CE frequently shows a molecular weight of 200,000 or even higher, thus CE probably forms a complex with albumin.

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

Albumin

Because commercial plasma protein preparations contain over 83% albumin, we studied the effect of purified, fat-free albumin on the CE. Purified CE of human lung and hog kidney are equally inhibited by albumin. The $K_{iO}$ values with Hip-Gly-Gly substrate were $2 \times 10^{-4}$ M and $1.9 \times 10^{-4}$ M. (The concentration of human enzyme was 42 mU/ml).

The inhibition of human lung enzyme was non-competitive. The $K_{i}$ determined in the Dixon plot (fig. 3) was $3 \times 10^{-4}$ M.

Fragments of Albumin

Since the commercial plasma protein solutions inhibited CE relatively more than accounted for by albumin content, which is an order lower than the $K_{iO}$ value of purified albumin, 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. Following one of the several conflicting nomenclatures, 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 $K_{iO}$ values were $2.2 \times 10^{-6}$ M, $7.4 \times 10^{-6}$ M, $2.5 \times 10^{-6}$ M for Fragments A, B and C, respectively.

Fragment C

Fragment C has most of the ligand binding properties of human plasma albumin, thus we studied the inhibition of CE by this protein. In electrophoresis, Fragment C in presence of SDS migrated as a single band of a molecular weight of 18,000 ± 800. The amino acid composition of Fragment C is shown in table 1. The values for single amino acids obtained are in good agreement with the theoretical value calculated from the available data, indicating a high degree of purity of Fragment C.

To establish whether the inhibition depends on the secondary and tertiary structure of the fragment or on free SH groups, we reduced and carboxymethylated the disulfide bridges in Fragment C (CMFC) formed...
TABLE 1. Amino Acid Composition of Fragment C of Human Serum Albumin and its Reduced and Carboxymethylated Derivatives

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Fragment C</th>
<th>CMFC 1 (Method 1)</th>
<th>CMFC 2 (Method 2)</th>
<th>CMFC 3 (Method 3)</th>
<th>Expected values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residues per mol Fragment C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASP</td>
<td>15.8</td>
<td>16.7</td>
<td>17.5</td>
<td>15.1</td>
<td>14</td>
</tr>
<tr>
<td>Thr</td>
<td>5.8</td>
<td>5.3</td>
<td>5.6</td>
<td>5.4</td>
<td>6</td>
</tr>
<tr>
<td>Ser</td>
<td>8.7</td>
<td>9.1</td>
<td>8.8</td>
<td>8.5</td>
<td>9</td>
</tr>
<tr>
<td>Glu</td>
<td>30.6</td>
<td>30.0</td>
<td>29.2</td>
<td>27.8</td>
<td>24</td>
</tr>
<tr>
<td>Pro</td>
<td>3.9</td>
<td>2.6</td>
<td>2.9</td>
<td>4.3</td>
<td>5</td>
</tr>
<tr>
<td>Gly</td>
<td>3.3</td>
<td>3.0</td>
<td>3.0</td>
<td>3.1</td>
<td>3</td>
</tr>
<tr>
<td>Ala</td>
<td>23.3</td>
<td>23.3</td>
<td>23.3</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>31.7*</td>
<td>—</td>
<td>—</td>
<td>25.3*</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>4.6</td>
<td>4.8</td>
<td>5.0</td>
<td>5.6</td>
<td>5</td>
</tr>
<tr>
<td>Ile</td>
<td>2.7</td>
<td>3.0</td>
<td>2.9</td>
<td>3.2</td>
<td>4</td>
</tr>
<tr>
<td>Leu</td>
<td>18.6</td>
<td>17.7</td>
<td>17.6</td>
<td>18.4</td>
<td>19</td>
</tr>
<tr>
<td>Tyr</td>
<td>5.0</td>
<td>5.5</td>
<td>5.9</td>
<td>6.2</td>
<td>6</td>
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<tr>
<td>Phe</td>
<td>8.8</td>
<td>9.8</td>
<td>10.2</td>
<td>10.5</td>
<td>10</td>
</tr>
<tr>
<td>His</td>
<td>3.8</td>
<td>3.7</td>
<td>3.9</td>
<td>4.2</td>
<td>4</td>
</tr>
<tr>
<td>Lys</td>
<td>20.0</td>
<td>18.4</td>
<td>18.7</td>
<td>19.6</td>
<td>19</td>
</tr>
<tr>
<td>Arg</td>
<td>7.8</td>
<td>7.8</td>
<td>7.8</td>
<td>11.0</td>
<td>9</td>
</tr>
<tr>
<td>Trp</td>
<td>—‡</td>
<td>—‡</td>
<td>—‡</td>
<td>—‡</td>
<td>1</td>
</tr>
<tr>
<td>H-Ser</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>CM-Cys</td>
<td>—</td>
<td>12.1†</td>
<td>12.8†</td>
<td>9.6</td>
<td></td>
</tr>
</tbody>
</table>

* Cys and Ala could not be separated under conditions applied.
†Trp is destroyed upon acid hydrolysis.
‡Upon reduction and carboxymethylation of Fragment C (CMFC) cysteine residues are completely or partially converted into CM-Cys, depending on the conditions of reduction.

TABLE 2. Inhibition of Human Lung Converting Enzyme by Fragment C and its Reduced and Carboxymethylated (CM) Derivatives

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cysteine content (mol/mol)*</th>
<th>CM-cysteine (mol/mol)</th>
<th>Ki (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment C</td>
<td>8.7</td>
<td>—</td>
<td>$1.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>CM Fragment C (Method 1; CMFC 1)</td>
<td>—</td>
<td>12.1</td>
<td>$7.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>CM Fragment C (Method 2; CMFC 2)</td>
<td>—</td>
<td>12.8</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>CM Fragment C (Method 3; CMFC 3)</td>
<td>2.3</td>
<td>9.6</td>
<td>$3 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* Determined by subtracting the theoretical value for alanine from the sum of alanine and cysteine obtained by amino acid analysis.
Reduced and carboxymethylated Fragment C from 1.7 x 10^{-8} M to 3 x 10^{-8} M. Carboxymethylation of all cysteine residues (Method 1) decreased the inhibition by raising the $K_i$ to 7.5 x 10^{-8} M.

When Fragment C was completely denatured by urea and by reducing all the disulfide bridges (Method 2), the resulting protein inhibited less than Fragment C, and the $K_i$ was 8 x 10^{-8} M.

The inhibition of Fragment C and CMFC 3 was not due to binding of a metal co-factor of human CE, because addition of 2 x 10^{-8} M CoCl$_2$ did not reverse the inhibition.

**Bioassay**

Fragment C inhibited the inactivation of bradykinin by human lung CE 60% at 7 x 10^{-8} M and CMFC 3 inhibited 73% at 4 x 10^{-8} M.

Fragment C also inhibited the enzyme on the surface of intact suspended cultured human endothelial cells as shown in Table 3. Here also CMFC 3 was a better inhibitor of the endothelial enzyme than was Fragment C.

**Albumin as Substrate of CE**

Purified human lung CE (0.01 U) was incubated with human serum albumin (4 x 10^{-8} M) and Fragment C (3.6 x 10^{-8} M) at pH 7.4 for 1 and 6 hours, respectively. After the reaction was stopped by acid precipitation of proteins there was no increase in ninhydrin-positive material over the zero time blank. Similarly, when the reaction mixture was examined by the amino acid analyzer, no peaks corresponding to dipeptides could be detected.

These experiments demonstrated that neither albumin nor Fragment C were substrates for CE. In addition, the experiments indicate that albumin and Fragment C, but not their breakdown products, are the inhibitors of CE.

**Discussion**

Various reports have described severe hypotensive reactions in patients, which followed the administration of plasma protein fractions manufactured by several companies. The reactions are particularly severe in patients with cardiopulmonary bypass. The plasma proteins are prepared by methods which include alcohol precipitation and heating over 10 hours at 60°C. They contain over 83% albumin, plus acetyltryptophan and other additives. The plasma kallikrein-kinin system is believed to be involved in the hypotensive reaction since the preparations are frequently contaminated by bradykinin and by a prekallikrein activator, Hageman factor fragment.

Inhibition of CE prolongs the hypotensive effects of kinins and abolishes the vasoconstrictor effect of renin by blocking the release of angiotensin II.

Bradykinin is inactivated mainly by two enzymes, kininase I and kininase II. The former enzyme is carboxypeptidase N, which is mainly responsible for cleaving the peptide in blood plasma, while the latter enzyme is identical with the angiotensin I converting enzyme. It is present in blood plasma although its more important location is on the surface of endothelial and epithelial cells.

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, hypothesitically it is high enough to inhibit sufficiently much of the activity of CE. Furthermore, albumin may interfere with assaying of CE activity in human plasma. Although in gel filtration CE exhibits a molecular weight about three times higher than albumin, albumin was eluted in the peak containing CE, indicating binding. Albumin present in partially purified human CE preparation may account for the low CE activity of these preparations. Digests of serum proteins can also inhibit other enzymes that inactivate bradykinin such as pancreatic carboxypeptidase B, a very potent kininase.

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 plasma preparations. These preparations inhibit CE more than indicated by their albumin content alone. They also potentiate the effect of bradykinin on the isolated rat uterus (Erdős and Robinson, unpublished data). A convenient way to obtain large fragments of proteins is to cleave them with CNBr at the methionine residues. All three fragments obtained by this method from human serum albumin inhibit CE more than albumin itself. The $I_{50}$ values were at least three times lower for the fragments than for serum albumin. Fragment C, which consists of the residues 124–298 of the polypeptide chain of albumin, is of particular interest because it has most of the ligand-binding potency of albumin. In addition to inhibiting soluble, purified human lung enzyme, Fragment C and its derivative also inhibit bradykinin hydrolysis by CE present on the surface of intact cultured human endothelial cells.

Changes in the structure of Fragment C affect its inhibitory potency as shown by reduction and carboxymethylation of all cysteine residues (Method 1) decreased the inhibition by raising the $K_i$ to 7.5 x 10^{-8} M.

**Table 3. Inhibition by Albumin Fragments of the Inactivation of Bradykinin by Cultured Human Endothelial Cells**

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment C</td>
<td>10^{-4}</td>
</tr>
<tr>
<td>Reduced and carboxy methylation of Fragment C (CMFC 3)</td>
<td>10^{-4}</td>
</tr>
</tbody>
</table>

Activity: 9.3 - 14.1 nmoles of bradykinin/hr/10^{6} cells.
boynamethylation of this fragment. The Kᵢ of CMFC 3, which has one disulfide bridge intact, is one sixth of that of Fragment C. Another protein, CMFC 1, is the product of reaction with a great excess of mercaptoethanol and all of the cysteine residues are derivatized (Method 1). Its Kᵢ is one-half of the value for underivatized Fragment C. These data suggest that introduction of carboxyl groups enhances the inhibitory properties of Fragment C, particularly when one intact disulfide bridge preserves the original con-

formation of a certain region within the polypeptide chain. As yet this bridge in the peptide chain has not been identified. In contrast, when derivatization is carried out under denaturing conditions, the CMFC 2 fragment obtained inhibits less than Fragment C itself. Since the CM-cysteine content of fragments obtained with Methods 1 and 2 is identical and CMFC 2 has a ten times higher Kᵢ than CMFC 1, preservation of the original secondary structure seems to have greater influence on the inhibition constant than the incorporation of carboxyl groups.

In conclusion, by inhibiting CE on vascular endothelial cells and in plasma, albumin, its fragments, and other plasma proteins may potentiate the hypotensive effects of bradykinin, which is present in or liberated by infused plasma protein preparations. The presence of kinins, prekallikrein ac-

tivator and kininase II inhibitors may explain the severe hypotension encountered in some cases after infu-

sion of such preparations, especially when the lung is excluded from the circulation, since the pulmonary vascular bed is very active in cleaving kinins. This study of the inhibition of CE by albumin fragments should help to clarify the nature of complexing of the enzyme by albumin and possibly by other plasma proteins.

Acknowledgments

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