Primary Structure of the Reactive Site of Human CT-Inhibitor*

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Guy S. Salvesen‡, Joseph J. Cataneses, Lawrence F. Kress§, and James Travis¶

From the 1Department of Biochemistry, University of Georgia, Athens, Georgia 30602 and the 2Department of Experimental Biology, Roswell Park Memorial Institute, Buffalo, New York 14263

Human CT-inhibitor (CT-Inh) forms an equimolar complex with complement proteinase Cts that is resistant to dissociation by sodium dodecyl sulfate. The formation of this stable complex results in the cleavage of a peptide bond near the carboxyl terminus of the inhibitor and, whereas the bulk of CT-Inh remains covalently bound to the light chain of Cts, the complex inhibitor peptide can be isolated under denaturing conditions. We have sequenced the amino-terminal region of this peptide and deduced that it represents the carboxyl-terminal side of the reactive site of CT-Inh.

Limited proteolysis of CT-Inh by Crotalus atrox protease results in an active derivative lacking an amino-terminal peptide of 36 residues. Further proteolysis of this derivative with Pseudomonas aeruginosa elastase inactivates the inhibitor and a peptide is released. The amino-terminal sequence of this peptide overlaps with that of the complex peptide and indicates that the residue imparting primary specificity to the inhibitor is arginine.

Human CT-Inhibitor (CT-Inh) is a single-chain glycoprotein of M, approximately 100,000 and containing 35% carbohydrate (1–3). As its name implies, CT-Inh was originally identified as an inhibitor of the activated form of the first component of complement (4, 5) and is the only known plasma inhibitor of CT and Cts (6). In addition, plasma kallikrein (7), Factors XI and XIIa (8), and plasmin (9) are inactivated by this inhibitor.

Cts and plasma kallikrein have both been shown to form an equimolar, denaturation-resistant, inactive complex with CT-Inh. Four other plasma proteinases, αi-proteinase inhibitor (αiPI), αi-antichymotrypsin (αiAChy), anti-thrombin III (AT III), and αi-antiplasmin (αiAP), share this property with their target enzymes (10), and in this way, at least, are functionally related to CT-Inh. αiPI, αiAChy, and AT III are sufficiently related in a structural sense to be assigned to the same protein superfamily (which also contains chicken ovalbumin) (11, 12) and data are beginning to accumulate suggesting that αiAP may also be a member (13). Although Harrison (14) found no sequence homology between the 40 amino-terminal residues of CT-Inh and any of the other inhibitors, we feel that the functional relationship between the proteins may reflect a structural one and we have examined this possibility by determining the sequence of at least part of the reactive site of human CT-Inh.

MATERIALS AND METHODS

Human Cts was provided by Dr. David Bing, Center for Blood Research, Boston, MA. The burst titrant, thiobenzyl guanidino-benzoate, and the Cts substrate, benzoyl-L-arginyl-L-lysine, were gifts of Dr. James Powers, Georgia Institute of Technology, Atlanta, GA. The Pseudomonas aeruginosa elastase was provided by Dr. K. Morihara, Shionogi Co. Ltd., Osaka, Japan. Crotalus atrox metaloproteinase (EC 3.4.24.1) was modified by a modification of a published method (15), with the incorporation of a final step on CM-Sepharose to remove contaminants. The kallikrein substrate B-Pro-Phe-Arg-p-nitroanilide (S-2233) was obtained from Kabi. DFP was from Sigma and was stored as a 0.2 M stock solution in dry propan-2-ol. Reagents for sequencing were from Beckman. All other chemicals were of reagent grade.

Sequence Analysis—Amino-terminal sequence analysis was performed with a Beckman Model 890C Sequencer, modified with a cold trap, according to the procedure of Edman and Begg (16). The 0.1 M Quadrol program, described by the manufacturer, was routinely utilized in this study. All samples for sequencing were first desalted on a 100-ml bed of Sephadex G-25. Phenylthiohydantoin derivatives were identified by isocratic separation on high pressure liquid chromatography (17), with identification of the anhydro derivatives of Ser and Thr at 313 nm. In case of ambiguities, samples were also back-hydrolyzed to the free amino acids (18) and identified by analysis on a Beckman Model 119CL amino acid analyzer.

Polyacrylamide Gel Electrophoresis (PAGE)—Nondenaturing PAGE in pore-limit gels of increasing matrix concentration (4–26%) and NaDodSO4-PAGE in gels of 7 or 12.5% uniform matrix concentration were carried out as described by Barrett et al. (19); linear gradient NaDodSO4 gels of matrix concentration 5–12% were run in the same buffer system.

Enzyme Assays—Human plasma kallikrein, purified as described by Nagase and Barrett (20), was assayed using B-Pro-Phe-Arg-p-nitroanilide, as described by the manufacturer, but in 0.1 M Tris–HCl, pH 7.8. The concentration of active kallikrein was determined using the burst titrant thiobenzyl guanidino-benzoate, as described by Cook and Powers (21). Human Cts (E1/2 280 nm = 9.4) (22) was assayed using Z-Ala-Arg-thiobenzoyl ester in the presence of 4,4′-dithiobispyridine, following McRae et al. (23), in 0.1 M Tris–HCl, pH 7.8.

Assay for CT-Inh—The concentration of active CT-Inh in solution was measured by its ability to inhibit Cts or plasma kallikrein. Forty-µg portions of Cts or plasma kallikrein in 20 µl of 0.1 M Tris–HCl, pH 7.8, were allowed to react with various volumes of purified CT-Inh or plasma fractions containing the inhibitor and brought to a total volume of 180 µl with Tris buffer. After 30 min at room temperature, duplicate 20-µl portions of the reaction mixture were assayed for remaining Cts or plasma kallikrein activity. Amounts of CT-Inh used in this study were thus based on estimates of the active protein as determined by titration with active-site standardized antibodies.

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† To whom correspondence should be addressed.

‡ The abbreviations used are: CT-Inh, Cts-esterase inhibitor; αiPI, αi-proteinase inhibitor; αiAChy, αi-antichymotrypsin; AT III, antithrombin III; αiAP, αi-antiplasmin; NaDodSO4, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; PEG, polyethylene glycol 20,000; PAGE, polyacrylamide gel electrophoresis. Nomenclature for complement components is that recommended by the World Health Organization (Immunochemistry (1970) 7, 137).

plasma kallikrein, assuming an equimolar binding ratio (3).

**Purification of CT-Inhibitor**—CT-Inh was isolated from outdated plasma by a procedure involving precipitation with polyethylene glycol 6000 (PEG), DEAE ion-exchange chromatography, and chromatography on Cibacron Blue-Sepharose.

Four hundred ml of human plasma was made 12.5% (w/v) in PEG by concentration of the appropriate volume of a 50% (w/v) solution of PEG in water. The mixture was stirred slowly at 23°C for 30 min, and the resulting precipitate was removed by centrifugation. A 50% solution of PEG (w/v) was added slowly to the supernatant to give a final concentration of 30% PEG (w/v), the mixture was again stirred slowly at 23°C for 30 min, and the precipitate was collected by centrifugation and redissolved in 200 ml of 0.02 M sodium phosphate buffer, pH 7.0, containing 0.05 M NaCl. The remaining steps were all performed at 4°C. The dissolved pellet was applied to a column (3.0 x 15 cm) of DEAE-Sephalac equilibrated with the pH 7.0 buffer, and the charged column was washed with 10 bed volumes of equilibration buffer. The column was then developed with a linear gradient (total volume of 600 ml) from 0.05 M NaCl to 0.20 M NaCl, in the same buffer, and fractions of 6.0 ml were collected and tested for their ability to inhibit CTs.

Peak inhibitory fractions were pooled and made up to a total volume of 200 ml with 0.05 M Tris-HCl, 0.05 M NaCl, pH 7.8. It was noted that all peak activity fractions co-eluted in a reproducible manner with ceruloplasmin, and it was found expedient to pool all the visibly blue fractions from the ion-exchange step.

A column (3.0 x 15 cm) of Cibacron Blue-Sepharose (24), equilibrated with the Tris buffer, was charged with the diluted CT-Inh pool and washed with 5 bed volumes of equilibration buffer containing 0.05 M NaCl. Pure CT-Inh was then eluted stepwise by addition of equilibration buffer containing 0.2 M NaCl.

**Formation of CT-Inhibitor Complex with CIs**—A complex was formed by incubating 100 nmol of CT-Inh and 80 nmol of CIs for 4 h at room temperature, in 5.0 ml of 0.1 M Tris-HCl, pH 7.8. The complex mixture was made 5 mM in dithiothreitol and 1% (w/v) in NaDodSO4. Following incubation for 15 min at 60°C, the reduced, denatured mixture was carboxymethylated with a 4-fold molar excess of iodoacetic acid over dithiothreitol, and the mixture was further incubated for 10 min at room temperature. Components were separated by gel filtration on a bed (2.0 x 150 cm) of Sephacryl S-300, equilibrated with the pH 7.8 Tris buffer, containing 1% NaDodSO4. The column was run at a flow rate of 12 ml/h and 4.0-ml fractions were collected. Fractions were combined into several pools, and each pool was freeze-dried, desalted, freeze-dried again, and applied to the sequenator.

**Limited Proteolysis of CT-Inhibitor and Isolation of Digestion Products**—CT-Inh (208 nmol) was modified by limited proteolysis with C. atrox α-protease (5.5 nmol) for 60 min in 0.05 M Tris-HCl, 0.2 M NaCl, pH 7.8. Aliquots were taken at various times during the digestion, treated with 0.005 M EDTA, and the products (modified inhibitor and peptide) were separated by gel filtration on Sephadex G-100 using the above buffer. The peak corresponding to 89-kDa modified inhibitor was pooled, concentrated, and dialyzed against the above buffer.

The modified CT-Inh (189 nmol) was reacted with P. aeruginosa elastase (0.24 nmol) for 90 min in 0.05 M Tris-HCl, 0.2 M NaCl, pH 7.8. Aliquots were analyzed by gradient electrophoresis (26). The reaction was terminated by addition of EDTA to a final concentration of 0.005 M. The products were reduced and carboxymethylated as described above and separated by gel filtration on Sephacryl S-200, using buffers containing 0.5% NaDodSO4, to completely dissociate all components. Protein peaks were pooled and prepared for sequenator determinations as already described.

**RESULTS**

**Characterization of CT-Inhibitor**—Estimates of CT-Inh molecular mass varied slightly, depending upon the gel systems used, but were near 100 kDa. However, because of the high carbohydrate content of this glycoprotein (33%), this value should only be taken as an approximation. The purified protein migrated as a single component in NaDodSO4-PAGE (Fig. 4), and as a single, slightly diffuse band in nondenaturing PAGE (not shown). Purity of the final product was also checked by double immunodiffusion against commercial antisera raised against α2-macroglobulin, α1PI, α1AChy, AT III, albumin, transferrin, and ceruloplasmin. Some early preparations of CT-Inh contained α1AChy, but this was eliminated by ignoring the CTs-inhibitory peak tail (pooling only the visibly blue tubes) from the DEAE-Sephalac column. Recoveries of CT-Inh were generally about 50%, based on CTs inhibitory capacity.

Sequence analysis (20 cycles) of 100 nmol of the purified protein revealed a single N-terminal sequence, in agreement with that reported by Harrison (14).

CT-Inh was found to be fully active by titration with active site-standardized plasma kallikrein (Fig. 1). This finding confirms the recently revised estimate of the E50,1cm = 280 am, of 3.6 (14). Preformed complexes of CT-Inh with plasma kalli- krein were unable to inhibit CTs, even after prolonged (4 h) incubations at 37°C. Similarly, CT-Inh:CTs complexes were unable to inhibit plasma kallikrein, and we take these results to be evidence for a single inhibitory site (reactive site) on CT-Inh which is recognized by either enzyme.

**Characterization of the CT-Inhibitor:CTs Complex**—Varying amounts of CT-Inh (5–200 µg) were added to a standard quantity of CTs (100 µl of a 1 mg/ml solution in 0.1 M Tris-HCl, pH 7.8), and portions of the mixture were assayed after 30 min and 8 h for residual enzyme activity. The decrease in enzyme activity was directly proportional to the amount of added inhibitor, and activity was abolished by a 0.86:1 molar ratio of inhibitor to enzyme at each time point tested (Fig. 1). As the CT-Inh was fully active (see previous section), we assume that the CTs was 86% active and conclude that the resultant complex contains an equimolar amount of enzyme and inhibitor.

CT-Inh was allowed to react for 30 min with a 0.5- and a 2.0-fold molar ratio of CTs, under the conditions described above, and the mixtures were run in a NaDodSO4-gel electrophoresis system (Fig. 2), after treatment with 5% 2-mercaptoethanol, 1% NaDodSO4 (final concentrations), at 100°C, for 5 min. One major enzyme:inhibitor complex band (M, = 120,000), presumably representing a complex between the native inhibitor and the light chain of CTs, and two minor complex bands were clearly distinguished, but it is also clear that not all of the native CT-Inh was complexed in an NaDodSO4-stable form in the presence of an excess of CTs.
have shown that the CT-Inh preparation used in this study was fully active. Therefore, we suggest that the free inhibitor seen in lane B of Fig. 2 represents CTs-complexed material that is not bound through an NaDodSO4-stable linkage to the enzyme after 30-min incubation. Explanations for this are dealt with under “Discussion.”

Bond Cleavage in the CT-Inhibitor:CTs Complex—When a preformed complex of CT-Inh with CTs was applied to the sequenator, a novel sequence was determined, in addition to those expected for the two chains of CTs (6) and the inhibitor (Table I). The origin of the material representing the new sequence was deduced from the results of the following experiments. When CTs was inactivated with DFP and then incubated with CT-Inh under conditions used for complex formation, only sequences corresponding to the amino-terminal structure of the two chains of CTs and the native CT-Inh were detected (Table I). When CTs was incubated alone under the same conditions, only amino-terminal sequences corresponding to the two chains of this proteinase were detected (not shown). Thus, the novel sequence is not caused by autolysis of the enzyme, nor does it represent any contaminants within either of the preparations. We therefore conclude that the new sequence arises from cleavage of the inhibitor at an X-Thr bond, following the formation of a complex with the enzyme, and we suggest that we have observed CTs-mediated reactive site cleavage of CT-Inh.

Analysis of the CT-Inh Postcomplex Peptide—Material containing the novel amino-terminal sequence identified as described in the previous section was isolated by gel filtration (see “Materials and Methods”). The pool of fractions eluting between 55 and 60% bed volume of the S-300 column contained a single sequence (Table II) identical to the novel one described in the previous section. This material had an apparent Mr of 5,000 in NaDodSO4-gel electrophoresis (Fig. 3), but this estimate must be examined with caution as the relationship between Mr and rate of migration in NaDodSO4 gels is nonlinear below about 8,000.

Other pooled material contained sequences corresponding only to the expected amino-terminals of native CT-Inh and the two chains of CTs, and we conclude that a single cleavage has occurred to give rise to a postcomplex peptide of low molecular weight comprising the carboxyl-terminal region of the inhibitor.

Limited Proteolysis of CT-Inhibitor and Isolation of Overlap Peptide—Incubation of intact CT-Inh (104 kDa) with C. atrox α-protease resulted in formation of modified CT-Inh (89 kDa) as shown in Fig. 4). The modified inhibitor was fully active, and it could be obtained in a homogeneous form by gel filtration on Sephadex G-100 (not shown). The amino-terminal sequence of modified CT-Inh was Ile-Glu-Val-X-X-Leu-Pro-Thr-Thr, which differs from the amino-terminal sequence of intact CT-Inh (14). However, the first four residues of this new sequence are identical with residues 37–40 of CT-Inh, clearly indicating that the protease had cleaved the native inhibitor in the amino-terminal region of the molecule.

Incubation of modified CT-Inh with P. aeruginosa elastase resulted in formation of an 83-kDa CT-Inh product (Fig. 4).

<table>
<thead>
<tr>
<th>Table I</th>
<th>Amino acid sequence of CTs:CT-Inh complex components</th>
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<tbody>
<tr>
<td>Cycle number</td>
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<tr>
<td>1</td>
<td>Glu, Asn, Ile</td>
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<td>2</td>
<td>Pro, Ile</td>
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<td>3</td>
<td>Thr, Gly</td>
</tr>
<tr>
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<td>Met, Ala, Gly</td>
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<td>5</td>
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<tr>
<td>6</td>
<td>Gly, Ser, Asp</td>
</tr>
<tr>
<td>7</td>
<td>Glu, Ser, Ala, Glu, Ser, Ala, Val</td>
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</tbody>
</table>

CTs (20 nmol), or CTs previously inactivated in the presence of 5 mM DFP, was mixed with 25 nmol of CT-Inh in 5 ml of 0.1 M Tris-HCl, pH 7.8. Taking into account the activity of the CTs (86%), the molar ratio of active CTs to CT-Inh was approximately 1:1.5. After 4 h at room temperature, the mixtures were desalted and applied to the sequenator for seven cycles each. Amino acid residues were identified as described in the text. The presence of multiple sequences precluded calculations of repetitive yields.

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<table>
<thead>
<tr>
<th>Table II</th>
<th>Sequence analysis of postcomplex peptide from CT-inhibitor:CTs complex interaction and from limited proteolysis of CT-inhibitor by P. aeruginosa elastase</th>
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<tr>
<td>Cycle number</td>
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<td>1</td>
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<tr>
<td>12</td>
<td>Gln</td>
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</table>

* Amino acid residues were identified as described in the text. A repetitive yield of 95% (based on phenylthiohydantoin-Val in cycles 4 and 7) was calculated for the postcomplex peptide sequence, while that for the elastase-generated peptide was 91% (based on phenylthiohydantoin-Val in cycles 7 and 10).
CT-Inhibitor Reactive Site

As methylated products were separated on Sephacryl described in the text was chromatographed on Sephacryl S-300 (not shown) in the presence of 1% NaDodSO4. Fractions containing the desired proteins were pooled and subjected to sequence analysis. Shown above are: A, the amino-terminal of CT-Inh plus a trace of CTs heavy chain; B, CT-Inh postcomplex peptide; C, Mr standards. Right panel: modified CT-Inh (89 kDa) was inactivated with P. aeruginosa elastase as described in the text, and the reduced, carboxymethylated products were separated on Sephacryl S-200 (not shown) in the presence of 1% (w/v) NaDodSO4. Fractions containing the desired proteins were pooled and subjected to sequence analysis. Shown above are: D, inactivated CT-Inh; E, CT-Inh reactive-site overlap peptide; F, Mr standards.

As well as total loss of inhibitory activity. The reaction products were isolated by gel filtration on Sephacryl S-300 and their homogeneity is shown in Fig. 3. The amino-terminal sequence of the 83-kDa inactivated CT-Inh was identified to that of the 89-kDa modified CT-Inh, indicating that no further cleavage had occurred in the amino-terminal region of the inhibitor molecule. The sequence of the released peptide overlapped that of the postcomplex peptide (Table II) and established Arg-Thr as the reactive site P1 and P′1 residues of human CT-Inh, according to the nomenclature of Schechter and Berger (31).

Discussion

In confirmation of previous reports (3, 27) we have found that CT-Inh forms an equimolar, denaturation-resistant complex with CTs, resulting from a presumed covalent link between the CTs light chain reactive-site serine residue and the P′α-carboxyl group of the inhibitor. However, after a 30-min incubation of CT-Inh with an excess of CTs, not all of the inhibitor was bound covalently to the enzyme, despite the fact that the reaction was performed under conditions that should have led to the complete complexing of the inhibitor. We suggest two explanations for this. First, the rate of formation of an apparent covalent complex, under the described conditions, may be slower than the rate of formation of an initial complex in which the inhibitor is inactive. We cannot comment further on the magnitude of this rate, except to say that it would be reflected in the unimolecular k2 = 3.5 x 10−1 s−1, as determined by Nilsson and Wiman (27) for the reaction of CTs with CT-Inh. Alternatively, an equilibrium may exist between the complex between covalently and noncovalently attached CTs to inhibitor. This may be equivalent to the equilibrium between virgin (reactive-site peptide bond intact) and modified (reactive-site peptide bond cleaved) soybean trypsin inhibitor during its reaction with proteinases, as described by Laskowski and Kato (28), although in the case of CT-Inh the protein does not dissociate from the complex.

The recent finding (29) that an amino-terminal threonine residue is detectable after complex formation between CTs and CT-Inh has been extended in the present work in which we suggest the reactive-site sequence of P2−P′2 in CT-Inh (Table III). We conclude that the reactive-site peptide bond is between the arginine and threonine residues and deduce that arginine in the P2 position dictates the primary specificity of the inhibitor. Our deduction is supported by the fact that those proteinases (CTr, CTs, and plasma kallikrein (10)) which form tight complexes with CT-Inh have a preference for arginine in their S1 subsites (23).

That the peptide bond cleavage which has enabled us to determine the location of the reactive site of CT-Inh is caused by the proteolytic activity of CTs, rather than a potential contaminating proteinase, is suggested by two lines of evidence. First, the fact that CTs and not DFP-inactivated CTs is able to form a covalent complex with the inhibitor (3) strongly indicates that the reactive-site serine of the enzyme has become acylated by the carbonyl function of a peptide bond of the inhibitor (10). Taking into account our knowledge of the mechanisms of peptide bond cleavage, this indicates that the first leaving group of the hydrolysis is the postcomplex peptide, containing a free amino terminus. This, in itself, implies that it is CTs bound to the inhibitor through its specificity pockets which produces peptide bond cleavage. Second, considering that a hypothetical contaminating proteinase would have to be present in very small quantities, for we cannot detect it by NaDodSO4-gel electrophoresis or by sequence analysis, it should catalytically and force peptide bond cleavage as did the P. aeruginosa elastase. However, our data show that a significant amount of intact inhibitor remains when we subject our equimolar complex to NaDodSO4-gel electrophoresis.

Several authors have commented previously on the high carbohydrate content of CT-Inh (3, 14), and for the purpose of comparison with other plasma proteinase inhibitors we rely...
on the deglycosylated $M_0$, of 78,000 estimated by Harrison (14). The plasma proteinase inhibitors CT-Inh, $\alpha$PI, $\alpha$Achy, AT III, and $\alpha$AP are all functionally related in their capacity to form equimolar, denaturation-resistant complexes with their target enzymes (10). They are also structurally related by, at least, the following criteria: (a) they are all single-chain glycoproteins; (b) their single reactive sites are close to their respective carboxyl termini; (c) at least four of the inhibitors, $\alpha$PI, $\alpha$Achy, AT III, and $\alpha$AP, contain regions of homology throughout their primary structures (10, 12, 13). The aminoterminal 40 residues of CT-Inh bear no homologies with the other inhibitors (14), nor does the reactive site of the protein show much homology with the corresponding region of members of the $\alpha$PI superfamily of proteins (Table III). We are not surprised that the reactive site sequences of these proteins show little homology with CT-Inh, as variation in the reactive site of an inhibitor is the usual way of changing its specificity (28). Indeed, homologies between the members of the superfamily are much greater outside this region than within (11). We await the appearance of more complete sequence data on CT-Inh to see whether, in light of the structural and functional similarities with members of the superfamily, CT-Inh also merits inclusion in the manner defined by Hunt and Dayhoff (11).

Acknowledgement—We thank Helana Hoover-Litty for expert technical assistance.

**REFERENCES**


**alignment of CT-inhibitor reactive sites with those determined for other members of the $\alpha$PI superfamily**

Alignments are based on the reactive site regions of human $\alpha$PI, human AT III, and chicken ovalbumin as given by Hunt and Dayhoff (11), $\alpha$Achy as given in Ref. 12, and rat angiotensinogen as in Ref. 30. No attempt has been made to increase apparent homologies between the proteins, other than the assignment of gaps as given in Ref. 11.

<table>
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<th>Protein</th>
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