Limited proteolysis of T-kininogen by heterologous and homologous endopeptidases (bovine trypsin, human leukocyte elastase, rat submaxillary gland endopeptidase k, and rat mast cell chymase) produced similar fragmentation.

Amino-terminal sequence analysis of whole T-kininogen lysates and purified proteolytic fragments identified four susceptible regions which contained all the preferential cleavage sites for these proteinases. Two of these susceptible regions were close to the junction between heavy chain cystatin-like domains, the third was in the kinin-containing region, and the fourth was close to the carboxyl terminus of the T-kininogen light chain.

There was only one primary site for each proteinase in the kinin-containing region, which explains why catalytic amounts of these proteinases did not release immunoreactive kinin from this kininogen. However, preferential cleavage of T-kininogen close to the junction between cystatin-like domains released fragments which, provided they included cystatin-like domains 2 and/or 3, strongly inhibited papain and cathepsin L.

The fragments were inhibitory even when parts of the amino-terminal ends of the domains were lacking. The highly conserved glycyl residue, thought to be involved in the inhibitory reactive site of cystatin-like inhibitors, was not required in purified domain 3 for inhibition of cathepsin L.

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Type 3 cystatins, which include all types of kininogens (1), are both tight-binding inhibitors of papain-like cysteine proteinases and potential precursors of bradykinin or related vasoactive peptides. These peptides are generally specifically excised from the single kininogen peptide chain by tissue or plasma kallikreins (2).

T-kininogen is an exception to the general rule for kininogens since it is not susceptible to kallikrein hydrolysis (3). T-kininogen is present in rat plasma as several molecular varieties (4-6) and was later found to be a cysteine proteinase inhibitor (4, 12); it is also the only kininogen described to date whose concentration increases during inflammation (13). Therefore, T-kininogen has also been called thiostatin, a name thought to better fit its presumed biological function (8, 14, 15). However, several proteinases can liberate T-kinin (Ile-Ser-bradykinin) from T-kininogen, but none of them is specific for this excision (3, 16-18).

We have previously shown that complete fragmentation of T-kininogen occurs before any kinin is released and that this fragmentation first gives rise to three major fragments of about 60, 40, and 20 kDa, suggesting that all cleavages occur in regions particularly prone to hydrolysis (18, 19). Human low M, kininogen can be similarly fragmented by several endopeptidases, and the elucidation of the multidomain structure of kininogens has shown that these susceptible regions lie at or close to interdomain regions of the kininogen heavy chain (20, 21). This cleavage may be physiologically important as some kininogen fragments are potent inhibitors of cysteine proteinases. The fragments might therefore react more readily with their potential target proteinases (20, 22) which are not found in extracellular spaces, at least in the normal physiological state (23). This interaction between kininogen fragments and proteinases has not yet been clearly demonstrated under physiological conditions, although low M, fragments cross-reacting with antibodies to low M, kininogen have been found in human urine and serum (24, 25). However, no enzyme would release these inhibitory fragments in vivo has been found in humans.

As part of our attempt to find a specific T-kininogenase, we have investigated two rat proteinases, mast cell chymase and submaxillary gland endopeptidase k (18), which are both potentially able to react with T-kininogen under physiological conditions. The submaxillary gland endopeptidase k is one of the major serine proteinases from submaxillary glands; it is structurally related to tissue kallikrein, but differs from the thiol-activated T-kininogenase first reported by Barlas et al. (26). Both chymase and endopeptidase k release T-kininogen fragments with similar M, max, which resemble those obtained after trypsin hydrolysis under experimental conditions in which no kinin is liberated (18, 27).

In the present study, we have identified the sites in T-kininogen which are preferentially cleaved by trypsin, chymase, endopeptidase k, and leukocyte elastase. We have also investigated the inhibitory properties of the released fragments toward cysteine proteinases to determine the structural requirements for inhibitory activity.

EXPERIMENTAL PROCEDURES

RESULTS

Proteolysis of T-kininogen—The proteolytic activities of bovine trypsin, human leukocyte elastase, rat submaxillary
gland endopeptidase k, and rat mast cell chymase were assayed using purified T-kininogen. All four proteinases readily induced similar, although not identical, fragmentation of T-kininogen, as judged by analytical SDS-PAGE (Fig. 1). All the proteinases first produced a 62-kDa fragment when in-...
According to the multidomain nomenclature of kininogens proposed by Müller-Esterl et al. (30), two of them were in the heavy chain, close to the junction between domains 1 and 2, and between domains 2 and 3; the third was in domain 4 which includes the bradykinin sequence, and the fourth was in the middle part of domain 5, which roughly corresponds to the carboxyl-terminal light chain. Sequence analyses of whole proteolytic lysates of T-kininogen indicated that these latter two regions were preferentially cleaved by all four proteases. Endopeptidase k preferentially cleaved after Arg residues, particularly those in the light chain Arg<sup>396</sup>-Ala<sup>397</sup> bond and the Arg<sup>368</sup>-Lys<sup>369</sup> bond of the bradykinin-light chain junction (Fig. 3). Subsequent cleavage occurred in the 2-3 interdomain region, at the Arg<sup>390</sup>-Glu<sup>391</sup> or Lys<sup>390</sup>-Asn<sup>391</sup> bonds (depending on the molecular variety of T-kininogen (7)). This was in complete agreement with the results of SDS-PAGE analysis which revealed an initial 62-kDa fragment (corresponding to domain 1-2-3 and part of the 4th domain), with no approximately 6-kDa complementary peptide (this had been preferentially cleaved, and its products were not distinguishable on the gel), followed by two fragments of 43 kDa and 20 kDa, corresponding to the cleavage between domain 1-2 and domain 3 of the heavy chain (18).

The initial tryptic cleavage occurred at the same position as that of endopeptidase k, in the carboxyl-terminal domain 5, but subsequent cleavages of the heavy chain were in the interdomain 1-2 region the Lys<sup>154</sup>-Lys<sup>157</sup> and Lys<sup>157</sup>-Thr<sup>158</sup> bonds, giving rise to a major fragment of 43 kDa after reduction and SDS-PAGE (Fig. 2). As shown previously (19), domain 2-3 can be further cleaved by large amounts of trypsin to liberate T-kinin plus a single inhibitory peptide from this double-headed inhibitor.

Mast cell chymase initially cleaved T-kininogen in the same regions as endopeptidase k and trypsin, although at different sites (Fig. 3). The peptide bonds most susceptible to chymase hydrolysis were the light chain Tyr<sup>398</sup>-Lys<sup>399</sup> bond (domain 5) and the Phe<sup>397</sup>-Arg<sup>398</sup> bond in the bradykinin sequence. This explains why T-kininogen digested with mast cell chymase followed by endopeptidase k or trypsin produced no immunoactive kinin (27). The third bond cleaved by chymase, Phe<sup>399</sup>-Glu<sup>400</sup> in interdomain 2-3, gave rise to two fragments, one of 45 kDa (domain 1-2) and a second of 20 kDa (domain 3), in perfect agreement with the SDS electrophoresis data.

Catalytic amounts of leukocyte elastase released a 62-kDa peptide domain 1-2-3) from T-kininogen, but no further cleavage in interdomain regions was observed before nonspecific proteolysis had split the molecule into small peptides (not shown). In keeping with this observation, the initial leukocyte elastase cleavage site was at the Ile<sup>398</sup>-Ser<sup>399</sup> bond, the first peptide bond in T-kinin.

Fig. 3 shows the 9 peptide bonds highly susceptible to hydrolysis by the four endopeptidases tested; one of them was common to two different enzymes. The four preferential endopeptidase cleavage regions appear clearly on this figure, suggesting that they are exposed to proteolytic attack. This is in agreement with predictive studies on the secondary structure based on knowledge of the sequence (31). Both heavy chain interdomain sequences and the kinin-containing domain include sequences with the highest probabilities of bend occurrence and of β-turn conformations, which facilitate enzyme accessibility (32). The cleavage sites of all the proteinases are always located in regions flanking those with the highest probabilities of β-turns (Fig. 4).

### Inhibitory Properties of T-kininogen Fragments

The total papain inhibitory capacity of T-kininogen is not modified under conditions which allow complete conversion of native T-kininogen to 20- and 40-kDa fragments, suggesting that the proteolytic fragments remain active once they are released from their precursor (18, 19).

The kinetic parameters, including inhibition constants (K<sub>i</sub>) and rate constants for association and dissociation, have been reported previously for some peptide products of trypsins and endopeptidase k hydrolysis (22). Low K<sub>i</sub> values were obtained in all cases, demonstrating that T-kininogen fragments may be as good if not better inhibitors of papain and cathepsin L than is the native molecule.

T-kininogen and cathepsin L was the endopeptidase k domain 3 fragment which lacks the 17 amino-terminal residues of the whole domain 3 (1). This fragment appeared to be the best inhibitor of rat liver cathepsin L, based on its inhibition constant K<sub>i</sub>. Curiously enough, this fragment did not include the highly conserved glycyl residue which is thought to be part of the reactive site of human cystatin C and chicken cystatin (33). This would indicate that the glycyl residue is not essential for the inhibitory function of the T-kininogen cystatin-like domain 3, or that of other inhibitory domains in kininogens from the rat and other species, since all of them have a high degree of homology in these particular regions of their heavy chain (1).

### DISCUSSION

Kininogen is released from kininogens by specific cleavage of two peptide bonds in their single chain by tissue or plasma kallikreins. This generates an amino-terminal heavy chain and a carboxyl-terminal light chain linked by a disulfide bridge. Kallikreins do not release kinins from rat T-kininogen, although massive (not physiological) amounts of several other proteinases will do so (3, 16–19). Proteolysis of T-kininogen, to give rise to peptides of similar Mr<sub>app</sub> whatever the endopeptidase, always occurs before any release of kinin. This suggested that the molecule contains preferential cleavage regions. Identification of cleavage sites by amino-terminal analyses revealed that T-kininogen contains four preferential cleavage regions. Although catalytic amounts of any of these
proteinases released immunoreactive kinin, the bradykinin-containing region was found to be preferentially attacked by at least three of the four endopeptidases used in this study. Only one peptide bond within or at either end of the T-kininogen moiety was cleaved by catalytic amounts of each proteinase. These enzymes therefore behave either as kininases (chymase) which inactivate the kinin before its excision from the precursor or as “half-cystatinogenases” (endopeptidase k, leukocyte elastase) which may help to release a kinin provided another enzyme cleaves a second peptide bond at the other end of the kinin moiety. There is no indication, as yet, that these cleavages occur in vivo.

The carboxyl-terminal end of the T-kininogen light chain is also highly susceptible to proteolysis. A stretch of 10 residues finishing 15 amino acids upstream from the carboxyl terminus contains two cleavage sites for three proteinases. There is no evidence, so far, for such a susceptible region in low M₉ kininogens from other species, nor for this cleavage having a biological significance. The function of the low M₉ kininogen light chain remains to be determined, and more information is necessary before speculating about a possible relationship between the high susceptibility of the light chain to proteolysis and any biological function.

Other sites of preferential cleavage by proteinases lie at the interdomain junction of the T-kininogen heavy chain, as in human L-kininogen (20, 21). This result was obtained, in part, using proteinases, 21 the same species. They may, therefore, be able to interact with T-kininogen in vivo. Acute phase conditions, which favor the release of proteinases including chymase and leukocyte elastase and induce a dramatic increase in T-kininogen concentration at inflammatory sites (5), seem to be most appropriate for generating inhibitory fragments from T-kininogen. Kininogen-derived inhibitory peptides have been found in human plasma (25) and urine (24), but no enzyme responsible for this release was identified. The present study indicates that endopeptidases of submaxillary glands, mast cells or leukocytes are possible candidates. This is supported by the observation (25) that an unknown enzyme, which generates an M₉ = 15,000 peptide in plasma, cleaves the human kininogen heavy chain at a site which is identical with that cleaved by endopeptidase k acting on T-kininogen. The high susceptibility to proteolysis of the T-kininogen interdomain regions may be due to the peculiar structure shared by all kininogens. Their heavy chain is made up of three repeated cystatin-like units which are thought to have evolved from a single cystatin-like ancestor (34) and seem to be highly resistant to proteolysis, except at the NH-terminal end of the inhibitory domains, as also shown for rat cystatin C.³ On the other hand, predictive studies on secondary structure have shown that the junctions between the cystatin-like domains of the T-kininogen heavy chain have a

³ F. Esnard, A. Esnard, and F. Gauthier, unpublished experiments.

The mechanism of inhibition of type 2 cystatins (33), was not an absolute requirement for T-kininogen domain 3 to be a potent inhibitor of papain and cathepsin L. The same conclusion can also be drawn from the results of Brzin et al. (35), who found a kininogen fragment in human plasma which lacked the critical glycol residue, but nevertheless had inhibitory activity. More recently, Abe et al. (36) also reported that the amino-terminal part of oryzacystatin, a structurally related molecule found in rice, was not essential for its papain-inhibitory activity. However, these three cystatin-like fragments in the human, rat, and plant cystatins all include the QVAG consensus sequence which was first reported to be implicated in cysteine proteinase inhibition by members of this superfamily (37). Further work is therefore needed to explain the mechanism of cysteine proteinase inhibition by kininogens and to determine whether it differs from that of cystatin family 2, from which they probably evolved (34). The most striking structural difference between family 2 cystatins and kininogen inhibitory domains is the presence in the latter of a small disulfide loop which includes the critical glycol residue, but one cannot yet say whether this may be related to the inhibitory function of kininogens.

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**Supplementary Material included:** Limiting Proteolytic Activity of T-Kininogen (Presented: RECOGNITION OF SOME HEMOGLOBIN POLYMORPHICS)

Richard EQUIAN, Wingeill GUTMAN, Bidek PERKES, and Fremis CAUTHEIN

**Experimental Procedure**

*Expression:* Rat tail cell lysates (EC 3.4.21.13) were purified from unselected cell cultures as described previously. K. and N. were used as substrates for EC 3.4.21.13 as described in previous work.

*Assay:* The peptide release was measured by a specific assay and quantitated by a specific assay and quantitated by a specific assay.

*Inactivation:* T-kininogen was purified from a specific assay and quantitated by a specific assay.

*Acknowledgment:* T-kininogen was purified from rat cells as described previously. K. and N. were used as substrates for EC 3.4.21.13 as described in previous work.

*Materials and Methods:* T-kininogen, synthesized by recombinant DNA technology, was used as a substrate for EC 3.4.21.13 as described in previous work.

*References:* T-kininogen was purified from specific assay and quantitated by a specific assay.

*Abstract:* T-kininogen was purified from specific assay and quantitated by a specific assay.

*Footnotes:* T-kininogen was purified from specific assay and quantitated by a specific assay.
Fragmentation of T-kinogen

Fig. 5: Elution of T-kinogen fragments by FPLC on Superox 6. T-kinogen (25 μg) was treated with a) endopeptidase K (10 μg), b) chymotrypsin (10 μg), c) tryppsin (20 μg), d) thrombin elution (0.5 μg) in appropriate buffers as required for Proteolysis of T-kinogen. Fractions (300 μl) were applied to a column. Fractions corresponding to eluted areas were pooled, stored, and freeze-dried.

The two major peaks of endopeptidase K and chymotrypsin corresponding to Fm ≈ 45 kDa and 30 kDa were pooled and concentrated by ultrafiltration 1/1000 of the native. Fractions were used for further purification by reverse phase chromatography. For native terminal sequence analysis and for terminal masses with similar mass but lower molecular mass, peak eluting fractions were collected from both columns. Fractions obtained from the analysis of 20 kDa containing peaks are shown in Fig. 6. These fragments were always eluted as two peaks, both had the same native terminal sequence and corresponded to the same fragment in TI and II-kinogens which are present in the initial preparation.

Fig. 6: Fractionation of T-kinogen fragments by HPLC on Superox 12. T-kinogen fragments were separated on Superox 12 after endopepsin K (a) and thrombin (b) hydrolysis. Fractions from each peak were pooled, concentrated and used for sequencing and NT determination.

Fig. 7: FPLC on Superox 12 of the endopepsin eluates from T-kinogen. Fractions from the analysis corresponding to Fm 25 kDa were pooled, concentrated and used for sequencing and NT determination.

- Spectrometric analysis: All terminal amino acid sequences were determined using an Applied Biosystems model 477A peptide mass spectrometer and stored in the mass spectra database. The terminal masses were determined using an Applied Biosystems model 477A peptide mass spectrometer and stored in the mass spectra database.

For the analysis of 20 kDa containing peaks, the fractions were pooled and concentrated by ultrafiltration 1/1000 of the native. Fractions were used for further purification by reverse phase chromatography. For native terminal sequence analysis and for terminal masses with similar mass but lower molecular mass, peak eluting fractions were collected from both columns. Fractions obtained from the analysis of 20 kDa containing peaks are shown in Fig. 6. These fragments were always eluted as two peaks, both had the same native terminal sequence and corresponded to the same fragment in TI and II-kinogens which are present in the initial preparation.

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