DNA Strongly Impairs the Inhibition of Cathepsin G by α₁-Antichymotrypsin and α₁-Proteinase Inhibitor

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Jérôme Duranton, Christian Boudier, Didier Belorgey, Philippe Mellet, and Joseph G. Bieth‡

From the Laboratoire d’Enzymologie, INSERM Unité 392, Université Louis Pasteur de Strasbourg, F-67400 Illkirch, France

This paper explores the possibility that neutrophil-derived DNA interferes with the inhibition of neutrophil cathepsin G (cat G) and proteinase 3 by the lung antiproteinases α₁-proteinase inhibitor (α₁PI), α₁-antichymotrypsin (ACT), and mucus proteinase inhibitor (MPI). A 30-base pair DNA fragment (30bpDNA), used as a model of DNA, tightly binds cat G (Kₐ 8.5 nm) but does not react with proteinase 3, α₁PI, ACT, and MPI at physiological ionic strength. The polynucleotide is a partial noncompetitive inhibitor of cat G whose Kᵢ is close to Kᵦ. ACT and α₁PI are slow binding inhibitors of the cat G G₃₀bpDNA complex whose second-order rate constants of inhibition are 2300 m⁻¹ s⁻¹ and 21 m⁻¹ s⁻¹, respectively, which represents a 195-fold and a 3190-fold rate deceleration. DNA thus renders cat G virtually resistant to inhibition by these irreversible serpins. On the other hand, 30bpDNA has little or no effect on the reversible inhibition of cat G by MPI or chymostatin or on the irreversible inhibition of cat G by carboxbenzoxyl-Gly-Leu-Phe-chloromethylketone. The polynucleotide neither inhibits proteinase 3 nor affects its rate of inhibition by α₁PI. These findings suggest that cat G may cause lung tissue destruction despite the presence of antiproteinases.

Neutrophils are phagocytic cells recruited at sites of infection and inflammation. Their azurophil granules, which participate in phagocytosis, store a number of hydrolytic enzymes including elastase, cat G, and proteinase 3, three serine proteinases whose tertiary structure has been elucidated recently. These 25–30-kDa glycoproteins contain a large number of basic amino acid residues that are responsible for their cationic character. Their specificity is directed against small aliphatic amino acid residues (elastase and proteinase 3) or more bulky ones (cat G). In vitro, these enzymes are able to cleave lung extracellular matrix proteins such as elastin, collagen, fibronectin, and laminin. They also cause extensive lung tissue damage in the animal (1).

Ideally, digestion of phagocytosed material should take place within the neutrophil in a phagocytic vacuole filled with the above proteinases. Actually, however, part of the lysosomal enzymes reaches the extracellular space as a result of incomplete closure of the phagolysosome or of frustrated phagocytosis of large particules. In addition, neutrophils are short lived cells that release the bulk of their proteinases when they die at sites of inflammation. Tissues are normally protected against these enzymes by a number of proteinase inhibitors. For example, lung secretions contain at least three antiproteinases, namely α₁-proteinase inhibitor (α₁PI), α₁-antichymotrypsin (ACT), and mucus proteinase inhibitor (MPI) (2). The 53-kDa α₁PI and the 68-kDa ACT are glycoproteins synthesized in the liver and transported into the lung via the blood circulation. They belong to the serpins, a superfAMILY of proteins that have developed by divergent evolution over 500 million years. The serpins have a highly conserved secondary structure comprising nine α-helices and three β-sheets with a very flexible reactive site loop. They form denaturant-stable complexes with their target proteinases and behave kinetically like irreversible inhibitors. This irreversible binding is due to the formation of a nonhydrolysable acyl enzyme adduct between the serine residue of the enzyme’s active site and the P₁ residue of the serpin. The P₁ residues of α₁PI and ACT are Met and Leu, respectively (3). α₁PI inhibits the three aforementioned proteinases, whereas ACT reacts only with cat G. The second-order rate constants for these serpin-proteinase associations range from 10⁵ to 10⁷ m⁻¹ s⁻¹ (1).

MPI is a 11.7-kDa basic protein synthesized and secreted by bronchial epithelial cells. It is formed of a single chain of 107 amino acid residues organized in two domains of similar size and architecture. It has a Leu residue at the P₁ position of its reactive center. MPI belongs to the class of canonical inhibitors that have a rigid reactive site loop that forms a reversible “lock and key” complex with the target proteinase. This complex is stabilized by a large number of noncovalent bonds, which account for the high enzyme-inhibitor affinity (4). MPI is a tight binding reversible inhibitor of elastase (Kᵢ 32 pm (5)). It is less efficient on cat G (Kᵢ 35 nM (6)) and does not inhibit proteinase 3 (7).

Because of their ability to cleave lung extracellular matrix proteins in vitro, neutrophil proteinases are thought to cause tissue destruction in inflammatory lung diseases. This raises, however, the following question: why does proteolysis occur despite the presence of the aforementioned inhibitors? Oxidative inactivation of proteinase inhibitors, transient excess of proteinase over inhibitor, and close neutrophil/matrix contact are conditions that favor proteolysis in the presence of inhibitors (1). Binding of proteinases to DNA released from neutrophils following cell death may also promote proteolysis in an inhibitory environment. We have recently shown that polynucleotides bind neutrophil elastase and impair its inhibition by MPI and α₁PI (8, 9). Here we study the influence of...
neutrastatin DNA on the inhibition of neutrophil cat G and proteinase 3 by α1PI, ACT, and MPI. To this end, we used a 30-base pair DNA fragment (30bpDNA) as a handy model of DNA.

**MATERIALS AND METHODS**

Human neutrophil cat G was isolated and active site titrated as described previously (10). Human neutrophil proteinase 3 and human plasma ACT were purchased from Athens Research and Technology (Athens, GA). Human recombinant α1PI and MPI were obtained through the courtesy of Dr. H.P. Schnelli, Novartis, Switzerland. α1PI and MPI were active site titrated with elastase (11), whereas ACT was titrated with cat G (12). Proteinase 3 was titrated with α1PI (7). Recombinant human pancreatic DNase I (Pulmozyme, Genentech, San Francisco, CA) was obtained from the Pharmacy of the University of Strasbourg. The p-nitroanilide and the thionbenzyester substrates came from Bachem (Switzerland) and E.S.P. (Livermore, CA), respectively. Stock solutions of the substrates and of 4,4-dithiodipyridine (Sigma) were made in dimethylformamide (final concentration, 2% (v/v)). Z-Gly-Leu-Phe CH2Cl and chymostatin came from E.S.P.-CH2Cl may be described by Equation 2 (15, 16),

\[
[P] = \frac{v_o}{1 - e^{-\frac{t}{k}}}
\]  

(Eq. 2)

where [P] is the product concentration at any time \( t \), \( v_o \) is the velocity at \( t = 0 \), and \( k \) is the pseudo-first-order rate constant of inhibition. The progress curves were fitted to Equation 2 by nonlinear least square analysis to obtain the best estimates of \( k \). The variation of \( k \) as a function of inhibitor or substrate concentration was analyzed assuming either that the inhibition conforms to a simple bimolecular reaction \( E + I \rightarrow EI \) or that it takes place in two-steps.

\[
E + I \rightarrow EI \rightarrow EI.
\]  

(Eq. 3)

The rate constant of inhibition is given by Equation 4 (one-step inhibition) or Equation 5 (two-step inhibition).

\[
k = \frac{k_o[I]}{1 + [S]/K_m}
\]  

(Eq. 4)

\[
k = \frac{k_o[I]}{[I] + K_i(1 + [S]/K_m)}
\]  

(Eq. 5)

Equations 4 and 5 show that a linear or a hyperbolic plot of \( k \) versus \( [I] \) will diagnose one-step or two-step inhibition, respectively. Two-step inhibition does not, however, necessarily yield a hyperbolic dependence of \( k \) versus \( [I] \); if the largest \( [I] \) used is significantly lower than \( K_i(1 + [S]/K_m) \), Equation 5 reduces to \( k = k_o/I + K_i(1 + [S]/K_m) \), a linear equation that resembles Equation 4.

The progress curves for the inhibition of 30bpDNA-bound cat G by the reversible inhibitors MPI and chymostatin may be described by Equation 6 (15, 16),

\[
[P] = \frac{v_o}{1 - e^{-\frac{t}{k}}}
\]  

(Eq. 6)

where \( v_o \) is the steady-state velocity, and the other symbols have the same meaning as in Equation 2. The variation of \( k \) as a function of inhibition concentration was analyzed assuming one-step inhibition:

\[
k = \frac{k_o[I]}{1 + [S]/K_m} + k_{\text{diss}}
\]  

(Eq. 7)

for which \( k \) is given by:

\[
k = \frac{k_o[I]}{1 + [S]/K_m} + k_{\text{diss}}
\]  

(Eq. 8)

**Kinetics of Elastin Solubilization by cat G**—Rate constants of inhibition were measured by adding cat G \( \pm 30bpDNA \) to a mixture of inhibitor + substrate \( \pm 30bpDNA \) and recording the release of product as a function of time. When the progress curves lasted for more than 10 min, an ordinary spectrophotometer (Uvikon 941, Kontron) was used to record them. When the reaction was terminated in 2 min or less the Uvikon spectrophotometer was equipped with a SFA-11 fast mixing accessory (High-Tech Scientific, Salisbury, UK). When faster reactions were followed, mixing of the reagents and data acquisition were performed with a FSPF-53 stopped flow apparatus (High Tech Scientific). All experiments were done under pseudo-first-order conditions, that is \( [I] \ll 10[I] \). The concentration of product at the end of the inhibition experiments was always lower than 5% of the initial substrate concentration so that the latter was virtually not depleted during the inhibition process. Under these two conditions the progress curves describing the enzyme-catalyzed hydrolysis of substrate in the presence of irreversible inhibitors such as ACT, α1PI, or Z-Gly-Leu-Phe-CHCl may be described by Equation 2 (15, 16),

\[
[P] = \frac{v_o}{1 - e^{-\frac{t}{k}}}
\]  

(Eq. 2)
concentrations were: cat G = 5 μM, 30bpDNA = 2 μM, elastin = 3 mg/ml.

RESULTS

Binding of cat G to 30bpDNA—Fig. 1A shows that at low ionic strength (50 mM Hepes, pH 7.4) Sepharose-30bpDNA binds ACT and cat G but does not significantly bind α1PI. MPI was weakly bound and was eluted with a low NaCl concentration (not shown). Whereas cat G was tightly bound to the affinity support, ACT was eluted at a NaCl concentration lower than that contained in the buffer used for the enzyme kinetic assays, namely 50 mM Hepes, 150 mM NaCl, pH 7.4. The weak binding of ACT to the affinity column is not surprising, because ACT is known as a DNA-binding protein whose binding domain has been identified recently (17). To further confirm the weak binding of MPI to the polynucleotide, we have chromatographed 30bpDNA on a Sepharose-MPI column. Fig. 1B shows that the polynucleotide elutes from this column with a NaCl concentration of about 100 mM. Thus, cat G is the only protein that binds 30bpDNA in the aforementioned buffer.

In an attempt to quantitate the cat G-30bpDNA affinity we have measured the effect of polynucleotide concentration on the fluorescence intensity of fluorescently labeled cat G. Fig. 2A shows that this intensity decreases up to a limiting value F_min. Fig. 2B is a replot of these data in accordance with Equation 1 from which K_d, the equilibrium dissociation constant of the cat G-30bpDNA complex, was calculated by nonlinear regression analysis. K_d was found to be 8.5 ± 3.2 nM.

Partial Inhibition of cat G Activity by 30bpDNA—Reaction of constant amounts of cat G with increasing amounts of 30bpDNA resulted in partial inhibition of the enzymatic activity on Suc-Ala2-Pro-Phe-pNA, whether the enzyme concentration was low (24 nM) or high (300 nM) (Fig. 3). When the inhibition of 24 nM cat G was measured using different substrate concentrations, similar results were obtained (data not shown). Partial inhibition may be analyzed using the scheme adapted from Ref. 18, where E, S, and I stand for enzyme, substrate, and 30bpDNA, respectively. K_i is the inhibition constant and α and β are dimensionless numbers. The inhibition is said to be partially competitive if α > 1 and β = 1 and partially noncompetitive if α = 1 and 0 < β < 1 (18). To decide between these two mechanisms we have measured the kinetic parameters for the hydrolysis of Suc-Ala2-Pro-Phe-pNA by free cat G to get k_cat and K_m, and by 30bpDNA-saturated cat G to obtain βk_cat and αK_m. The following constants were found: k_cat, 3.1 ± 0.3 s⁻¹;
curves recorded following mixing constant concentrations of cat G, 30 nM, and DNA (see Equation 2) were calculated from progress curves recorded following mixing constant concentrations of cat G, 30 nM, and substrate (Suc-Ala2-Pro-Phe-pNA) and variable concentrations of 30bpDNA. A, cat G, 30 nM; ACT, 0.76 μM; substrate, 2.5 mM; B, cat G, 5 nM; α1PI, 16 μM; substrate, 2 mM.

The pseudo-first-order rate constant for the inhibition of cat G by ACT and α1PI (see Equation 2) were calculated from progress curves recorded following mixing constant concentrations of cat G, 30 nM; ACT, 0.76 μM; substrate, 2.5 mM; and DNA (see Equation 2) were calculated from progress curves recorded following mixing constant concentrations of cat G, 30 nM; ACT, 0.76 μM; substrate, 2.5 mM; and DNA (see Equation 2) were calculated from progress curves recorded following mixing constant concentrations of cat G, 30 nM, and substrate (Suc-Ala2-Pro-Phe-pNA) and variable concentrations of 30bpDNA. A, cat G, 30 nM; ACT, 0.76 μM; substrate, 2.5 mM; B, cat G, 5 nM; α1PI, 16 μM; substrate, 2 mM.

FIG. 4. Influence of 30bpDNA on the inhibition of cat G by ACT (A) and α1PI (B) at pH 7.4 and 25 °C. The pseudo-first-order rate constants of inhibition k (see Equation 2) were calculated from progress curves recorded following mixing constant concentrations of cat G, 30 nM, and substrate (Suc-Ala2-Pro-Phe-pNA) and variable concentrations of 30bpDNA. A, cat G, 30 nM; ACT, 0.76 μM; substrate, 2.5 mM; B, cat G, 5 nM; α1PI, 16 μM; substrate, 2 mM.

As shown in Fig. 5, the rate constant for the inhibition of 30bpDNA-bound cat G varies hyperbolically with the ACT concentration, suggesting two-step inhibition. Indeed, the data could be fit to Equation 5 by nonlinear regression analysis. In contrast, the linear variation of k with the α1PI concentrations indicates one-step inhibition. The kinetic constants are reported in Table I together with those collected previously with free cat G (12). It can be seen that 30bpDNA lowers the second-order rate constant for the inhibition of cat G by ACT and α1PI by a factor of 195 and 3200, respectively.

Two experiments were run to further illustrate the dramatic effect of 30bpDNA on the rate inhibition of cat G by α1PI. First, we have incubated constant concentrations of free or polynucleotide-bound cat G with increasing concentrations of α1PI for 1 h, a time largely sufficient to ensure full inhibition of the free enzyme. The inhibitor yielded a linear inhibition curve with free cat G, whereas the 30bpDNA-cat G complex was fully resistant to inhibition (data not shown). Second, we have studied the influence of 30bpDNA on the inhibition of the elastolytic activity of cat G by α1PI. We have found that α1PI fully inhibited the elastolytic activity of cat G in the absence of 30bpDNA, whereas in the presence of the polynucleotide, there was only 28% inhibition.

Effect of 30bpDNA on the Inhibition of cat G by MPI—Fig. 6 shows that the progress curves run in the presence of cat G + MPI + 30bpDNA are biphasic, as predicted for reversible inhibition (Equation 6). The rate constant of inhibition is linearly related to the MPI concentration, indicating one-step inhibition
TABLE I

Kinetic constants describing the inhibition of neutrophil cat G by ACT and α-PI in the absence and presence of 30bpDNA at pH 7.4 and 25 °C

| Inhibitor 30bpDNA | Inhibition kinetics | \( k^a \) | \( k_2 \) | \( k/K^a \) or \( k_{\text{ass}} \) |
|-------------------|---------------------|----------|---------|---------------------|
| ACT^a | two-step | \( 6.2 \times 10^{-5} \) s^{-1} | \( 2.8 \times 10^{-2} \) s^{-1} | \( 4.5 \times 10^3 \) |
| ACT | two-step | \( 2.8 \times 10^{-6} \) s^{-1} | \( 6.0 \times 10^{-3} \) s^{-1} | \( 2.3 \times 10^5 \) |
| α-PI^a | two-step | \( 8.1 \times 10^{-7} \) s^{-1} | \( 5.5 \times 10^{-2} \) s^{-1} | \( 6.7 \times 10^4 \) |
| α-PI | one-step | \( >1.7 \times 10^{-5} \) s^{-1} | \( >3.6 \times 10^{-4} \) s^{-1} | 21 |

^a From Duranton et al. (12).

The progress curves recorded in the presence of 30bpDNA were used to calculate the pseudo-first-order rate constant of inhibition, \( k_{\text{ass}} \), and \( k_{\text{diss}} \). The rates of inhibition were measured using 5–10 nM cat G, 0.5 μM 30bpDNA, 0.6 mM Suc-Ala-Pro-Phe-thiobenzylester, and variable concentrations of inhibitor. For both compounds the rate constant of inhibition \( k \) was found to be linearly related to the inhibitor concentration, indicating one-step-inhibition. Z-Gly-Leu-Phe-CH2Cl inhibited cat G with a \( k_{\text{ass}} \) of 25 m^{-1} s^{-1} and 23.9 m^{-1} s^{-1} in the absence and presence of polynucleotide, respectively. The kinetic parameters for the cat G-chymostatin interaction were found to be: \( k_{\text{ass}} \)

= 2.4 \times 10^4 \, \text{M}^{-1} \, \text{s}^{-1}, \, k_{\text{diss}} = 1.2 \times 10^{-2} \, \text{s}^{-1}, \, k_{\text{ass}} = 1.8 \times 10^3 \, \text{M}^{-1} \, \text{s}^{-1}, \, k_{\text{diss}} = 1.6 \times 10^{-2} \, \text{s}^{-1} in its presence. The polynucleotide does not, therefore, significantly affect the inhibition of cat G by these synthetic inhibitors.

**Effect of 30bpDNA on the Activity and Inhibition of Proteinase 3—**Proteinase 3 did not bind to the Sepharose-30bpDNA column even at low ionic strength (50 mM Hepes, pH 7.4). Also, its activity on methoxysuccinyl-lysyl (2-picolinoyl)-Ala-Pro-Val-pDNA and on elastin was unaffected by 30bpDNA. On the other hand the polynucleotide did not significantly change its rate of inhibition by α-PI (data not shown).

**DISCUSSION**

We have explored the possibility that DNA released from neutrophils at sites of inflammation interferes with the inhibition of neutrophil cat G and proteinase 3 by their endogenous inhibitors. A 30bpDNA fragment used as a model of DNA was able to tightly bind cat G to form an enzymatically active complex. This complex was almost as active on synthetic substrates and on elastin as free cat G and could be easily inhibited by MPI and synthetic cat G inhibitors. In contrast, the 30bpDNA-cat G complex was virtually resistant to inhibition by the two serpins ACT and α-PI. ACT inhibits both free (12) and 30bpDNA-bound cat G via a two-step reaction (see Eq. 3).

The polynucleotide decreases both the affinity of the Michaelis-type complex EI (\( K^a \) increases 42-fold) and the rate constant for its conversion into the final complex E1 (\( k_2 \) decreases ~15-fold) (Table I). Whereas α-PI inhibits free cat G in two steps (12), it reacts in one step with the 30bpDNA-cat G complex. The value of \( k_{\text{ass}} \) (21 m^{-1} s^{-1}) is, however, several orders of magnitude lower than the maximum rate constant for a bimolecular diffusion-controlled reaction (21). It may, therefore, be assumed that the reaction involves an intermediate even if the latter is not seen kinetically. This assumption predicts that the largest inhibitor concentration used (50 μM) must be lower than \( K^a (1 + |S|/K_m) \) (see Equation 5). Hence, \( K^a > 17 \, \mu\text{M} \) and \( k_2 > 3.6 \times 10^{-4} \, \text{s}^{-1} \). Comparison of these limits with \( K^a \) and \( k_2 \) previously determined with free cat G (12) indicates that 30bpDNA increases \( K^a \) at least 21-fold and decreases k_2 at least 153-fold. Thus, the dramatic decrease in the rate of inhibition of cat G by ACT and α-PI is due in both cases to an unfavorable effect of 30bpDNA on \( K^a \) and \( k_2 \).

We have previously shown that polynucleotides also depress the rate of neutrophil elastase inhibition by α-PI. For instance, tRNA and polydeoxycytosine decrease the second-order rate constant of inhibition by factors of 3 and 31, respectively (9). We have confirmed these mild effects using 30bpDNA, which depressed the second-order rate constant by a factor of 9 (data not shown). Thus, polynucleotides affect much less the inhibition of elastase by α-PI than that of cat G by α-PI. This is reminiscent of previous findings showing that heparin, another anionic ligand, decreases the rate of inhibition of cat G by α-PI by a factor of 400 (22), whereas it lowers the rate of inhibition of elastase by α-PI by a factor of only 5 (23). DNA and heparin have thus similar effects on the two proteinase/α-PI systems; they enormously decrease the rate of inhibition of cat G but only marginally affect the rate of inhibition of elastase. This difference may be related to differences in the localization of the arginine residues in cat G and elastase. Arginine residues have the potential to form salt bridges with the anionic groups of heparin and DNA. Unlike elastase (24), cat G has three arginine residues in the immediate vicinity of its active site (25). As a consequence, heparin or 30bpDNA might bind closer to the active site of cat G than to that of elastase thus causing a more steric hindrance to the access of α-PI in cat G than in elastase.
Proteinase 3 is not inhibited by ACT or MPI but is rapidly inactivated by α₁PI ($k_{\mathrm{on}} = 8 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ (7)). This reaction rate is not altered by 30bpDNA. Thus, the inhibition of the three neutrophil serine proteinases by α₁PI is diversely affected by 30bpDNA; cat G almost fully resists inhibition, elastase is inhibited with a moderately reduced rate, and the inhibition of proteinase 3 is not affected at all by the polynucleotide.

We believe that our kinetic data have pathological bearing. In chronic bronchitis there is a continuous recruitment of neutrophils in airways. During activation or phagocytosis, neutrophils release part of their cat G, elastase, and proteinase 3 content in airway secretions. In addition, when these short lived cells die in situ they release both their proteinase and DNA content (26). We have shown that DNA forms a tight complex with cat G, and so it renders this proteinase virtually resistant to inhibition by the fast acting serpins α₁PI and ACT. Thus, DNA promotes cat G-mediated proteolysis of lung matrix proteins in an inhibitory environment.

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