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Inhibition of neutrophil elastase by the α_1-proteinase inhibitor-immunoglobulin A complex

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Abstract **Neutrophil elastase is thought to be involved in cartilage destruction occurring in rheumatoid arthritis despite the local presence of α_1-proteinase inhibitor. Part of synovial fluid α_1-proteinase inhibitor forms a mixed disulfide with immunoglobulin A, which has been postulated to lack inhibitory activity. We show here that the immunoglobulin-inhibitor complex tightly inhibits neutrophil elastase and cathepsin G, bovine pancreatic trypsin and chymotrypsin, and porcine pancreatic elastase. Although the rate constant of inhibition of neutrophil elastase by immunoglobulin A-bound α_1-proteinase inhibitor ($k_{ass} = 9.2 \times 10^5$ $M^{-1} \cdot s^{-1}$) is about 10-fold lower than that measured with the free inhibitor, it is high enough to enable efficient inhibition of elastase in vivo.**

Key words: Rheumatoid arthritis; Elastase; α_1-Proteinase inhibitor-immunoglobulin A complex; Western blot; Enzyme kinetics

1. Introduction

Rheumatoid arthritis is a debilitating disease characterized by an extensive degradation of the articular cartilage. Massive numbers of neutrophils accumulate in rheumatoid synovial fluids where they may release large amounts of NE [1], a serine proteinase that cleaves proteoglycans and type II collagen, the major constituents of articular cartilage [2,3]. NE is therefore believed to play a significant pathological role in arthritis.

Synovial fluid contains plasma-derived α_1PI, the major physiological inhibitor of NE. In rheumatoid arthritis, up to 30% of synovial fluid α_1PI is bound to IgA via a disulfide link between Cys-232, the sole cysteine residue of the inhibitor, and Cys-471, the penultimate cysteine residue of the α-chain of immunoglobulin [4]. Two lines of evidence suggest that binding of α_1PI exerts an unfavorable effect on the evolution of rheumatoid arthritis: (i) treatment of patients with D-penicillamine decreases the concentration of complex and ameliorates their clinical conditions [5,6]; (ii) the concentration of complex correlates directly with the development of articular erosions in patients with early rheumatoid arthritis [7]. These correlations strongly suggest that the α_1PI-IgA complex does not inhibit NE and significantly lowers the NE inhibitory capacity of synovial fluids, thus favoring intra-joint proteolysis [5,7]. Yet, no attempt has ever been made to check this hypothesis. We therefore prepared the complex in vitro and tested it on a number of serine proteinases including NE.

2. Materials and methods

The source and active site titration of human NE, cathepsin G and α_1PI, bovine trypsin and chymotrypsin were the same as before [8]. Published procedures were used to isolate [9] and titrate [10] porcine pancreatic elastase. The *p*-nitroanilide substrates (Bachem, Switzerland) were dissolved in dimethylformamide whose final concentration in the enzymatic reaction medium was 1% (v/v). Fluorescent labelling of NE and α_1PI was done with celite-bound FITC (Molecular Probes, OR) as described by Gennis et al. [11]. The molar ratio of bound FITC to α_1PI or NE was deduced from absorbance readings using $\varepsilon = 66\,800$ $M^{-1} \cdot cm^{-1}$ at 495 nm for protein-bound FITC [12], and protein concentration measurements using the method of Bradford [13]. The degree of labelling ranged from 0.9 to 1.1 for FITC-α_1PI and FITC-NE. Human serum IgA (Jackson, PA) was a mixture of monomeric (~90%) and dimeric (~10%) immunoglobulins. The calibration kits used for electrophoresis and gel filtration were from Pharmacia (Sweden).

The immunoassay of α_1PI was done using a double-sandwich ELISA. The plates were coated with goat anti-human α_1PI antibodies (Nordic, The Netherlands), and the bound α_1PI was revealed with successive addition of rabbit anti-human α_1PI antibodies (Behring, Germany) and peroxidase-labelled goat anti-rabbit immunoglobulin G antibodies (Nordic).

Electrophoresis and Western blotting were done with a Pharmacia Phastsystem apparatus. The samples were incubated with 0.1% SDS buffer for 15 min at 25°C and electrophoresed in SDS-polyacrylamide gels. After blotting on nitrocellulose, the calibration proteins were stained with amido black, while the antigens were revealed by the addition of rabbit anti-human α_1PI (Behring) and anti-human IgA α-chain (ICN, CA) antisera, followed by addition of alkaline phosphatase-labelled goat anti-rabbit immunoglobulin G antibodies.

2.1. Preparation of the α_1PI-IgA complex

α_1PI (2 mg/ml) was reacted with 10 mM reduced glutathione (Merck, Germany) for 12 h at 4°C in 50 mM HEPES, 100 mM NaCl, pH 7.4, in order to free its single cysteinyl groups [14]. The excess glutathione was removed by gel filtration through a Sephadex G25 column developed with 100 mM Tris-HCl pH 8.2. The α_1PI-containing fractions were concentrated by Centriprep-10 filtration (Amicon, MA), and the free thiol of reduced α_1PI (α_1PI-SH) was titrated with DTNB (Sigma, MO) [15], which showed that the inhibitor was fully reduced. IgA was reacted with 10 mM DTNB for 12 h at 4°C in the above pH 7.4 buffer, to facilitate its coupling to α_1PI. The mixed disulfide (IgA-SS-TNB) was then isolated using a Superose 6 column (Pharmacia) developed with the above pH 8.2 buffer, and concentrated by Centricon-100 filtration (Amicon). 3 mg α_1PI-SH and 12 mg IgA-SS-TNB were reacted for 12 h at 4°C in a total volume of 1.2 ml of the pH 8.2 buffer. 200 µl portions of this mixture were then gel filtrated on a 1×30 cm column of Superose 6 eluted with 50 mM HEPES buffer pH 7.4 at a flow rate of 25 ml/h. The S1 fractions (Fig. 1) were pooled and chromatographed on a 0.5×5 cm Mono Q 5/5 column equilibrated with 50 mM HEPES pH 7.4. Protein elution was

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Abbreviations: NE, neutrophil elastase; α_1PI, α_1-proteinase inhibitor, also called α_1-antitrypsin; α_1PI-SH, reduced α_1PI; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); IgA, immunoglobulin A; IgA-SS-TNB, mixed disulfide of IgA and 5-thio-(2-nitrobenzoate); FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; Suc-, *N*-succinyl-; MeOSuc-, *N*-methoxy-succinyl-; Bz-, benzoyl-; *p*NA, *para*-nitroanilide

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done with a linear NaCl gradient at a flow rate of 60 ml/h. The M2 fractions were pooled, diluted with an equal volume of 50 mM HEPES pH 7.4 and concentrated by Centriprep-100 filtration.

2.2. Enzymatic methods

All reactions were carried out in 50 mM HEPES, 100 mM NaCl buffer pH 7.4 and at 25°C. Titration of proteinases with the α_1PI-IgA complex was done as follows. Constant amounts of proteinases were reacted with increasing amounts of complex in a total volume of 990 µl of buffer. After 1 h at 25°C, 10 µl of stock solution of substrate was added to measure the residual enzyme activities. The proteinases and their final concentrations were: 50 nM NE, 200 nM cathepsin G, 20 nM bovine chymotrypsin, 200 nM bovine trypsin and 100 nM porcine pancreatic elastase. The corresponding substrates and their final concentrations were: 0.1 mM MeOSuc-Ala_2-Pro-Val-*p*NA, 1.5 mM Suc-Ala_2-Pro-Phe-*p*NA, 0.2 mM Suc-Ala_2-Pro-Phe-*p*NA, 2 mM Bz-Arg-*p*NA and 0.8 mM Suc-Ala_3-*p*NA.

The rate of NE inhibition was measured under pseudo-first-order conditions, i.e. $[I]_0 \geq 10\ [E]_0$, by reacting 30 nM NE with a mixture of α_1PI-IgA (variable concentration) and 1 mM MeOSuc-Ala_2-Pro-Val-*p*NA. The release of *p*-nitroaniline was recorded at 410 nm. The progress curves were analyzed as described previously [16].

3. Results and discussion

3.1. Preparation of the α_1PI-IgA complex

α_1PI-SH and IgA-SS-TNB were prepared and mixed as described in section 2. Their mixture was then gel filtrated on Superose 6, and the eluted proteins were divided into three fractions S1, S2 and S3 (Fig. 1A), each of which was electrophoresed, electroblotted and reacted with anti-α_1PI and anti-IgA antibodies (Fig. 1B). It can be seen that IgA-bound α_1PI is present in S1 whereas free α_1PI is present in S2 and S3. Fraction S1 was chromatographed on a Mono Q column

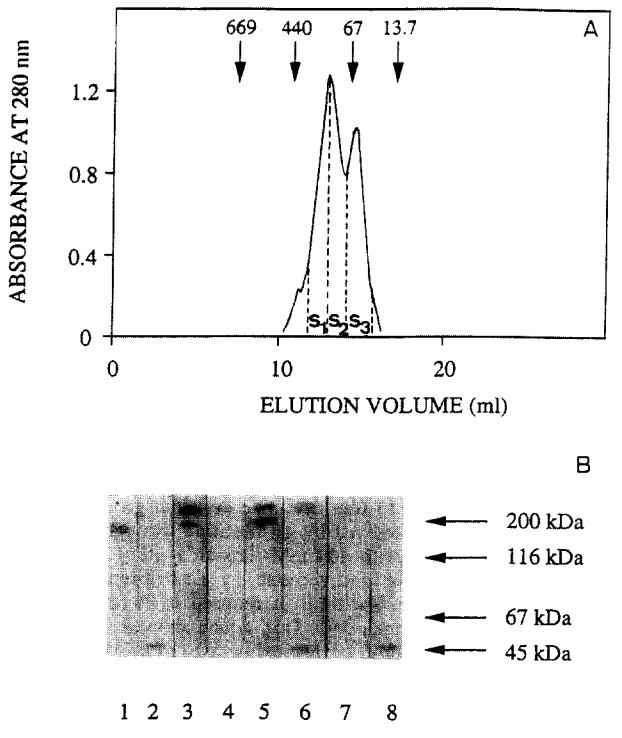

Fig. 1. A: Superose 6 gel filtration of the α_1PI+IgA-SS-TNB reaction mixture. The arrows indicate the elution volumes of marker proteins whose molecular mass are given in kDa. B: Western blot analysis of fractions S1 (lanes 3, 4), S2 (lanes 5, 6), and S3 (lanes 7, 8), and of IgA (lane 1) and α_1PI (lane 2) controls. The antigens were revealed with anti-IgA-α-chain antibodies (lanes 1, 3, 5, 7) or with anti-α_1PI antibodies (lanes 2, 4, 6, 8).

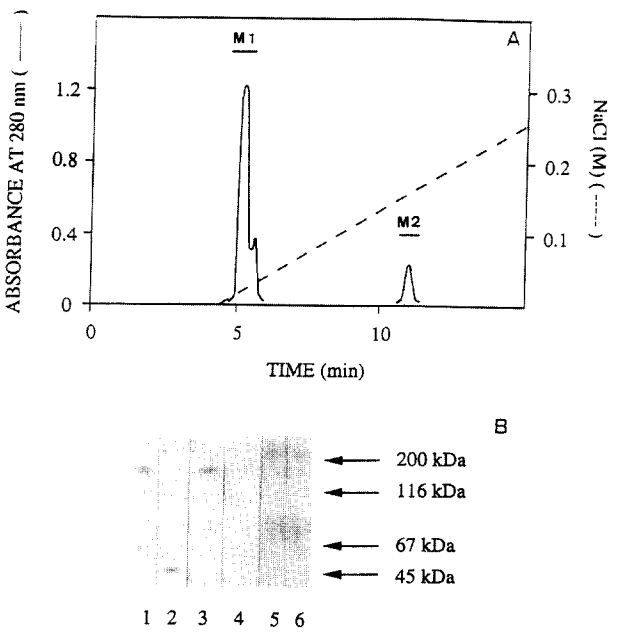

Fig. 2. A: Mono Q chromatography of fraction S1 from Fig. 1. B: Western blot analysis of fractions M1 (lanes 3, 4), M2 (lanes 5, 6), and of IgA (lane 1) and α_1PI (lane 2) controls. The antigens were revealed with anti-IgA-α-chain antibodies (lanes 1, 3, 5) or with anti-α_1PI antibodies (lanes 2, 4, 6).

which separated the proteins into two major fractions, M1 and M2 (Fig. 2A), each of which was blotted as in Fig. 1 (Fig. 2B). M1 contained free IgA whereas M2 contained the α_1PI-IgA complex. Reaction of 3 mg α_1PI with 12 mg IgA yielded about 120 µg of α_1PI-IgA complex. The adduct was pure as judged by electrophoresis on a 7.5% SDS-polyacrylamide gel (not shown).

This is the first report on the preparation of the complex

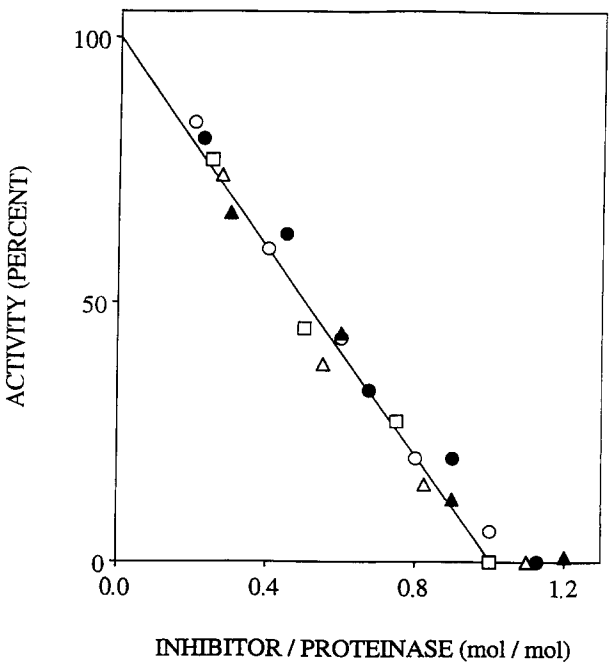

Fig. 3. Effect of increasing amounts of α_1PI-IgA complex on the activity of constant amounts of NE (○), cat G (●), bovine chymotrypsin (△), bovine trypsin (▲) and porcine elastase (□).

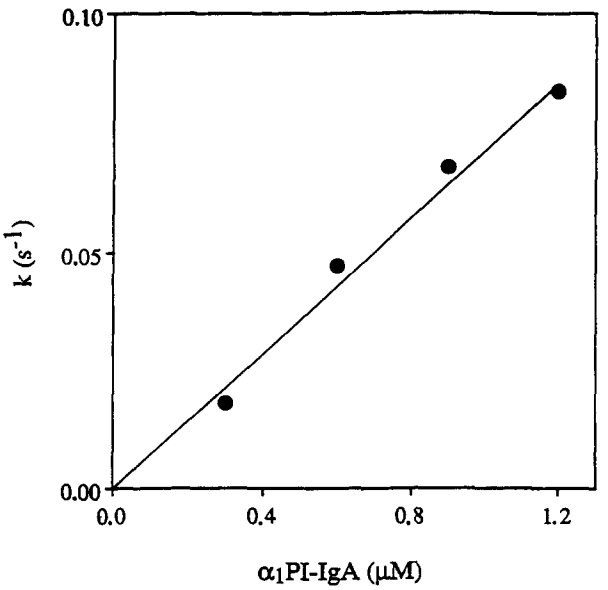

Fig. 5. Effect of the concentration of the α_1PI-IgA complex on k, the pseudo-first-order rate constant of NE inhibition at pH 7.4 and 25°C. The rate constant k was calculated as described in section 2.

from its individual components. Previous investigators have partially purified α_1PI-IgA from serum [4,17].

3.2. α_1PI:IgA stoichiometry and ε of the complex

Each α-chain of monomeric IgA has one free cysteine residue at its penultimate position. Thus, one molecule of IgA may theoretically bind two molecules of α_1PI. We therefore investigated the binding stoichiometry of the α_1PI-IgA complex. The complex migrated as a 220 kDa protein on SDS-polyacrylamide gel electrophoresis with immune (Fig. 1A) or Coomassie blue staining. It also eluted as a ca. 220 kDa protein on calibrated analytical Superose 6 gel filtration (not shown), suggesting that it is formed of equimolar amounts of α_1PI ($M_r = 53\,000$) and IgA ($M_r \approx 170\,000$).

On the other hand, UV absorption spectroscopy gave $A_{1\%}^{280nm} = 13.5$, as calculated with a formula published by van Iersel et al. [18]. Using the molecular mass of 220 kDa, the calculated molar extinction coefficient was $\varepsilon = 300\,000$ $M^{-1} \cdot cm^{-1}$ at 280 nm. This value was checked in two ways. First the molar concentration of the complex formed with FITC-labelled α_1PI-IgA complex was determined using $\varepsilon = 66\,800$ $M^{-1} \cdot cm^{-1}$ at 495 nm for protein-bound FITC [12] and assuming a 1:1 stoichiometry. This molar concentration was very close to that determined using $\varepsilon = 300\,000$ $M^{-1} \cdot cm^{-1}$ at 280 nm. Second, the α_1PI-IgA complex was reduced with a 10-fold molar excess of dithiothreitol and the concentration of free α_1PI was measured using an ELISA. This result confirmed that obtained with the FITC-α_1PI-IgA complex. The ε value was used to calculate the concentrations of complex solutions used for the enzymatic studies.

3.3. Inhibitory properties of the α_1PI-IgA complex

Titration of NE, cathepsin G, bovine chymotrypsin, bovine trypsin or porcine pancreatic elastase with the α_1PI-IgA complex yields linear and superimposable titration curves, whose intercept with the abscissa indicates that about 1 mol of α_1PI-IgA inhibits 1 mol of enzyme (Fig. 3). Thus α_1PI-IgA behaves like an irreversible serine proteinase inhibitor like free α_1PI [19].

The NE inhibition was investigated in more detail. Free and IgA-bound α_1PI were reacted with stoichiometric amounts of FITC-labelled NE, and the complexes were gel filtrated on analytical Superose 6. Fig. 4 shows that the two inhibitory complexes elute from the column as 250 kDa and 83 kDa proteins. This indicates that the inhibition of NE by α_1PI-IgA is due to the formation of a ternary NE-α_1PI-IgA complex, and not to a NE-induced dissociation of the α_1PI-IgA complex. When analyzed by SDS-12.5% polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol, the NE-α_1PI-IgA complex showed bands at 25 kDa and 50 kDa corresponding to the light and heavy chains of IgA and one band at ca. 80 kDa, also observed upon electrophoresis of the NE-α_1PI complex (not shown). The latter band corresponds to the classical denaturant-stable irreversible proteinase-inhibitor complex [20].

Fig. 5 shows that k, the pseudo-first-order rate constant for the inhibition of NE by α_1PI-IgA, varies approximately linearly with the inhibitor concentration, indicating that enzyme E and inhibitor I associate via a simple bimolecular reaction governed by the second-order association rate constant $k_{ass}(E + I \xrightarrow{k_{ass}} EI)$. As demonstrated previously,

$$k = \frac{k_{ass}[I]_o}{1 + [S]_o/K_m} \quad (1)$$

where $[S]_o$ and K_m are the initial substrate concentration and the Michaelis constant, respectively [16,21]. Non-linear regression analysis of the progress curves yielded $k_{ass}/(1 + [S]_o/K_m)$, from which k_{ass} was calculated using $K_m = 85 \pm 12$ μM for the NE/MeOSuc-Ala$_2$-Pro-Val-*p*NA system [16]. In a previous investigation we have shown that free α_1PI also inhibits NE via a bimolecular reaction characterized by a k_{ass} of $(1.05 \pm 0.15) \times 10^7$ $M^{-1} \cdot s^{-1}$ [16]. IgA therefore decreases k_{ass} by a factor of 11, indicating that the bulky immunoglobulin molecule does not dramatically disturb the enzyme-inhibitor binding. On the other hand, theory predicts that with a k_{ass} of ca. 10^6 $M^{-1} \cdot s^{-1}$, α_1PI-IgA still acts fast enough to prevent intra-joint proteolysis [22]. This contradicts the hypothesis that α_1PI is inactivated following its binding with IgA [5,7].

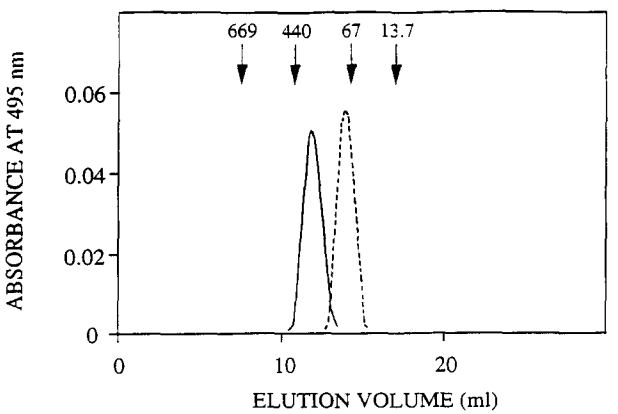

Fig. 4. Superose 6 gel filtration of the complexes formed between FITC-NE and α_1PI or α_1PI-IgA complex. The complexes were formed by reacting 2 μM FITC-NE with 2.4 μM inhibitor for 15 min at pH 7.4 and 25°C. Each complex was chromatographed separately by pouring a 100 μl aliquot on a 1 × 30 cm column developed with 50 mM HEPES pH 7.4. The arrows indicate the elution of the calibration proteins.

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