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INHIBITION OF HUMAN ELASTASE FROM POLYMORPHONUCLEAR LEUCOCYTES BY GOLD SODIUM THIOMALATE AND PENTOSAN POLYSULFATE (SP-54®)

ANTONIO BAICI*, PRATHIMA SALGAM, KURT FEHR and ALBERT BÖNI
Department of Rheumatology, University Hospital, Gloriastrasse 25, CH-8091 Zurich, Switzerland

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Abstract—Human lysosomal elastase, the serine proteinase from the azurophil granules of polymorphonuclear leucocytes, is inhibited by gold thiomalate and pentosan polysulfate (SP-54®). The kinetic mechanism of the inhibition was studied using succinyl–alanyl-alanyl-prolyl-valyl-4-methyl-7-coumarylamide and *t*-butyloxycarbonyl-alanyl-*p*-nitrophenylester as substrates. The degree of inhibition was also tested using insoluble elastin as substrate. Independent of the substrate, the maximal inhibition of elastase by gold thiomalate and pentosan polysulfate was 40% and 60%, respectively. Pentosan polysulfate behaved as a simple intersecting, hyperbolic, non-competitive inhibitor and K_i', the dissociation constant of the E·S·I complex, was 1.8×10^{-7}M. The interaction between inhibitor and enzyme is driven by electrostatic forces. Gold thiomalate showed a hyperbolic mixed type inhibition (intersecting, slope-hyperbolic, intercept-hyperbolic, non-competitive inhibition) with $K_i' = 5.4 \times 10^{-5}$M.

The overall kinetic mechanism of lysosomal elastase conforms to that of other serine proteinases. With both the ester and peptide substrates the rate limiting step of the reaction has been identified with the formation of the acyl-enzyme.

Human lysosomal elastase, a serine proteinase stored in the azurophil granules of polymorphonuclear leucocytes [1] is thought to be a possible mediator in the pathophysiology of connective tissue breakdown. Elastase has a broad spectrum of specificity, being the only known proteinase capable of degrading all the three major components of connective tissues, namely collagen, proteoglycans and elastin at neutral pH [2]. The enzyme is also able to degrade human immunoglobulins [3, 4] and was 'caught in the act' in the articular cartilage of patients with rheumatoid arthritis [5] supporting the hypothesis that it plays a role in the pathology of this disease.

The beneficial effects of gold therapy in rheumatoid arthritis have been known for a long time but no demonstration of the actual mechanism(s) of action has been found. An attractive hypothesis is that the drug can act as an inhibitor of lysosomal enzymes. Pentosan polysulfate, SP-54®, a semisynthetic polysulfated xyloside with a broad spectrum of action, including anti-inflammatory properties [6], was also found to be an inhibitor of elastase. In an attempt to analyze a possible role of anti-inflammatory and anti-rheumatic drugs as proteinase directed inhibitors, we recently described the inhibition of human lysosomal elastase by a glycosaminoglycan polysulfate [7]. The present paper reports the kinetic mechanism of inhibition of lysosomal elastase by gold thiomalate and pentosan polysulfate. A preliminary account of the results presented here has already been reported [8].

MATERIALS AND METHODS

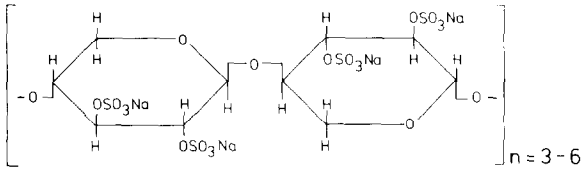
Enzyme. Human lysosomal elastase (EC 3.4.21.11) was purified from granulocytes by affinity chromatography on Sepharose–Trasylol [9]. With Boc–Ala–ONp as substrate in 50 mM phosphate buffer, pH 7.4, the elastase preparation used in the present study had a turnover number V/[E] of $13.8 \pm 0.8\,s^{-1}$. The enzyme active sites were titrated with the aza-peptide *N*-acetyl-L-Ala-L-Ala-NHN($CH_2CH_2CH_3$) CO-*p*-nitrophenylester [10]. The titration was carried out in a stopped flow apparatus by rapidly mixing enzyme and titrant and observing the appearance of *p*-nitrophenol at 345 nm, in 0.1 M citrate buffer, pH 6.0. An extinction coefficient of $6{,}250\,M^{-1}cm^{-1}$ was used for concentration calculations.

Chemicals. *N*-Acetyl-L-Ala-L-Ala-NHN-($CH_2CH_2CH_3$)CO-*p*-nitrophenylester was the generous gift of Prof. J. C. Powers (Atlanta, Ga., U.S.A.); Boc-Ala-ONp† from Sigma, St. Louis, Mo., U.S.A.; Suc–Ala–Ala–Pro–Val–AMC was the generous gift of Dr. T. Payne, Basel, Switzerland; gold thiomalate, sodium salt, was kindly supplied by

* Author to whom all correspondence should be addressed.

† Abbreviations used: Boc–Ala–ONp; α, *N*-tert. Butyloxycarbonyl-L-alanine-4-nitrophenyl ester. Suc–Ala–Ala–Pro–Val–AMC: succinyl-L-alanyl-L-analyl-L-prolyl-L valyl-4-methyl-7-amino-coumarylamide.

Byk Gulden (Constance, FRG) and was a pure sample of the commercially available Tauredon®; pentosan polysulfate, SP-54®, was provided by Bene-Chemie (Munich, FRG) (molecular weight ca. 2000); elastin from bovine neck ligament was obtained from Sigma (St. Louis, Mo., U.S.A.); Trasylol® was kindly supplied by Bayer, Leverkusen, FRG. The structural formula of pentosan polysulfate is given below.



Pentosan polysulfate
SP-54®

Enzyme assays. All inhibition studies were carried out in 50 mM phosphate buffer, pH 7.4, ionic strength 0.13. With Boc–Ala–ONp as substrate, the release of *p*-nitrophenol was monitored at 400 nm, the maximum of the absorption spectrum. For calculations, an extinction coefficient of 12,700 M^{-1} cm^{-1} was used. This value was calculated from the dissociation curve of *p*-nitrophenol using a p*K* value of 7.04 and an extinction coefficient of 18,380 M^{-1} cm^{-1} for the fully ionised species [11]. The sample cell in the spectrophotometer contained inhibitor plus enzyme and the reference cell contained inhibitor and buffer, the reaction was started by the addition of a small aliquot of substrate dissolved in acetonitrile to both the reference and sample cells in order to compensate for the spontaneous hydrolysis of the substrate. The end concentration of acetonitrile in all experiments was 1.6% (v/v).

In the experiments with Suc–Ala–Ala–Pro–Val–AMC, this substrate was preincubated with the inhibitor for 5 min and the reaction started by addition of enzyme. The release of 7-amino-4-methylcoumarin was monitored both spectrophotometrically at 360 nm ($\Delta\varepsilon = 6{,}700\ M^{-1}cm^{-1}$) and fluorimetrically with excitation and emission wavelengths of 383 and 455 nm respectively [12]. A sample of 7-amino-4-methylcoumarin of known concentration was used as the reference in the latter case. In the experiments followed spectrophotometrically, the reference cell contained buffer alone. The temperature was 25 ± 1°C.

The extinction coefficients of Suc–Ala–Ala–Pro–Val–AMC at various wavelengths and those of the leaving group 7-amino-4-methylcoumarin were determined with a Beckman Acta VI spectrophotometer using solutions of the compounds in 50 mM phosphate buffer, pH 7.4. Weight determinations were made with a Cahn electronic balance. The extinction coefficients at the absorption maxima were 17,300 $M^{-1}cm^{-1}$ at 324 nm for Suc–Ala–Ala–Pro–Val–AMC, and 9,770 $M^{-1}cm^{-1}$ at 342.5 nm for 7-amino-4-methylcoumarin. At 360 nm, a good wavelength for enzymatic assays, the extinction coefficients were 7200 $M^{-1}cm^{-1}$ and 500 $M^{-1}cm^{-1}$ for 7-amino-4-methylcoumarin and Suc–Ala–Ala–Pro–Val–AMC, respectively. The absolute fluorescence quantum yield of 7-amino-4-methylcoumarin was determined using quinine bisulfate in 1N H_2SO_4 as a reference [13], and was found to be 0.91.

The measurements with elastin as substrate were carried out according to Schwabe [14]. Briefly, 2 mg of elastin from bovine neck ligament were incubated with enzyme and inhibitor for 120 min at 37° in 50 mM phosphate buffer, pH 7.4, after preincubation of enzyme and inhibitor for 30 min at 37°. The solution was then made to 5% (w/v) with trichloroacetic acid and centrifuged. 0.1 ml of clear supernatant was added to 3.0 ml of 0.2 M sodium borate buffer, pH 8.5, followed by rapid addition with vigorous stirring of 0.1 ml of a fluorescamine solution (15 mg/100 ml acetone). The fluorescence of the labeled peptides was monitored at 480 nm after excitation at 390 nm. The fluorescence obtained in the absence of inhibitors was taken as the reference (100% activity).

Kinetic analysis. Michaelis constants (K_m) and maximum velocities (V) were evaluated with the plot of Eisenthal and Cornish-Bowden [15]. Inhibition experiments were analyzed by the use of various plots in order to have enough diagnostic tools for the determination of the inhibition type. Besides the well known Lineweaver–Burk plot, the Dixon [16] and Cornish-Bowden [17] plots have been used. Moreover, the Dixon plot for tightly bound inhibitors was used [18].

Since the inhibition by gold thiomalate and pentosan polysulfate gave saturating effects (partial or mixed-type inhibition), the Dixon and Cornish-Bowden plots could not be used for the determination of the kinetic parameters, since these plots are exactly applicable only for linear inhibition types. Nevertheless, these two plots offered an excellent diagnostic tool for the qualitative determination of the inhibition type, as was already discussed in the preceding paper [7]. The inhibitor concentration range chosen for the qualitative analysis never exceeded the K_i value, so that the plots were almost linear. The trend of the extrapolated curves was used for the diagnosis [17].

Apparatus. Stopped flow measurements were carried out with an apparatus described elsewhere [19]. Fluorescence was monitored using the ratio mode on an Aminco SPF-500 spectrofluorometer in thermostated 10 × 10 mm quartz cells. Initial velocity measurements with the spectrophotometric method were performed on a thermostated Beckman DB-GT spectrophotometer.

RESULTS

Pentosan polysulfate as inhibitor. The inhibitory effect of pentosan polysulfate on human lysosomal elastase was studied using three substrates: (1) the synthetic peptide Suc–Ala–Ala–Pro–Val–AMC; (2) the synthetic ester Boc–Ala–ONp; (3) insoluble elastin from bovine neck ligament. With the two soluble substrates, the mechanism of inhibition could be studied by performing initial velocity measurements with variable concentrations of substrate and inhibitor. All studies presented below were carried out in a buffer with ionic strength 0.13. An increase of ionic strength up to 0.5 by the addition of NaCl completely eliminated the inhibition effects.

The initial velocity experiments with Suc–Ala–Ala–Pro–Val–AMC as substrate were done both spectrophotometrically and fluorimetrically, and data obtained by the two techniques were considered for calculations. K_m for this substrate was $8.6 \pm 0.4 \times 10^{-4}$M, the turnover number, $V/[E]$, which for a monomeric enzyme as elastase coincides with k_{cat}, was $11.5 \pm 0.4\,s^{-1}$, and the ratio k_{cat}/K_m 13,400 $M^{-1}s^{-1}$. For substrate concentrations greater than 2 mM, substrate inhibition was observed. The inhibition constant of pentosan polysulfate was extremely small (tight binding). The tight binding of inhibitor to the enzyme prevented the use of the initial velocity equations and the usual plotting methods for calculating inhibition constants. In fact, the inhibitor concentrations which appear in the initial velocity equations, represent the free concentration which is considered equal to the total added concentration if there is no depletion of inhibitor by the enzyme. In our tightly bound system this is no longer valid, and therefore the method used for calculating K_i was that proposed by Dixon [18], and is shown in Fig. 1.

In order to determine the inhibition type, the Dixon and Cornish-Bowden plots were also drawn (not shown here) as a qualitative tool. The family of curves in both plots extrapolated from the linear part show a trend to a common intersection point on the inhibitor concentration axis. An increase in inhibitor concentration led to a saturation effect and replots of slope and intercepts of the double reciprocal plot (Lineweaver–Burk) were hyperbolic with respect to inhibitor concentration. All these results are in agreement with a partial noncompetitive inhibition mechanism (simple intersecting hyperbolic noncompetitive inhibition). In this case K_i and K_i', the dissociation constants of the EI and ESI complexes respectively, were numerically equal ($K_i = K_i' = 1.5 \times 10^{-7}$M). Scheme 1 shows the proposed mechanism. The α and β coefficients could be calculated by replots of 1/Δslope and 1/Δintercept of double reciprocal plots [20]. β can also be directly evaluated as the fraction of V when both inhibitor and substrate are saturating.

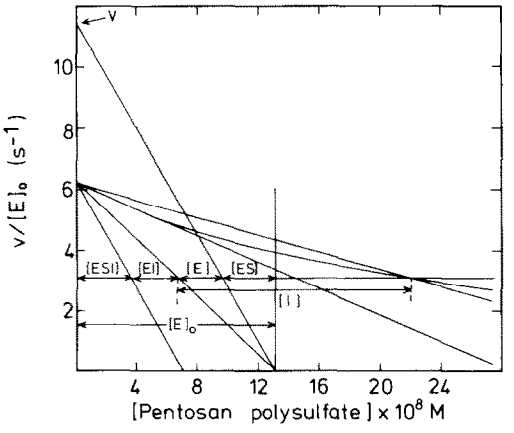


Fig. 1. Inhibition of lysosomal elastase by pentosan polysulfate. Dixon plot for tightly bound inhibitors [18]. The substrate was Suc–Ala–Ala–Pro–Val–AMC at a fixed concentration of 1.0 mM. The total enzyme concentration, $[E]_o$, was 13.1×10^{-8}M.

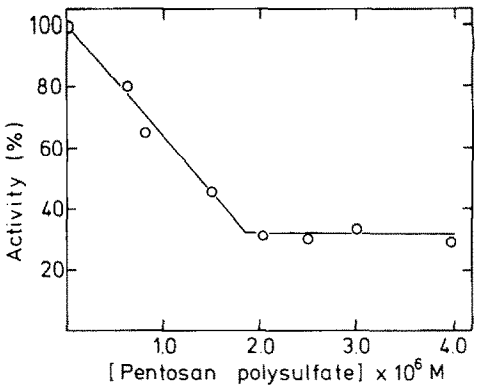


Fig. 2. Inhibition of lysosomal elastase by pentosan polysulfate. The substrate was insoluble elastin from bovine neck ligament, and the enzyme concentration was kept constant throughout (4.9×10^{-7}M).

The inhibition of lysosomal elastase by pentosan polysulfate when using the ester substrate Boc–Ala–ONp was identical to that observed with the peptide substrate described above. $K_i = K_i'$ was calculated from the Dixon plot for tightly bound inhibitors as described in Fig. 1 and was 1.8×10^{-7}M. The linear part of the Dixon and Cornish-Bowden plots extrapolated to an intersection point on the concentration axis (not shown here). K_m for Boc–Ala–ONp was $1.8 \pm 0.2 \times 10^{-4}$M and k_{cat} $13.8 \pm 0.8\,s^{-1}$; k_{cat}/K_m was 77,000 $M^{-1}s^{-1}$. Also in this case the slope and intercept replots of the double reciprocal plot were hyperbolic. Boc–alanine was found to be a pure competitive inhibitor against Boc–Ala–ONp ($K_i = 8.0 \pm 0.5 \times 10^{-4}$M).

Elastin is the substrate of choice for elastases. Since it is insoluble it is not suitable for kinetic studies. However it is very interesting to compare the inhibition effects of pentosan polysulfate obtained with the ester and peptide substrates and those obtained with elastin. Figure 2 shows an experiment obtained with various pentosan polysulfate concentrations. The maximal inhibition obtained is 60–70 per cent.

Gold thiomalate as inhibitor. For this inhibitor Boc–Ala–ONp was used at the substrate. Figure 3 shows the Lineweaver–Burk plot. The curves intersect above the concentration axis indicating a deviation from pure noncompetitive inhibition. Kinetic parameters were calculated from replots of the Lineweaver–Burk plot as was done above with the peptide substrate [20]. K_i' was 5.4×10^{-5}M. The Dixon plot showed an intersection point above the concentration axis, which was lower than $1/V$. The Cornish-Bowden plot presented an intersection point significantly below the concentration axis. The slope and intercept replots of the Lineweaver–Burk plot were hyperbolic. All these observations agree with a hyperbolic mixed type inhibition system (Scheme 1). With 1 mM Boc–Ala–ONp and gold thiomalate greater than 8×10^{-6}M an apparently higher inhibition was recorded, and reciprocal plots were curved upwards (Fig. 3). The plot of initial velocity versus

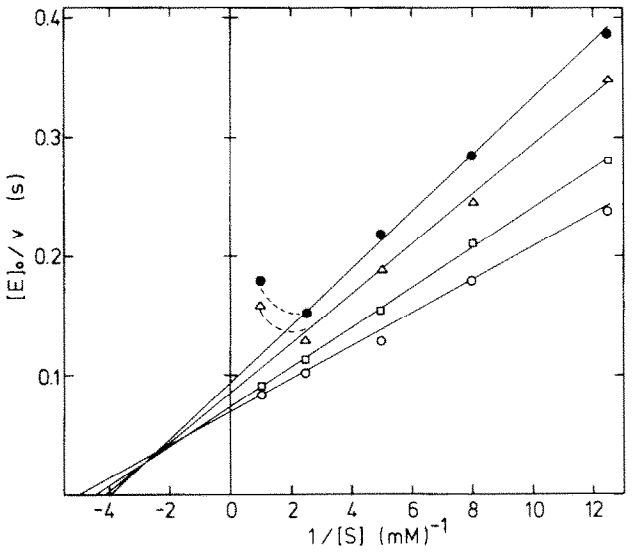


Fig. 3. Inhibition of lysosomal elastase by gold sodium thiomalate. Lineweaver-Burk plot. The substrate was Boc-Ala-ONp. Gold thiomalate concentrations (μM): ○ = 0.0; □ = 7.5; △ = 15.0; ● = 25.0.

inhibitor concentration was sigmoidal suggesting an interaction between the ester and gold thiomalate [20]. The depletion of substrate by the added inhibitor simulated higher inhibition. Thiomalic acid alone caused no inhibition of lysosomal elastase, according to the literature [21]. Gold thiomalate was also tested with elastin as substrate and a maximal inhibition of 40 per cent was observed. A summary of the kinetic constants is given in Table 1.

Stopped flow measurements. In order to locate the rate limiting step of the reaction, stopped flow measurements with both Boc-Ala-ONp and Suc-Ala-Ala-Pro-Val-AMC as substrates were performed. After rapidly mixing enzyme and substrate, the appearance of product was linear with time from the very beginning of the reaction and corresponded to the steady state turnover of substrate with a zero-order reaction law. This finding holds for both the ester and peptide substrate. The reaction trace for the peptide substrate is shown in Fig. 4(a). The lack of an initial burst of product release locates the rate limiting step to the dissociation of P_1 (Scheme 1) and k_2 is rate limiting. Actually, the amplitude of the burst reaction, π, on the basis of the chymotrypsin studies [22, 23], is given by $\pi = [E]_0\{k_2/(k_2+k_3)\}^2/\{1+K_m/[S]\}^2$ (for the meaning of k_2 and k_3 see Scheme 1). In the experiment of Fig. 4(a), the substrate concentration used was of the same

Table 1. Kinetic constants and inhibition of lysosomal elastase*

	Substrate		Inhibitor
	Boc-Ala-ONp	Suc-Ala-Ala-Pro-Val-AMC	
K_m (M)	$1.8 \pm 0.2 \times 10^{-4}$	$8.6 \pm 0.4 \times 10^{-4}$	
k_{cat} (s^{-1})	13.8 ± 0.8	11.5 ± 0.4	
k_{cat}/K_m ($s^{-1}M^{-1}$)	$77 \pm 11 \times 10^3$	$13.4 \pm 0.9 \times 10^3$	
α	1.6	—	Gold thiomalate
β	0.6	—	Gold thiomalate
K_i (M)	3.3×10^{-5}	—	Gold thiomalate
K_i'(M)	5.4×10^{-5}	—	Gold thiomalate
α	1	1	Pentosan polysulfate
β	0.4	0.4	Pentosan polysulfate
$K_i = K_i'$ (M)	1.8×10^{-7}	1.5×10^{-7}	Pentosan polysulfate

* The values given were obtained in 50 mM phosphate buffer, pH = 7.4, ionic strength 0.13, and 25°C.

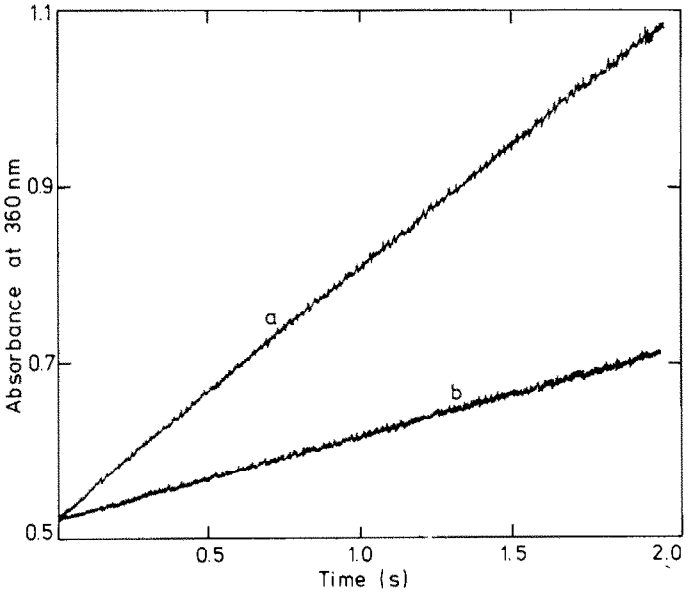
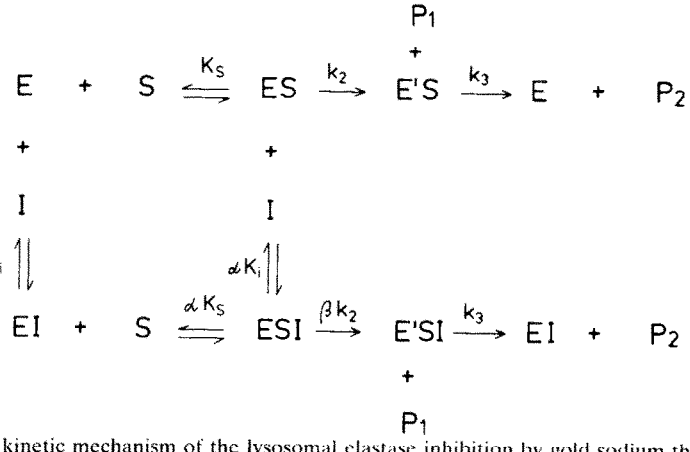


Fig. 4. Stopped flow kinetic measurements with lysosomal elastase. The curves represent computer traces of the reactions after rapidly mixing in the observation chamber an enzyme solution with substrate solutions (with or without inhibitor). The substrate was Suc–Ala–Ala–Pro–Val–AMC and the reaction progress was followed by measuring the release of 7-amino-4-methylcoumarin at 360 nm. (a) Enzyme pushed against substrate. (b) Enzyme premixed with pentosan polysulfate pushed against substrate. The final concentrations of reactants were: enzyme 9.1 μM, substrate 0.9 mM, pentosan polysulfate 70 μM.

order of magnitude as K_m. For higher substrate concentrations and with the highest amplification of the instrument, the reaction trace was always linear, both for the ester and peptide substrates, so that the situation $k_2 << k_3$ is valid. In order to detect some possible changes of the pre-steady-state phase of the reaction in the presence of pentosan sulfate experiments were performed as in Fig. 4(a), but including inhibitor. Curve b in Fig. 4 represents the appearance of product when having enzyme and pentosan polysulfate in one syringe of the stopped flow apparatus and substrate in the other one. A zero-order reaction is recorded from the very beginning and the ratio of the slope of curve b relative to curve a corresponds to 70 per cent inhibition, in agreement with the steady state measurements.

DISCUSSION

Human lysosomal elastase has a kinetic mechanism conforming to that of other serine proteinases (upper part of Scheme 1). In this scheme only the steps accessible to the present kinetic investigation are shown, whereas steps more relevant to the catalytic mechanism are omitted because they were not investigated. In the nomenclature of Cleland [24], the mechanism belongs to the Ping Pong Bi Bi class, and since the second substrate is water, present at a constant and always saturating concentration, the reaction can be regarded as Ordered Uni Bi. For such a mechanism the product P_2 must behave as a competitive inhibitor towards the substrate. With



Scheme 1. The kinetic mechanism of the lysosomal elastase inhibition by gold sodium thiomalate and pentosan polysulfate. E = enzyme, S = substrate, I = inhibitor, P_1 and P_2 = products.

Boc–Ala–ONp as substrate we tested the inhibitory effect of Boc–Ala and found it to be a pure competitive inhibitor.

The appearance or not of a burst reaction preceding the steady-state release of product is essentially dependent on the relative magnitude of the kinetic constants k_2 and k_3. With both the ester and peptide substrate the rate limiting step of the reaction has been located at the level of k_2 (formation of the acyl enzyme, $k_2 << k_3$). For the mechanism shown in Scheme 1 the following relationships are valid: $k_{cat} = k_2k_3/(k_2 + k_3)$; $K_m = K_sk_3/(k_2 + k_3)$. Since in the case of elastase $k_2 << k_3$, k_{cat} can be approximated to k_2 and $K_m = K_s$.

Both pentosan polysulfate and gold thiomalate behave as hyperbolic inhibitors, and the general inhibition mechanism is described in Scheme 1. With pentosan polysulfate as inhibitor and with both the ester and peptide substrates, the coefficient α was found to be 1, and β was between 0.3 and 0.4, i.e., the maximal inhibition was 60–70 per cent. The addition of inhibitor lowered the value of k_2 as demonstrated by the linearity of the reaction trace observed with the stopped flow apparatus in the first milliseconds of the reaction. On the basis of these observations, the inhibition of human lysosomal elastase by pentosan polysulfate is partial non-competitive, or in other words, a simple intersecting hyperbolic non-competitive inhibition. The same mechanism is valid for both the ester and peptide substrates. The driving forces of the interaction between pentosan polysulfate and elastase are electrostatic in nature since the inhibition is abolished by high salt concentrations. Lysosomal elastase is a basic enzyme with an isoelectric point near 10 [25], due to the high content of arginine residues [9], and is therefore positively charged at neutral pH. Pentosan polysulfate, with its negatively charged sulfate groups, is a good partner for a tight electrostatic binding with elastase (see also discussion in [7]).

For gold thiomalate, with reference to Scheme 1, the coefficients α and β were found to be 1.6 and 0.6 respectively. This picture conforms to a hyperbolic mixed type inhibition, i.e., it is a combination of partial competitive and partial non-competitive inhibition (also called intersecting, slope-hyperbolic, intercept-hyperbolic, non-competitive inhibition). The maximal inhibition capacity is 40 per cent. Janoff [26] first recognized the inhibitory action of gold thiomalate on the elastolytic activity of a crude leucocyte extract. Other papers reported inhibition by gold thiomalate greater than 40 per cent using Boc–Ala-ONp as substrate. Kruze *et al.* [27] described a 60 per cent and Starkey and Barrett [21] found 81 per cent inhibition with 0.5 mM gold thiomalate. These results are reproducible in our hands, but each inhibitory effect greater than 40 per cent is merely due to an interaction between gold thiomalate and the substrate. This interaction occurs at both substrate and inhibitor high concentrations, and depletes the substrate from the reaction mixture, thus lowering the initial velocity. The upper limit of gold thiomalate concentration with which linear reciprocal plots are obtained in kinetic studies with 0.5 mM Boc–Ala–ONp, is about 0.03 mM.

Recently Barg *et al.* [28] described the effect of pentosan polysulfate on the proteoglycan degradation by leucocyte neutral proteinases. The Authors obtained, with the proteoglycan substrate, the same degree of inhibition presented in this paper.

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