Oxidized mucus proteinase inhibitor: a fairly potent neutrophil elastase inhibitor

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N-chlorosuccinimide oxidizes one of the methionine residues of mucus proteinase inhibitor with a second-order rate constant of $1.5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Cyanogen bromide cleavage and NH_2 -terminal sequencing show that the modified residue is methionine-73, the P'_1 component of the inhibitor's active centre. Oxidation of the inhibitor decreases its neutrophil elastase inhibitory capacity but does not fully abolish it. The kinetic parameters describing the elastase-oxidized inhibitor interaction are: association rate constant $k_{\text{ass.}} = 2.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, dissociation rate constant $k_{\text{diss.}} = 2.9 \times 10^{-3} \text{ s}^{-1}$ and equilibrium dissociation constant $K_i = 1.1 \times 10^{-8} \text{ M}$. Comparison with the native inhibitor indicates that

oxidation decreases $k_{\text{ass.}}$ by a factor of 18.8 and increases $k_{\text{diss.}}$ by a factor of 6.4, and therefore leads to a 120-fold increase in K_i . Yet, the oxidized inhibitor may still act as a potent elastase inhibitor in the upper respiratory tract where its concentration is 500-fold higher than K_i , i.e. where the elastase inhibition is pseudo-irreversible. Experiments *in vitro* with fibrous human lung elastin, the most important natural substrate of elastase, support this view: $1.35 \mu\text{M}$ elastase is fully inhibited by $5\text{--}6 \mu\text{M}$ oxidized inhibitor whether the enzyme-inhibitor complex is formed in the presence or absence of elastin and whether elastase is pre-adsorbed on elastin or not.

INTRODUCTION

Living organisms have potent protein-oxidizing systems which serve a number of physiological functions and sometimes lead to disease. Oxidation converts surface-exposed methionine residues, the most sensitive targets of oxidants, into methionine sulfoxide. This chemical modification becomes biologically significant when it affects the activity of proteins. Thus, a number of hormones, chemotactic factors, proteinases and proteinase inhibitors undergo partial or total oxidative inactivation (for a review see Swaim and Pizzo, 1988).

Mucus proteinase inhibitor (MPI)¹ and α_1 -proteinase inhibitor, the two physiologic neutrophil elastase (NE)¹ inhibitors of the lung are examples of proteins whose activity is depressed by oxidants (for a review see Bieth, 1986). The oxidation of the latter has been studied in great detail from a chemical, an enzymic and a pathological point of view. Reaction of α_1 -proteinase inhibitor with *N*-chlorosuccinimide (NCS) or H_2O_2 + myeloperoxidase, secreted by triggered neutrophils, converts the P_1 and P_8 methionine residues of its active-site loop into methionine sulfoxides (Johnson and Travis 1979; Matheson et al., 1981). This modification considerably depresses the rate constant $k_{\text{ass.}}$ for the association of NE with the inhibitor: $k_{\text{ass.}}$ decreases from $\sim 2.10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ to $\sim 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Beatty et al., 1980; Padrines et al., 1989). Thus, oxidants present in the vicinity of activated neutrophils may transform α_1 -proteinase inhibitor into a slow-binding inhibitor that is unable to prevent NE-mediated proteolysis of connective tissue proteins such as elastin, collagen, proteoglycans, fibronectin and laminin. Oxidation of α_1 -proteinase inhibitor is therefore an important pathogenic factor of pulmonary emphysema and rheumatoid arthritis. This contention is supported by the observation that rheumatoid synovial fluid contains oxidized α_1 -proteinase inhibitor (Wong and Travis, 1980).

MPI is a 11.7 kDa protein produced by secretory cells and found in large amounts in bronchial and cervical mucus (Wallner and Fritz, 1974; Ohlsson et al., 1977), seminal plasma (Schiessler et al., 1976) and salivary gland secretions (Ohlsson et al., 1983). It also occurs at low levels in plasma (Ohlsson et al., 1977), cartilage (Böhm et al., 1991) and in the lower respiratory tract where it has been shown to be located near elastin fibres (Kramps et al., 1989). MPI is composed of a single polypeptide chain of 107 amino acid residues stabilized by eight disulphide bonds. Residues 1–54 and 55–107 show a high degree of identity, suggesting that the inhibitor has evolved by duplication of a single ancestral gene (Seemüller et al., 1986; Thompson and Ohlsson, 1986). X-ray crystallographic analysis of the MPI-chymotrypsin complex confirms this two-domain organization and indicates that the disulphides form intra-domain bridges only (Grütter et al., 1988). The two domains are encoded on separate exons (Steller et al., 1986). MPI has 15 lysine and 5 arginine residues which account for its basicity. It is likely that some of these residues are part of the heparin-binding site of the inhibitor (Faller et al., 1992).

MPI is a potent reversible inhibitor of NE, neutrophil cathepsin G, and pancreatic chymotrypsin and trypsin (Schiessler et al., 1976; Ohlsson et al., 1977; Thompson and Ohlsson, 1986; Boudier and Bieth, 1989). It has the unique property of inhibiting proteolysis at the neutrophil-elastin or neutrophil-fibronectin interface (Rice and Weiss, 1990). The chymotrypsin inhibitory loop encompasses residues 67–74 of the C-terminal domain, the P_1 - P'_1 residues being Leu-72–Met-73 (Grütter et al., 1988). Site-directed mutagenesis confirms the involvement of Leu-72 in the binding of chymotrypsin and suggests that NE and trypsin also bind at the same site (Eisenberg et al., 1990). At low enzyme and inhibitor concentration, the enzyme:inhibitor binding stoichiometry is 1:1. However, in the micromolar concentration range, MPI forms a 1:2 complex with cathepsin G, trypsin and

Abbreviations used: MPI, mucus proteinase inhibitor = secretory leucoprotease inhibitor (SLPI); NE, neutrophil elastase (leukocyte elastase: EC 3.4.21.37); Suc-Ala-3-pNA, succinyl-Ala-3-pNA; MeOSuc-Ala-2-Pro-Val-pNA, methoxysuccinyl-Ala₂-Pro-Val-pnitroanilide; NCS, *N*-chlorosuccinimide; TFA, trifluoroacetic acid.

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chymotrypsin and a 1:1 complex with NE. MPI has therefore an additional proteinase-binding site whose location is not known as yet (Boudier and Bieth, 1992).

MPI contains four Met residues. This may explain why oxidants depress its anti-NE capacities (Carp and Janoff, 1980; Smith et al., 1987; Kramps et al., 1990; Vogelmeier et al., 1991). The kinetic consequences of MPI oxidation on its interaction with NE have however never been investigated, and a possible anti-elastase function of the oxidized inhibitor *in vivo* was therefore impossible to predict. In addition, none of the above investigations attempted to localize the methionine residues that are modified in oxidized MPI.

EXPERIMENTAL PROCEDURES

Human NE was purified from purulent sputum as described by Martodam et al. (1979). The batch used in the present work was 90% active, as judged by active-site titration (Powers et al., 1984) done with acetyl-Ala-2-AzaAla-pnitrophenyl ester (Enzyme System Product, Livermore, CA, U.S.A.). Recombinant MPI was obtained from Synergen (Boulder, CO, U.S.A.) through the courtesy of Dr. H. P. Schnebli (Ciba-Geigy, Basel, Switzerland). The MPI concentration was determined by titration using NE (Boudier and Bieth, 1989). The enzyme and inhibitor concentrations indicated in this article refer to concentrations of active molecules. Human lung elastin (pass 100 mesh) was purchased from Elastin Product Company (Owenville, MI, U.S.A.). Succinyl-Ala-3-pnitroanilide (Suc-Ala-3-pNA) and methoxysuccinyl-Ala-2-Pro-Val-pnitroanilide (MeOSuc-Ala-2-Pro-Val-pNA) were obtained from Bachem, Bubendorf, Switzerland.

All enzymic measurements were done in 50 mM Hepes, 100 mM NaCl, pH 7.4, a solution called 'the buffer' throughout the text.

Oxidation of MPI

MPI was oxidized with NCS using the method of Shechter et al. (1977) that selectively converts surface-exposed methionine residues into methionine sulphoxide derivatives. MPI and NCS were reacted at room temperature in 100 mM Tris/HCl, pH 8.5. After a given period of time, the oxidation process was stopped by diluting 0.55 volume of reaction medium with 0.45 volume of 100 mM *N*-acetylmethionine dissolved in water. The samples were then chromatographed or used directly after dilution with buffer. The concentration of MPI in the oxidation mixture was 60 μ M throughout. The NCS concentration and the incubation time are given in the legends to the Figures. The oxidized inhibitor used to identify the modified methionine residues was prepared by reacting MPI with 2.7 mM NCS over a period of 2 h.

Location of methionine sulphoxide

The oxidized and the non-oxidized inhibitor samples were desalted by h.p.l.c. chromatography using a 250 mm $\times$ 4 mm Eurosil Bioselect 300-5 C4 column (Knauer, Berlin, Germany) equilibrated with 0.1% trifluoroacetic acid (TFA)/water (v/v) and eluted with 0.1% TFA/water/acetonitrile (v/v). Following lyophilization, the proteins were dissolved in 80% (v/v) formic acid and reacted for 24 h at room temperature with a 30-fold molar excess of cyanogen bromide over methionine. The reaction media were then chromatographed on the above h.p.l.c. column and the proteins eluted were lyophilized, sequenced (Knauer Model 810

automatic protein sequencer) and subjected to amino acid analysis.

Enzymic methods using synthetic substrates

The extent of oxidation of MPI was routinely checked by reacting 0.69 μ M inhibitor with 0.69 μ M NE for 15 min at pH 7.4 and 25 °C and measuring the residual enzyme activity as described in the legend to Figure 1. Under these conditions the equimolar mixture of NE and oxidized MPI is 27% active while native MPI fully inhibits the enzyme.

Kinetics of inhibition of NE by MPI was assessed using the progress curve method (Morrisson and Walsh, 1988). NE was added to a buffered solution of inhibitor and MeOSuc-Ala-2-Pro-Val-pNA. The absorbance at 410 nm was recorded using a CARY 1 spectrophotometer on-line with a Xerox PS 65 micro-computer. Non-linear regression analysis of the progress curves was done using the ENZFITTER Software (Biosoft, Cambridge, U.K.). The final concentrations were as follows: NE, 11.5 nM; native MPI, 150 nM or 450 nM; oxidized MPI, 110–700 nM; and substrate, 1.4 mM.

Rate of elastolysis

The time dependency of the NE-catalysed solubilization of human lung elastin was monitored by measuring the absorbance of the soluble elastin peptides at 280 nm (Boudier et al., 1981). Buffered suspensions (2 ml) of elastin (5 mg/ml), containing NE (1.35 μ M) alone or in a mixture with MPI (variable concentrations) were incubated at 37 °C under continuous stirring. Every 10 min a 250 μ l aliquot was removed, mixed with 750 μ l of 100 mM acetate buffer, pH 4.5, centrifuged for 10 min at 10000 g and read at 280 nm. The rate of elastin solubilization was derived from progress curves constructed with 5–6 absorbance/time pairs.

All other technical details are given in the legends to the Figures.

RESULTS

Effect of NCS on the activity of MPI

When increasing amounts of native MPI were reacted with a constant concentration of NE (0.69 μ M), a straight inhibition curve was obtained (Figure 1). In addition, the equimolar mixture of enzyme and inhibitor had no significant residual activity, as anticipated from the low K_i value of this system (Gauthier et al., 1982; Boudier and Bieth, 1989). In contrast, concave inhibition curves were obtained with MPI that had been previously treated for 2 h with NCS, indicating that the oxidizing agent lowers the enzyme-inhibitor affinity. The maximal effect was observed with $[NCS] \geq 1.2$ mM, i.e. $[NCS]/[MPI] \geq 20$. Under these conditions, the residual NE activity was $\sim 27\%$ at the equivalence point ($a = 0.27$, see Figure 1). When the titration of NE with oxidized MPI was repeated using a lower enzyme concentration (10 nM), the inhibition was much weaker: the residual NE activity $\sim 80\%$ at the equivalent point, typical behaviour for a reversible enzyme inhibitor (Figure 1).

Use was made of the NCS-induced increase in NE activity to assess the kinetics of MPI modification. The inhibitor was reacted for various periods of time with a 41-fold molar excess of NCS and assayed with an equimolar concentration of NE. Figure 2 illustrates the time-dependent appearance of NE activity. The data could be fit by nonlinear regression analysis to the following first-order equation:

$$a = a_{\infty}(1 - e^{-k_{\text{obs}}t}) \quad (1)$$

Figure 1 Titration of NE with native and oxidized MPI at pH 7.4 and 25 °C

Constant amounts of NE ($0.69 \mu\text{M}$) were reacted with increasing amounts of inhibitor in a total volume of $990 \mu\text{l}$ of buffer. After 15 min, $10 \mu\text{l}$ of 100 mM Suc-Ala-3-pNA dissolved in *N*-methylpyrrolidone were added and the absorbance at 410 nm was followed for about 15 min at 25°C . The rate of substrate hydrolysis was measured when E, I, S and their complex reached their equilibrium. The fractional activity, a , is the ratio of the reaction velocity in the presence of inhibitor to that in its absence. MPI was reacted with NCS for 2 h at room temperature. The NCS concentration in the oxidation medium was: 0.13 mM ($\diamond$), 0.27 mM ($\square$), 0.54 mM ($\blacksquare$), 1.2 mM , 2.7 mM and 13.6 mM . The three later concentrations gave similar rates represented by bars. The data obtained with these three NCS concentrations were averaged and used to calculate K_{app} by a non-linear least-squares fit to eqn. (2). The inhibition curve corresponding to these data was calculated using the best estimate of K_{app} (40 nM). The data obtained with native MPI ($\circ$) were indistinguishable from those obtained with MPI incubated with 0.13 mM NCS. In one experiment ($\bullet$), the NE concentration was 10 nM , the substrate was MeOSuc-Ala-2-Pro-Val-pNA ($[S_0] = 0.2 \text{ mM} \approx 2 \times K_m$) and MPI was oxidized with 2.7 mM NCS. Other conditions were the same as those described above.

Figure 2 Kinetics of the reaction of $60 \mu\text{M}$ MPI with 2.46 mM NCS

The reaction was stopped with *N*-acetylmethionine after appropriate intervals of time. After dilution with buffer, the elastase inhibitory capacity was measured by reacting $0.69 \mu\text{M}$ NE with an equimolar concentration of inhibitor as described in the legend to Figure 1. ($\circ$) Experimental points; (—) theoretical curve generated using eqn. (1) and $k_{\text{obs}} = 3.8 \times 10^{-3} \text{ s}^{-1}$.

where a is the fractional activity at any time t , a_∞ is the activity at infinite time and k_{obs} is the pseudo-first-order rate constant of MPI modification. The best estimate of k_{obs} was $3.8 \times 10^{-3} \text{ s}^{-1}$. There is a good fit between the experimental points and the

Figure 3 Reverse-phase h.p.l.c. chromatography of native (A) and NCS-treated MPI (B)

theoretical curve generated using eqn. (1) and this estimate (Figure 2).

Location of methionine sulfoxide in oxidized MPI

Reverse-phase chromatography, used to de-salt NCS-treated MPI, showed that the modified inhibitor eluted as a single, symmetrical peak, indicating that NCS fully converts MPI into a single molecular species. On the other hand, native and oxidized MPI have significantly different retention times (Figure 3).

The de-salted inhibitors were reacted with cyanogen bromide and re-chromatographed on reverse-phase h.p.l.c. Each reaction medium separated into two peaks, one containing unretained material and the other eluting at 35% acetonitrile. The latter material was lyophilized and subjected to N-terminal sequence analyses.

Cyanogen bromide selectively cleaves proteins at the carboxyl bond of non-oxidized methionine residues that are converted into homoserine (lactone) (Gross, 1967). The four cyanogen bromide cleavable bonds in native MPI are shown in Figure 4, A. The cleaving reagent should thus generate five peptides, four of which are linked together by disulphide bridges (MPI has eight disulphide bonds, see Grütter et al., 1988). These four peptides could be identified by sequence analysis of cyanogen bromide-cleaved native MPI (Figure 4, B). In contrast, the sequence Leu-74 NPPNF-E was not found with oxidized MPI (Figure 4, C), indicating that NCS treatment converted Met-73 into methionine sulfoxide.

Sequencing was stopped after nine cycles because the noise level became too important to allow accurate identification of further residues. The state of Met-94 in oxidized MPI was therefore ascertained in a different way. Cyanogen bromide will or will not release the dipeptide Gly-95-homoserine-96 depending upon whether Met-94 is virgin or oxidized. It was hypothesized that this dipeptide was present in the unretained fraction eluted from the above h.p.l.c. column. This fraction was therefore lyophilized and tested for amino acid composition. The material originating from both native and oxidized MPI was mainly composed of glycine, homoserine and homoserine lactone, indicating that NCS does not oxidize Met-94.

In summary, NCS oxidizes only one of the four methionine residues of MPI, namely Met-73, the P_1' residue of the active centre of the inhibitor. This result also allows the second-order rate constant of methionine oxidation, k_{2nd} , to be derived from

Figure 4 Location of methionine sulfoxide in NCS-oxidized MPI

A, complete amino acid sequence of native MPI (Thompson and Ohlsson, 1986). The arrows indicate the cyanogen bromide cleavage sites. The four sequences yielded by cyanogen bromide-cleaved native MPI are underlined. B and C, N-terminal amino acid sequence analysis of cyanogen bromide-cleaved native (B) and NCS-oxidized MPI (C). Cysteine (—) was not determined.

Table 1 Kinetic constants describing the inhibition of NE by native and oxidized MPI at pH 7.4 and 25 °C.

$K_{i,calc.} = k_{diss.}/k_{ass.}$; $K_{i,meas.} = K_i$ determined from equilibrium titrations (Figure 5). n.d. = not done.

	$k_{ass.} (M^{-1} \cdot s^{-1})$	$k_{diss.} (s^{-1})$	$K_{i,calc.} (M)$	$K_{i,meas.} (M)$
Native MPI	$(4.9 \pm 0.5) \times 10^6$	$(4.5 \pm 0.8) \times 10^{-4}$	$(9.2 \pm 2.5) \times 10^{-11}$ n.d.	
Oxidized MPI	$(2.6 \pm 0.3) \times 10^5$	$(2.9 \pm 0.5) \times 10^{-3}$	$(1.1 \pm 0.3) \times 10^{-8}$	$(1.0 \pm 0.2) \times 10^{-8}$

Figure 5 The effect of increasing Suc-Ala-3-pNA concentrations on $K_{i(app.)}$, the apparent equilibrium dissociation constant of the NE-oxidized MPI complex

$K_{i(app.)}$ was determined from equilibrium titration experiments as described in the legend to Figure 1 using MPI that was reacted for 2 h with 1.2 mM NCS.

the pseudo-first-order rate constant $k_{obs.} : k_{2nd} = k_{obs.}/[Met-73] = k_{obs.}/[MPI] = 1.5 M^{-1} \cdot s^{-1}$.

Kinetics of inhibition of NE by oxidized MPI

The equilibrium dissociation constant, K_i , of the oxidized MPI-NE complex was determined directly by adding substrate

to an equilibrium mixture of enzyme and inhibitor and measuring the rate of release of product. If E and I are in equilibrium, a , the ratio of the reaction rate in the presence of inhibitor to that in its absence, is given by the following equation (Bieth, 1984):

$$a = 1 - \frac{([E_0] + [I_0] + K_{i(app.)}) - \sqrt{([E_0] + [I_0] + K_{i(app.)})^2 - 4[E_0][I_0]}}{2[E_0]} \quad (2)$$

where $K_{i(app.)}$ is the apparent K_i .

The data from Figure 1 (vertical bars) were fit to eqn. (2) by non-linear regression analysis which gave a $K_{i(app.)}$ of 40 nM. The titration curve calculated using this value and eqn. (2) fits the experimental points well, indicating that eqn. (2) satisfactorily describes the inhibition data.

To investigate the substrate-dependence of $K_{i(app.)}$, further titrations were performed in the presence of substrate concentrations ranging from 0.22 mM to 1.5 mM (0.44 to $3.0 \times K_m$) and the data analysed as described above. Figure 5 shows the effect of $[S_0]$, the initial substrate concentration, on $K_{i(app.)}$. The linear increase of this parameter with $[S_0]$ strongly suggests that I and S truly compete for the binding of the enzyme, as does the native inhibitor (Boudier and Bieth, 1989). Hence, $K_{i(app.)}$, $[S_0]$ and the true K_i are related as follows:

$$K_{i(app.)} = K_i (1 + [S_0]/K_m) \quad (3)$$

This equation describes the linear curve shown in Figure 5, from which K_i was determined graphically (see Table 1).

The progress curve method (Cha, 1975; Morrisson and Walsh, 1988) was used to follow the time course of inhibition of NE by native and oxidized MPI. Enzyme was added to a mixture of inhibitor and substrate and the concentration of product (absorbance at 410 nm) was measured with time. Figure 6 shows typical progress curves recorded with identical concentrations of native (curve A) and NCS-treated MPI (curves B, C and D). These curves are biphasic: a pre-steady-state release of product precedes the steady state of the reaction. Oxidation of the inhibitor results in a strong elongation of the pre-steady state and an important increase of the steady-state velocity. The magnitude of these effects increases with the NCS+MPI incubation time, i.e. with the extent of inhibitor oxidation. Such kinetic alterations are interpreted to mean that oxidized MPI associates more slowly than does native MPI.

Scheme 1 Bimolecular mechanism for the binding of NE with oxidized MPI

Scheme 2 Two-step mechanism for the binding of NE with oxidized MPI

The progress curves describing NE inhibition by native or oxidized MPI were recorded under pseudo-first-order conditions ($[I_0] > 10[E_0]$). During the progress of the reaction the substrate was not depleted to a significant extent. Hence, progress curves A and D may be described by the following integrated equation (Morrison and Walsh, 1988):

$$[P] = v_s t + \frac{v_z - v_s}{k} (1 - e^{-kt}) \quad (4)$$

Where $[P]$ is the product concentration, v_z is the velocity at $t = 0$, v_s is the steady-state velocity and k is the apparent first-order rate constant for the approach to the steady state. The progress curves were fitted to eqn. (4) by non-linear least-squares analysis to obtain the best estimate of v_z , v_s and k . There is a good fit between the theoretical and the experimental curves, indicating that eqn. (4) adequately describes the inhibition kinetics.

Since oxidized MPI and substrate compete for the binding of NE, the inhibition process may be described by Scheme 1 or 2 (Tian and Tsou, 1982).

Where E is NE, I is oxidized MPI, S and P are the substrate and product, respectively. EI is the final enzyme-inhibitor complex and EI* is an intermediate reversible complex resulting from a fast pre-equilibrium governed by $K_i^* = k_{-1}/k_1$. The pseudo-first-order rate constant k is given by one of the following relationships:

$$k = \frac{k_{\text{ass.}}[I_0]}{1 + [S_0]/K_m} + k_{\text{diss.}} \quad (5)$$

$$k = \frac{k_2[I_0]}{[I_0] + K_i^*(1 + [S_0]/K_m)} + k_{\text{diss.}} \quad (6)$$

Figure 6 Progress curves for the inhibition of NE by native and oxidized MPI at pH 7.4 and 25 °C

Curve A, native MPI; curves B, C and D, MPI reacted with 1.5 mM NCS for 4 min, 10 min and 18 h, respectively. Curves B, C and D of the main Figure are the initial segments of the progress curves. The inset shows progress curve D in full. Curves A and D were fitted to eqn. (4) by non-linear regression analysis. The smooth lines represent the theoretical curves corresponding to the best fit. The final reactant concentrations were: NE, 11.5 nM; native or oxidized MPI, 450 nM.

Figure 7 The effect of oxidized MPI concentration on k , the pseudo-first-order rate constant governing NE inhibition at 25 °C and pH 7.4

k was measured using the progress curve method. (●) Experimental points; (—) theoretical line calculated by linear least-squares analysis.

To decide whether NE associates with oxidized MPI according to a simple bimolecular mechanism (Scheme 1) or a two-step mechanism (Scheme 2), NE inhibition was investigated using increasing inhibitor concentrations to detect the possible accumulation of the reaction intermediate EI*. Figure 7 shows that the rate constant, k , increases linearly when $[I_0]$ varies up to 700 nM, in agreement with eqn. (5). This suggests that scheme 1 adequately describes the mechanism of complex formation. The second-order rate constant, $k_{\text{ass.}}$, can therefore be calculated for the slope of the linear curve shown in Figure 7 using $[S_0] = 1.4$ mM and $K_m = 90$ μ M. The intercept of this curve with the ordinate yields $k_{\text{diss.}}$, the first-order dissociation rate constant. As can be seen from Table 1, the calculated K_i is very close to that determined directly. This provides internal consistency of the kinetic data. With inhibitor concentrations higher than 700 nM,

the rates of inhibition could no longer be followed by ordinary spectrophotometric means.

The parameters describing NE inhibition by native MPI have already been reported in a previous article (Boudier and Bieth, 1989). Since the experimental conditions used in the present investigation were slightly different, the kinetic constants $k_{\text{ass.}}$ and $k_{\text{diss.}}$, governing the interaction between the native inhibitor and NE, were re-determined as described above. With 450 nM native MPI, v_s is close to zero (curve A of Figure 6), i.e. the inhibition is said to be pseudo-irreversible (Bieth, 1984) and $k_{\text{diss.}}$ cannot be measured accurately. We therefore ran a series of progress curves using 150 nM MPI. v_s had a significant and reproducible value (data not shown) so that both association and dissociation constants could be determined reliably.

As shown in Table 1, oxidation adversely affects the two rate constants: $k_{\text{ass.}}$ decreases by a factor of 18.8, while $k_{\text{diss.}}$ increases 6.4-fold. As a result, the calculated K_i increases 120-fold.

Inhibition of the elastolytic activity of NE by oxidized MPI

Constant amounts of NE were reacted with increasing amounts of oxidized MPI and the mixtures were allowed to incubate with a continuously stirred suspension of human lung elastin. The kinetics of elastin solubilization was assessed by withdrawing samples from these suspensions and assaying them for soluble peptides. The inset of Figure 8a shows that elastin solubilization is linear with time, which allows the initial rate of elastinolysis to be calculated in a straightforward manner. All rates shown in Figure 8 were measured in this way.

The above experiment yielded the titration curve shown in Figure 8a. This curve is much more concave than the one that would have been obtained with Suc-Ala-3-pNA, the synthetic substrate used in Figure 1 (see dashed curve in Figure 8a). Nevertheless, a 5-fold molar excess of oxidized MPI over NE is sufficient to stop elastinolysis fully. Also, the observation that all progress curves of elastin solubilization were linear indicates that the enzyme-inhibitor complex does not dissociate during the 60 min period of the assay.

In a second set of experiments, oxidized MPI was incubated with elastin before addition of NE. The resulting inhibition curve (Figure 8b, open circles) can be superimposed on that of Figure 8(a). This indicates that oxidized MPI favourably competes with elastin for the binding of NE. The oxidized inhibitor was, however, significantly less potent than the native molecule in such a competition experiment (Figure 8b, open triangles).

In a third set of experiments, NE was absorbed on elastin before addition of increasing amounts of oxidized MPI. Figure 8b shows that the resulting data points are on the same curve as those of the preceding experiment. This indicates that oxidized MPI inhibits elastin-bound NE as efficiently as it blocks free NE.

DISCUSSION

We have shown that oxidized MPI is still a potent NE inhibitor (Figures 1 and 8). This result is new and does not fully agree with previous reports demonstrating full oxidative inactivation of the inhibitor (Carp and Janoff, 1980; Kramps et al., 1990; Vogelmeier et al., 1991). These authors used NE concentrations in the nanomolar range to assess the elastase inhibitory capacity of the modified inhibitor. Under these conditions, oxidized MPI cannot significantly inhibit NE since the equilibrium dissociation constant K_i of the complex (which was not measured by other workers) is also in the nanomolar range ($K_i = 10$ nM, Table 1). This is clearly illustrated by the titration curves shown in Figure

Figure 8 Inhibition of the elastolytic activity of NE by oxidized MPI

(a) Titration of NE with oxidized MPI. Reaction mixtures (500 μ l) containing 5.4 μ M NE and increasing concentrations of oxidized MPI were allowed to incubate for 5 min at 37 °C before being diluted into 1.5 ml of elastin suspension. The inset shows the progress curves corresponding to the four data points marked with an arrow. The rates of elastin solubilization were derived from these curves. The dotted line is the titration curve that would have been observed if the substrate were Suc-Ala-3-pNA instead of elastin. It was calculated using eqn. (2) with $[E_0] = 1.35$ μ M and $K_{i(\text{app.})} = 40$ nM. (b) Effect of pre-mixing elastin with oxidized MPI or NE on the inhibition of elastinolysis. Elastin suspensions were incubated for 15 min at 37 °C with increasing amounts of oxidized ($\circ$) or native MPI ($\triangle$), after which NE was added and the rates of elastinolysis measured. In another set of experiments ($\bullet$), elastin was pre-mixed for 15 min at 37 °C with constant amounts of NE, after which increasing amounts of oxidized MPI were added and the rates of elastinolysis measured. The final NE concentration was 1.35 μ M throughout. Oxidized MPI was prepared by reacting the inhibitor with 1.5 mM NCS for 3 h.

1: for $[E_0] = [I_0] = 0.69$ μ M and 10 nM there is 73 % and 20 % NE inhibition, respectively. Thus, as expected for a reversibly associating enzyme-inhibitor system, the smaller the NE concentration, the lower the inhibition.

The decrease in the anti-elastase function of MPI correlates with the oxidation of one (of a total of four) methionine residues, namely Met-73, the P'_1 component of the inhibitory site. The selectivity of this modification is probably due to the fact that the active-site loop of MPI, although formed of hydrophobic amino acid residues, is highly exposed to solvent (Grütter et al., 1988), rendering Met-73 particularly sensitive to modification. Other serine proteinase inhibitors also undergo partial oxidative inactivation, namely, α_1 -proteinase inhibitor (Johnson and Travis, 1979), chicken ovomucoid inhibitor (Schechter et al., 1977) and plasminogen activator inhibitor I (Lawrence and Loskutoff, 1986). Although there is a methionine residue at the P'_1 position of plasminogen activated inhibitor I, inactivation is not due to methionine oxidation, but to an oxidant-induced conformational change of the protein, as demonstrated with a mutant inhibitor in which valines replaced methionines (Strandberg et al., 1991). In this context it is worthwhile noticing that mutant MPI, in which the four methionines are replaced by leucines, no longer undergoes oxidative impairment of anti-elastase activity (Rudolph et al., 1991a). This indicates that the effect of NCS is not an indirect one, as in the case of the plasminogen activator

inhibitor, but results directly from the oxidation of the P₁' residue of the inhibitor.

Serine proteinase inhibitors react with cognate enzymes in a substrate-like fashion, the binding of P₁ (inhibitor) as S₁ (proteinase) being most important for specificity and binding energy. The S₁'-P₁' interaction appears to be much less significant (Laskowski and Kato, 1980). This may help to explain why oxidation of P₁ = Met-358 in α_1 -proteinase inhibitor depresses the rate constant k_{ass} for the inhibition of NE by 2000-fold (Beatty et al., 1980; Padrines et al., 1989), whereas oxidation of P₁' = Met-73 in MPI results in a 18.8-fold decrease in k_{ass} only (Table I). Oxidation of α_2 -antiplasmin has no effect at all on the inhibition of plasmin and trypsin, although the inhibitor has a methionine residue at P₁' (Shieh and Travis, 1987). This observation strongly supports the expressed view above.

NE is thought to be responsible for lung tissue destruction occurring in inflammatory lung diseases such as cystic fibrosis. Airways proteins are protected against the deleterious action of this enzyme by MPI, the dominant anti-elastase of the upper respiratory tract (Bieth, 1986). The chronic pulmonary infection that characterizes cystic fibrosis leads to a permanent recruitment of neutrophils in airways. During phagocytosis these cells produce H₂O₂ and release myeloperoxidase, which, in the presence of Cl⁻, generates hypochlorite, a highly oxidant species (Weiss, 1989) that may oxidize the bulk of MPI present in airway secretions. Also, the generation of oxidants is closely paralleled by a release of NE and other lysosomal proteinases. The question is then: does oxidized MPI act as an efficient antielastase in inflammatory airways?

The efficiency *in vivo* of a reversible enzyme inhibitor is best defined by the ratio of $[I]_0$, the inhibitor concentration *in vivo*, to K_i , the equilibrium dissociation constant of the enzyme-inhibitor complex: the higher this ratio, the better the inhibition (Bieth, 1984). Since $[I]_0 \approx 5 \mu\text{M}$ in airways secretions (Tournier et al., 1983; Kramps et al., 1984) and K_i for oxidized MPI is $\approx 10 \text{ nM}$, one gets $[I]_0/K_i \approx 500$. For such a high ratio there is only 1.4 % of free NE present in an equimolar mixture of $5 \mu\text{M}$ NE and $5 \mu\text{M}$ oxidized MPI. Theory therefore predicts that oxidized MPI may be an efficient anti-elastase in airway. Experiments *in vitro* with elastin entirely confirm this prediction (Figure 8).

Fibrous elastin is the most important substrate of NE from a pathological point of view, since elastin fragmentation always accompanies lung tissue destruction (Bieth, 1986). Previous studies have shown that this insoluble substrate significantly decreases the efficiency of NE inhibitors. For instance, if NE is pre-adsorbed on elastin it is partly shielded from the action of α_1 -proteinase inhibitor (Reilly and Travis, 1980), but not from that of MPI (Bruch and Bieth, 1986). On the other hand, elastin strongly decreases the rate constant for the inhibition of NE by synthetic irreversible inhibitors and sharply increases the K_i of synthetic and natural inhibitors. Among others, the K_i of the NE-MPI complex is 29-fold higher in the presence of elastin than in the presence of a synthetic substrate (Padrines and Bieth, 1991). These findings led us to study the effect of oxidized MPI on the elastinolytic activity of NE. The enzyme-inhibitor affinity was found to be significantly lower in the presence of elastin than in the presence of a synthetic substrate (Figure 8). Yet, the elastolytic activity of $1.35 \mu\text{M}$ NE was fully inhibited by $5\text{--}6 \mu\text{M}$ oxidized MPI, the inhibitor concentration of airways secretions (Tournier et al., 1983; Kramps et al., 1984). Moreover, oxidized MPI was found to inhibit elastin-bound NE as efficiently as free NE. It is therefore likely that the oxidized inhibitor is able to prevent efficiently the NE-induced elastinolysis of airways elastin whether NE is free or adsorbed on elastin.

Recombinant MPI may soon be used as a drug in emphysema

and cystic fibrosis. Animal experiments indicate that the inhibitor has a good bioavailability in the lung (Gast et al., 1990; McElvaney et al., 1992) and protects this organ against NE-induced emphysema (Lucey et al., 1990; Rudolph et al., 1991b). Local administration of MPI as an aerosol (McElvaney et al., 1992) might result in fast oxidation of the bulk of the inhibitor because of the important neutrophil recruitment and activation that takes place in patients with acute lung inflammation. Our data show that even in such an extreme case, MPI might still be an efficient anti-elastase drug provided that a high local therapeutic concentration ($\geq 5 \mu\text{M}$) can be achieved.

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