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Different selectivities of oxidants during oxidation of methionine residues in the α-1-proteinase inhibitor

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Oxidation of the reactive site methionine (Met) in α-1-proteinase inhibitor (α-1-PI) to methionine sulfoxide (Met(O)) is known to cause depletion of its elastase inhibitory activity. To estimate the selectivity of different oxidants in converting Met to Met(O) in α-1-PI, we measured the molar ratio Met(O)/α-1-PI at total inactivation. This ratio was determined to be 1.2 for both the myeloperoxidase/H_2O_2/chloride system and the related compound NH_2Cl. With taurine monochloramine, another myeloperoxidase-related oxidant, 1.05 mol Met(O) were generated per mol α-1-PI during inactivation. These oxidants attack preferentially one Met residue in α-1-PI, which is identical with Met 358, as concluded from the parallelism of loss of elastase inhibitory activity and oxidation of Met. A similar high specificity for Met oxidation was determined for the xanthine oxidase-derived oxidants. In contrast, the ratio found for ozone and *m*-chloroperoxybenzoic acid was 6.0 and 5.0, respectively, indicating oxidation of additional Met residues besides the reactive site Met in α-1-PI, i.e. unselective action of these oxidants. Further studies were performed on the efficiency of oxidants for total depletion of the elastase inhibitory capacity of α-1-PI. Ozone and *m*-chloroperoxybenzoic acid were 10-fold less effective and the superoxide anion/hydroxyl radicals were 30–50-fold less effective to inactivate the elastase inhibitory activity as compared to the myeloperoxidase-derived oxidants. The myeloperoxidase-related oxidants are discussed as important regulators of α-1-PI activity in vivo.

Proteinase inhibitor, α-1-; Methionine; Methionine sulfoxide; Myeloperoxidase; Xanthine oxidase; Ozone; Chloroperoxybenzoic acid, *m*-; Sulfite

1. INTRODUCTION

α-1-Proteinase inhibitor (α-1-PI) is a well characterized compound of the antiproteolytic system in plasma. One major function of α-1-PI is the inhibition of neutrophil elastase [1–4]. This function is related to the reactive site which contains in position 358 one of the nine Met residues of the protein [5,6]. Oxidation of this Met residue to Met(O) strongly reduces the elastase inhibitory activity [7–9]. Severe genetic deficiency in α-1-PI is frequently associated with genesis of pulmonary emphysema [1,10]. Oxidative inactivation of α-1-PI in the lungs is also considered to be a possible mechanism of emphysema. Carp et al. [11] reported that the elastase inhibitory capacity in bronchoalveolar lavage fluids from smokers is decreased by about 40%. The authors showed, that oxidation of Met residues in α-1-PI is responsible for this loss of inhibitory capacity. It has been reported, that the inactive α-1-PI isolated from smokers' bronchoalveolar lavage fluid contained 4 mol Met(O)/mol protein. Johnson and Travis [5] demonstrated the oxidative inactivation of α-1-PI by *N*-chlorosuccinimide. The authors found that 2 Met residues were oxidized in the completely inactivated inhibitor. One of these residues turned out to be identical with the Met in the reactive center. The elastase inhibitory activity of α-1-PI was also shown to be depleted by the action of ozone

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Abbreviations: Met, methionine; Met(O), methionine sulfoxide; α-1-PI, α-1-proteinase inhibitor

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[12,13]. In addition to air-borne oxidants such as ozone and organic peroxy compounds, endogenous generated reactive oxygen species, which are released into lung tissue and airways, may impair the antiproteolytic system in the lungs. Especially the myeloperoxidase/H_2O_2/chloride system from neutrophils was recently shown to cause a rapid inactivation of α-1-PI [14,15].

The present study was prompted by the need for detailed information on the action of relevant endogenous and exogenous oxidants on α-1-PI. We were particularly interested in the selectivity of oxidants with respect to oxidation of Met residues in α-1-PI and in their efficiency to block the elastase inhibitory capacity.

We found, that the myeloperoxidase-derived oxidants are highly specific in attacking Met residues and very efficient in inactivation of the elastase inhibitory activity of α-1-PI. The xanthine oxidase-derived oxygen radicals are also very specific, however, by a factor of 30–50 less efficient as compared to the myeloperoxidase-derived oxidants. Ozone and 3-chloroperoxybenzoic acid unselectively oxidize Met residues in α-1-PI. The efficiency of these oxidants to kill the elastase inhibitory activity was about 10-fold lower as compared to that of the myeloperoxidase system.

2. MATERIALS AND METHODS

2.1. *Chemicals*

L-Amino acid calibration mixture, *o*-phthaldialdehyde reagent solution, sample diluent buffer (1 M potassium borate, pH 10.4) and cyanogen bromide were from Pierce (Rockford, IL); acetonitrile (HPLC gradient grade) and tetrahydrofurane (HPLC gradient grade) were from Baker (Groß-Gerau); homoserine, porcine pancreas elastase, human α-1-PI and succinyl-$(Ala)_3$-*p*-nitroanilide were from Sigma (Taufkirchen); myeloperoxidase was from Calbiochem (Frankfurt); xanthine oxidase from cow milk was from Bochringer (Mannheim); all other chemicals were from Merck (Darmstadt).

2.2. *Assay for elastase inhibitory activity of α-1-PI*

Elastase inhibitory activity of α-1-PI was assayed with porcine pancreas elastase and succinyl-$(Ala)_3$-*p*-nitroanilide as substrate [16]. Briefly, α-1-PI was preincubated in the presence of 25–35% excess of pancreas elastase in the assay buffer (0.1 M Hepes/0.5 M NaCl (pH 7.5) mixed with dimethyl sulfoxide in a ratio of 9:1 (v/v)) for 30 min at 25°C. An aliquot of 350 μl of the preincubation mixture was then added to 650 μl assay buffer containing 1.17 mg substrate. Elastase inhibitory activity was calculated from the difference in absorbance/min at 410 nm between the elastase control incubation and the elastase incubation with α-1-PI.

2.3. *Determination of total Met and Met(O) in α-1-PI*

Total Met was determined with a Biotronik amino acid analyzer LC 6000 using a single-column system [17,18] after hydrolysis of α-1-PI with 6 N HCl in the presence of 5 mM dithioerythritol for 24 h at 110°C. For quantitation of Met(O), the cyanogen bromide procedure [19,20] was used. During this procedure, the non-oxidized Met is converted to homoserine/homoserine lactone. Aliquots of the pretreated α-1-PI (50–100 μg) were dissolved in 840 μl of 75% (v/v) formic acid and 60 μl of 3 M cyanogen bromide were added giving a final reagent concentration of 0.2 M [21]. The reaction was performed under N_2-saturation in darkness at room temperature for 24 h. The reaction products were hydrolyzed with 6 N HCl at 110°C for 48 h in the presence of 5 mM dithioerythritol. Under these conditions, the remaining Met(O) is reduced back to Met, which is determined by amino acid analysis.

2.4. *Inactivation studies on α-1-PI*

The decrease of elastase inhibitory activity of α-1-PI was followed during treatment with different oxidants. Concomitantly, formation of Met(O) in the protein was measured. Aliquots of the samples used for Met(O) analysis were lyophilized immediately after finishing incubation with oxidants.

2.4.1. Myeloperoxidase/H_2O_2/Cl^- system

Inactivation was performed by the procedure from Clark et al. [15], which was slightly modified. The incubation mixture (2 ml) contained 4 mg α-1-PI, 0.1 M sodium chloride and 112 mU myeloperoxidase in 20 mM sodium phosphate buffer (pH 7.0). The reaction was started at 37°C by addition of 70 μM H_2O_2. Aliquots of 0.3 ml were taken at 0, 2, 5, 10, 30 and 60 min and added to 325 U catalase to stop oxidation.

2.4.2. Hypochlorous acid, taurine monochloramine, NH_2-Cl, chlorinated poly(Lys) and chlorinated albumin

Chlorinated amines are obtained by reaction of hypochlorous acid with amines [22]. In our studies chlorination was carried out with a 40-fold molar excess of the amines (10 mM) over hypochlorous acid in 100 mM potassium phosphate buffer (pH 7.6). Under these conditions, hypochlorous acid reacts quantitatively with the amines to monochloramine derivatives. Chlorinated samples of poly(Lys) and albumin were subsequently desalted on a Sephadex G-25 column. The concentrations of the oxidants used for inactivation of α-1-PI were determined by oxidation of 5-thio-2-nitrobenzoic acid [23,24]. Inactivation of α-1-PI was performed in 20 mM potassium phosphate buffer (pH 7.0) at 25°C for 1, 2 and 5.5 h. The molar ratios of oxidants/inhibitor ranged between 0 and 2.0.

2.4.3. Xanthine oxidase system

Generation of superoxide anion was performed by the xanthine oxidase system according to [25,26]. Briefly, hypoxanthine (0.3 mM), α-1-PI (0.1 mg) and xanthine oxidase from cow milk (4.2 μg/ml) were incubated in 0.1 M potassium phosphate buffer (pH 7.6) at 25°C in the absence and presence of 0.1 mM Fe(III) in a total volume of 21 ml (the buffer was initially equilibrated with 100% oxygen for 10 min). After 60 min another aliquot of 4.2 μg xanthine oxidase/ml was added. The reaction was followed by monitoring increase of uric acid at 293 nm (ϵ = 12.0 × 10^3 $M^{-1} \cdot cm^{-1}$). During oxidation of 1

molecule hypoxanthine via xanthine to uric acid four electrons are released, which are available for reduction of molecular oxygen. According to Fridovich [25] 20% of the electron flux is used to generate the superoxide anion by monovalent reduction of oxygen, as measured by reduction of ferricytochrome *c* at pH 7.0. We could confirm this recovery for monovalent reduction of oxygen with our incubation system at pH 7.6. Samples of 2 × 2 ml were taken at 0, 30, 60 and 120 min and 5 μg superoxide dismutase/ml were added to stop the inactivation reaction.

2.4.4. Ozone

Ozone obtained by electric discharge using a Fischer ozone generator was sampled in phosphate buffered saline (pH 7.0). The ozone concentration in this stock solution was determined according to Hendricks and Larsen [27], which was immediately used for the inactivation experiments. Portions of 0.375 mg α-1-PI/ml phosphate buffered saline (pH 7.0) were incubated in the presence of 3.8, 7.6, 15, 35 and 76 μM ozone at 37°C for 10 min.

2.4.5. *m*-Chloroperoxybenzoic acid

The incubation volume of 1.5 ml contained 0.6 mg α-1-PI and *m*-chloroperoxybenzoic acid in concentrations up to 100 μM in 0.1 M potassium phosphate buffer (pH 7.5). The reactions were performed at room temperature for 1 h.

2.4.6. Sulfite

The incubation volume (1 ml) contained 1 mg α-1-PI and sulfite in concentrations between 0 and 4 mM in 20 mM potassium phosphate buffer (pH 7.0). The incubations were performed for 16 h at 37°C.

3. RESULTS

3.1. *Selectivity of oxidation of Met residues in α-1-PI*

During depletion of the elastase inhibitory activity of α-1-PI by action of different oxidants, we concomitantly measured formation of Met(O). The selectivity of oxidants to oxidize Met residues was judged by the number of Met(O) residues formed per molecule of inactivated α-1-PI (mol Met(O)/mol α-1-PI).

Elastase inhibitory activity of α-1-PI is efficiently depleted by the myeloperoxidase-catalyzed reaction [15]. Relevant oxidants generated by the myeloperoxidase/H_2O_2/Cl^- system in neutrophils are hypochlorous acid [28,29] and the chlorinated derivatives of NH_3 and taurine [22,30]. For total depletion of the elastase inhibitory activity of α-1-PI by taurine monochloramine, 1.05 mol Met/mol inhibitor had to be oxidized (table 1). A similar specific attack at Met was demonstrated for the myeloperoxidase-catalyzed reaction and for NH_2-Cl. The corresponding value found by oxidation with hypochlorous acid was 1.8, indicating a lower specific attack at Met (table 1).

Table 1

Effect of different oxidants on Met during inactivation of the elastase inhibitory activity of α-1-PI

Oxidant	Met(O) content in α-1-PI at total inactivation[a] (mol Met(O)/mol α-1-PI)
Myeloperoxidase/H_2O_2/Cl^-	1.2
NH_2-Cl	1.2
Taurine monochloramine	1.05
Hypochlorous acid	1.8
Xanthine oxidase	
− Fe(III)	1.3
+ 0.1 mM Fe(III)	1.1
Ozone	6.0
m-Cl-peroxybenzoic acid	5.0
Sulfite	1.6

[a] Calculated by linear extrapolation of values for Met(O) determined at 50% inactivation of α-1-PI assuming that at total inactivation the double amount of Met is oxidized

A further candidate to affect the activity of α-1-PI is the superoxide anion, which is released in high amounts by activated phagocytes [31]. We used for these studies the xanthine oxidase/hypoxanthine system as a source of the superoxide anion [25,26]. In the presence of Fe(III) ions, the hydroxyl radical may be additionally formed from the superoxide anion by the so-called iron-catalyzed Haber-Weiss reaction [32,33]. We therefore followed inactivation of α-1-PI and Met oxidation by the xanthine oxidase/hypoxanthine system in the absence and presence of Fe(III) ions. In the absence of Fe(III) ions, 1.3 mol Met/mol α-1-PI were found to be oxidized by the xanthine oxidase-catalyzed reaction for total loss of elastase inhibitory activity. In the presence of Fe(III) ions the molar ratio Met(O)/α-1-PI was determined to be 1.1 for complete inactivation (see table 1).

Ozone, one of the most common airborne oxidants, has been recently described to inactivate α-1-PI [12,13]. For total inactivation by ozone 6.0 mol Met/mol α-1-PI were shown to be oxidized, which indicates a rather unspecific attack at Met residues (table 1).

Organic peroxy acids belong to a group of oxidants which may arise in the atmosphere by photochemical reactions, as known for the peroxyacetyl nitrate [34,35]. Instead of peroxyacetyl nitrate,

which is rather an unstable compound, we used *m*-chloroperoxybenzoic acid, which is stable for 10 min in solution, to study oxidation of Met residues in α-1-PI. This oxidant was shown to oxidize 5 mol Met/mol α-1-PI for total inactivation (table 1).

Sulfite has been reported to mediate oxidation of Met in the presence of oxygen [36,37]. Our studies revealed that the elastase inhibitory activity of α-1-PI is markedly depleted by sulfite in concentrations between 0.1 and 2 mM at pH 7.0 within 16 h (data not shown) and that 1.6 mol Met/mol α-1-PI are oxidized at total inactivation (table 1).

3.2. *Efficiency of oxidants for inactivation of α-1-PI*

The efficiency of oxidants to deplete elastase inhibitory activity of α-1-PI was defined as the molar ratio of α-1-PI/oxidant at total inactivation. The chlorinated derivatives obtained from ammonia and taurine exhibit high efficiency ratios of 0.80 and 0.90, respectively (table 2). Hypochlorous acid showing an efficiency ratio of 0.22 appeared to be a less effective oxidant for inactivation of α-1-PI. We also studied the effect of high-molecular mass oxidants, like chlorinated albumin and chlorinated poly(Lys) on the elastase inhibitory activity of α-1-PI. Both chlorinated compounds readily inactivate the inhibitor (data not shown). The efficiency ratio measured for albumin-NH-Cl was 0.22 and that for poly(Lys)-NH-Cl was 0.27 (table 2). For inactivation by ozone and *m*-chloroperoxybenzoic acid, the efficiency ratios were determined to be 0.10 and 0.11, respectively. The xanthine oxidase/hypoxanthine system turned out to be very inefficient in inactivating α-1-PI. The efficiency ratio measured in the absence of Fe(III) was <0.018 and in the presence of 0.1 mM Fe(III) was <0.028.

Table 2

Efficiency of different oxidants for inactivation of the elastase inhibitory activity of α-1-PI

Oxidant	Efficiency for total inactivation[a] (mol α-1-PI/mol oxidant)
Taurine monochloramine	0.90
NH_2-Cl	0.80
Hypochlorous acid	0.22
Albumin-NH-Cl	0.22
Poly(Lys)-NH-Cl	0.27
Xanthine oxidase	
− Fe(III)	≤0.018
+ 0.1 mM Fe(III)	≤0.028
Ozone	0.10
m-Cl-peroxybenzoic acid	0.11

[a] Calculated by linear extrapolation of the efficiency determined for 50% inactivation of α-1-PI assuming that for total inactivation the double amount of oxidant is necessary

4. DISCUSSION

Both the myeloperoxidase-derived oxidants taurine monochloramine and NH_2-Cl attack one Met residue out of the 9 Met residues in α-1-PI during inactivation. This residue is very likely identical with the Met in position 358, which is essential for elastase inhibition [5]. Oxidation of the reactive site Met in α-1-PI is the only way of explaining the simultaneous loss of elastase inhibitory activity. Taurine monochloramine and NH_2-Cl were shown to be the most efficient oxidants to inactivate α-1-PI. For both oxidants, the efficiency ratios for inactivation of α-1-PI (mol α-1-PI/mol oxidant) are close to the theoretical value of 1.0. The theoretical efficiency ratio of 1.0 is achieved when the oxidation of Met by *N*-chloramines follows to a 1:1 stoichiometry during inactivation of α-1-PI. Such a 1:1 stoichiometry for the reaction of Met with chloramines was recently shown with *N*-formylleucyl-methionyl-phenylalanine (FMLP), a chemotactic factor for neutrophils [38]. For oxidation of Met in α-1-PI with NH_2-Cl, we calculated a stoichiometric ratio of 0.97:1. Oxidation of other amino acids aside from Met in α-1-PI by the myeloperoxidase-derived chloramines was not observed by amino acid analysis. Modification of additional amino acids by reaction with NH_2-Cl or taurine monochloramine during inactivation of α-1-PI can be excluded because these oxidants are quantitatively consumed by oxidation of Met as shown above. Albumin-NH-Cl and poly(Lys)-NH-Cl also readily inactivate α-1-PI (table 2). This finding indicates, that the reactive site Met in α-1-PI is accessible to oxidants with high molecular masses. We therefore conclude that this Met is an 'exposed' residue located on the surface of the protein.

The xanthine oxidase-derived oxidants exhibit a selectivity to oxidate Met residues in α-1-PI, which

is comparable to that of the myeloperoxidase-derived oxidants (table 1). However, the efficiency of the superoxide anion is estimated to be about 50-fold lower, as compared to that of taurine monochloramine or NH_2-Cl. There is no marked change in the efficiency when Fe(III) is present in the reaction mixture (table 2). The complete system which generates O_2^- and H_2O_2 is believed to give rise to hydroxyl radicals or other reactive oxygen species through a metal-catalyzed Haber-Weiss reaction [39]. We conclude from our findings that the superoxide anion as well as the hydroxyl radical are of minor relevance to the inactivation of α-1-PI in vivo by oxidation of the reactive site Met.

Nevertheless, the hydroxyl radical is known to oxidize amino acids in proteins resulting in a decrease of their biological activity. As recently reported, L-glutamine synthetase and alkaline phosphatase are readily inactivated by the xanthine oxidase system in the presence of O_2 and of a suitable donor (xanthine or acetaldehyde) when Fe(III) ions are available [39,40]. In both enzymes, a site-specific attack of the reactive oxygen species at or near the active center is assumed to be responsible for loss of enzyme activity. Inactivation of L-glutamine synthetase could be correlated to the loss of one His residue out of 16 His residues by mixed-function oxidation with ascorbate, Fe(III) and O_2 [41,42].

The exogenous compounds *m*-chloroperoxybenzoic acid and ozone were shown to oxidize the Met residues in α-1-PI very unselectively (table 1). We conclude from this finding that the lipophilic *m*-chloroperoxybenzoic acid easily attacks Met residues which are located in the hydrophobic interior of the protein (buried Met residues). Our studies also revealed that ozone behaves like a lipophilic agent when acting on α-1-PI. The efficiency of *m*-chloroperoxybenzoic acid and ozone to suppress the elastase inhibitory activity of α-1-PI is significantly lower as compared to that of NH_2-Cl and taurine monochloramine. This is partially due to the simultaneous oxidation of several Met residues during inactivation of the elastase inhibitory activity of α-1-PI. We could not detect modification of other amino acids, as Met in α-1-PI, after treatment with *m*-chloroperoxybenzoic acid. In contrast, His and Tyr residues were found to be modified by low ozone concentrations, however, to a lower extent than Met. Oxidation of Trp and Cys residues in α-1-PI have not been investigated. Oxidation of His, Tyr and Trp residues in α-1-PI by ozone has already been reported [12,13].

Among the oxidants generated by phagocytes, the myeloperoxidase-derived oxidants were shown to be highly efficient in inactivation of the elastase inhibitory activity of α-1-PI. We believe that these oxidants generated by neutrophils during inflammatory lung diseases, are of great importance in lung pathology. Oxidative inactivation of α-1-PI contributes to an imbalance between elastase and the naturally occurring proteinase inhibitor, which is considered to be a mechanism for pathogenesis of pulmonary emphysema [43]. In contrast, the superoxide anion and related oxygen radicals are obviously not directly involved in this pathobiochemical mechanism, because of their low efficiency to deplete α-1-PI activity. Exogenous oxidants, like ozone or organic peroxy compounds may also contribute to an imbalance of the antiproteolytic system in the lungs by inactivation of α-1-PI, however, to a lower extent than the myeloperoxidase-derived oxidants. Since sulfite mediates inactivation of α-1-PI only slowly when present in high concentrations, it is unlikely that this air pollutant impairs the antiproteolytic system in the lungs.

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