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Enzymatic reduction of oxidized α-1-proteinase inhibitor restores biological activity

(emphysema/methionine sulfoxide-containing peptide reductase)

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ABSTRACT **The major serum inhibitor of proteolytic activity, α-1-proteinase inhibitor (α-1-PI), (or α-1-antitrypsin) can be readily inactivated by oxidation [Carp, H. & Janoff, A. (1978) *Am. Rev. Resp. Dis.* 118, 617–621]. This inactivation appears to be due to the oxidation of a critical methionine(s) in α-1-PI that is required for the inhibition of elastase activity. An enzyme from *Escherichia coli* that reduces methionine sulfoxide residues in protein [Brot, N., Weissbach, L., Werth, J. & Weissbach, H. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2155–2158] can restore the biological inhibitory activity of canine oxidized α-1-PI.**

An enzyme has been purified from extracts of *Escherichia coli* that can reduce methionine sulfoxide [Met(O)] residues in *E. coli* ribosomal protein L12 (1). The enzymatic reduction requires a reducing agent such as reduced thioredoxin or dithiothreitol (1). A similar enzymatic activity was found in extracts of *Euglena gracilis*, *Tetrahymena pyriformis*, spinach, and various rat tissues (1). Oxidized [Met]enkephalin ([Met(O)]enkephalin) was also reduced by the *E. coli* enzyme, suggesting a nonspecificity for the enzyme with regard to the peptide-bound Met(O) residue (1). It should be noted that the *E. coli* reductase for Met(O)-containing peptide [Met(O)-peptide reductase] is different from another enzyme system described in yeast (2) and *E. coli* (3) that reduces free Met(O) to methionine.

The Met(O)-peptide reductase could have an important physiological role if it is involved in reducing essential Met(O) residues in proteins that have become oxidized (1). One such example is α-1-proteinase inhibitor (α-1-PI), the major serum proteinase inhibitor (4–8). This protein has been postulated to play a critical role in modulating the activity of the neutral proteinase, elastase, in lung (4–11). Inactivation of α-1-PI has been implicated in the excessive destruction of lung tissue seen in emphysema, and there is evidence that limited exposure of α-1-PI to oxidizing agents results in oxidation of methionine residues to Met(O) and inactivation of the protein's function (4–12). A similar oxidation may explain the lower α-1-PI activity seen in lung washings from cigarette smokers and rats exposed to cigarette smoke (13, 14). In addition, an inactive form of the protein that contains Met(O) residues has been isolated from synovial fluid from patients with rheumatoid arthritis (15).

The present study shows that oxidized, functionally inactive canine α-1-PI regains its biological inhibitory activity after reduction with a partially purified preparation of *E. coli* Met(O)-peptide reductase.

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MATERIALS AND METHODS

Ampholines were obtained from LKB Instruments; Sephacryl S-200 and phenyl-Sepharose, from Pharmacia Fine Chemicals; Iso-Gel and GelBond, from FMC Marine Colloids Division, Rockland, ME; succinyl-(L-Ala)$_3$-*p*-nitroanilide, from the Protein Research Foundation, Osaka, Japan; and Bio-Gel P-10, from Bio-Rad Laboratories. Thioredoxin was the gift of A. Holmgren of the Karolinska Institutet, Stockholm.

Dog neutrophil elastase was purified to homogeneity as described (8). Purified porcine pancreatic elastase was a gift of Harold James of Temple University. Met(O)-peptide reductase from *E. coli* was purified as described by Brot *et al.* (1).

Preparation of Canine α-1-PI. Canine α-1-PI was prepared from plasma by a modification of the method of Abrams *et al.* (16) with a hydrophobic chromatographic step in the purification scheme. A unit of inhibitory activity is defined as the minimum amount of α-1-PI required to completely inhibit 1 μg of porcine pancreatic elastase per μl under the conditions described herein. Approximately 80 mg (9000 units) of partially purified α-1-PI (16) was loaded onto a 2 × 30 cm column of phenyl-Sepharose in a solution containing 0.05 M phosphate buffer, pH 6.5/3 M NaCl/1 mM 2-mercaptoethanol, and the protein was eluted with 400 ml of a decreasing linear salt gradient from 3 M NaCl to no NaCl. The α-1-PI was eluted at a concentration of approximately 1 M NaCl. The active fractions (4.8 mg, 1680 units) were pooled and then chromatographed as described (16) on a column of Sephacryl S-200 (2.6 × 85 cm), and fractions containing the α-1-PI were concentrated by ultrafiltration. The protein appeared homogenous when electrophoresed on alkaline polyacrylamide gels (17) and when examined by isoelectric focusing. In a standard assay, the final preparation had a specific activity of 350 units/mg of α-1-PI.

Oxidation of α-1-PI. Purified canine α-1-PI (12 μM) was exposed to 12 mM chloramine T at 20°C for 15 min. These conditions completely destroy the inhibitory activity of α-1-PI (4). The reaction mixture was rapidly chilled to 4°C and then chromatographed on a Bio-Gel P-10 column (1.6 × 40 cm) at a flow rate of 10 ml/hr in 0.2 M Tris·HCl buffer (pH 8.0). The void volume of the column contained the oxidized, inactivated α-1-PI, which was separated from unreacted chloramine T. The oxidized α-1-PI fractions were pooled and dialyzed against 0.01

Abbreviations: α-1-PI, α-1-proteinase inhibitor; MalNEt, *N*-ethylmaleimide; MalNEt-α-1-PI, *N*-ethylmaleimide-treated α-1-proteinase inhibitor; Met(O), methionine sulfoxide; Met(O)-peptide reductase, *E. coli* protein that reduces Met(O)-containing peptide.

M Tris (pH 8.0). The dialyzed solution was lyophilized, and the protein residue was dissolved in 0.05 M Tris (pH 8.0). This preparation of oxidized α-1-PI did not inhibit either dog neutrophil elastase or porcine pancreatic elastase, under the conditions used, but retained approximately 20% of the original trypsin inhibitory activity.

Met(O)-Peptide Reductase. The conditions and reaction components for reduction of oxidized α-1-PI were similar to those used previously for the reduction of oxidized *E. coli* ribosomal protein L12 [Met(O)-L12] (1). The incubation medium contained, in a total volume of 30 μl, 33 mM Tris·HCl (pH 7.4), 13 mM $MgCl_2$, 13 mM dithiothreitol, 120 pmol of thioredoxin, 10.2 μg of oxidized α-1-PI (or as indicated), and 3.2 μg of partially purified *E. coli* Met(O)-peptide reductase (1). The reaction mixture was incubated at 37°C for 60 min unless otherwise indicated, and the reaction was stopped by placing the tubes in ice. The assay for the reduction of the oxidized α-1-PI was based on the ability of the reduced α-1-PI to inhibit dog neutrophil elastase. The elastase activity was monitored by measuring the initial rate of hydrolysis of the synthetic substrate succinyl-(L-Ala)$_3$-*p*-nitroanilide (4, 18). To each 30-μl reaction mixture were added 200 mM Tris·HCl, pH 8.0/1.4 μg of elastase (dog neutrophil)/1.0 mg of succinyl-(L-Ala)$_3$-*p*-nitroanilide in a total volume of 1.0 ml. The α-1-PI sample and elastase were preincubated for 2 min prior to addition of the substrate. The release of *p*-nitroaniline was followed spectrophotometrically at 410 nm as a function of time.

Reaction of α-1-PI with *N*-ethylmaleimide (MalNEt) and Preparation of Oxidized MalNEt-Treated α-1-PI. α-1-PI (17 μM) was allowed to react with 2 mM MalNEt at 25°C for 90 min in 1.0 ml of 0.2 M Tris buffer (pH 8.0). By this procedure, the cysteine residue in α-1-PI is alkylated without destroying its ability to inhibit elastase activity. The solution was chilled to 4°C, and the MalNEt-treated protein (MalNEt-α-1-PI) was separated from the unreacted reagent by chromatography on a 1.6 × 40 cm Bio-Gel P-10 column (flow rate, 10 ml/hr) in the same buffer. The material in the void volume was concentrated by ultrafiltration and the MalNEt-α-1-PI (4.4 μM) was oxidized by treatment with 4.4 mM chloramine T. After chromatography on Bio-Gel P-10, the fractions containing the oxidized MalNEt-α-1-PI were pooled, dialyzed against 0.01 M Tris·HCl buffer (pH 8.0), lyophilized, and dissolved in 0.05 M Tris·HCl (pH 8.0). After oxidation the MalNEt-treated α-1-PI had no elastase inhibitory activity.

Isoelectric Focusing and Crossed Immunoelectrophoresis. Agarose slab gels for isoelectric focusing (1.5 mm thick) were prepared as described (19). The electrofocusing range of 4–7 was obtained with pH Ampholines 3.5–5, 4.0–6.0, 5.0–8.0, and 7.0–9.0. The anode electrolyte was 1 M phosphoric acid, and the cathode electrolyte was 1 M NaOH; the focusing was performed for 60 min at 10°C and 10 W. The pH gradient was determined directly in the gel with internal pI reference proteins for the pH range of 2.5–6.5. Strips from the focused gel were oriented 90° to the original electrophoresis direction, imbedded in 1% Isogel (containing 0.05 M barbital buffer) and electrophoresed at 15°C for 18 hr (4.0 V/cm) into agarose-containing goat anti-canine α-1-PI antisera (16). The resulting precipitin lines were fixed and stained.

RESULTS

It has been shown that extracts of *E. coli* contain an enzyme that can reduce Met(O) residues in protein to methionine (1). A partially purified enzyme preparation (1) was used to determine whether oxidized α-1-PI could be enzymatically reduced and reactivated. As noted, the reduction of oxidized α-1-PI could be measured by the restoration of inhibitory activity towards elastase.

Table 1. Requirements for the reduction of oxidized α-1-PI

System	% inhibition
Complete	28
Without Met(O)-peptide reductase	3
Without dithiothreitol	0
Without thioredoxin	29
Without oxidized α-1-PI	0

The percentage of inhibition of 1.4 μg of dog neutrophil elastase due to reactivation of oxidized α-1-PI was assayed after a 60-min incubation as described.

Table 1 shows the dependencies for the reduction of inactive oxidized α-1-PI to a functional inhibitor of elastase. In the absence of dithiothreitol or the *E. coli* Met(O)-peptide reductase, there is essentially no reduction of oxidized α-1-PI as measured by inhibition of elastase activity. Because thioredoxin stimulated the reduction of Met(O)-containing protein L12 (1), it was routinely added to the incubations. However, it had no effect on the reduction of oxidized α-1-PI if dithiothreitol was present. On the other hand, it was observed that in the presence of NADPH and thioredoxin reductase, thioredoxin could replace dithiothreitol in the enzymatic reduction of oxidized α-1-PI (data not shown). When oxidized MalNEt-α-1-PI was used as substrate, it could also be reactivated in this system (data not shown). This result eliminates the possibility that a thiol interchange between dithiothreitol and the —SH group is responsible for the restoration of the inhibitory activity of α-1-PI.

Fig. 1 *A* and *B* shows the effect of concentration of oxidized α-1-PI and Met(O)-peptide reductase on the reduction of oxidized α-1-PI. Linear responses were obtained with up to 15 μg of oxidized α-1-PI and 9.6 μg of Met(O)-peptide reductase. The reduction of oxidized MalNEt-α-1-PI was also linear with increasing oxidized protein. The effect of time of incubation on the reduction of oxidized α-1-PI is seen in Fig. 2. The reaction was linear for 120 min, and routinely a 60-min incubation was used.

It is known that α-1-PI forms a tight complex with elastase and that oxidized α-1-PI is not only inactive as an elastase inhibitor but cannot form a tight complex with elastase (6, 8). Two-

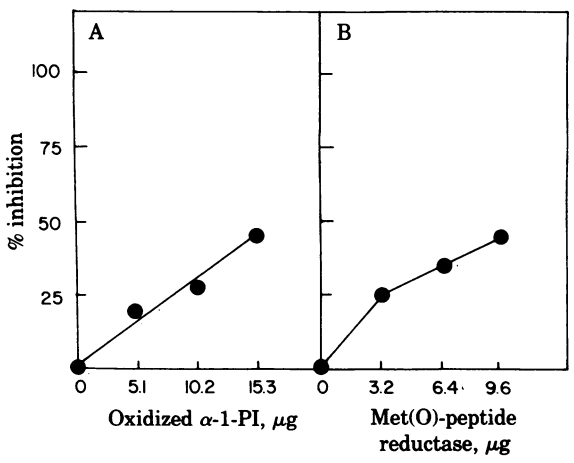

FIG. 1. The inhibition of elastase by reactivated α-1-PI is shown as a function of oxidized α-1-PI concentration with 3.2 μg of Met(O)-peptide reductase (*A*) and as a function of increasing Met(O)-peptide reductase with 10.2 μg of oxidized α-1-PI (*B*).

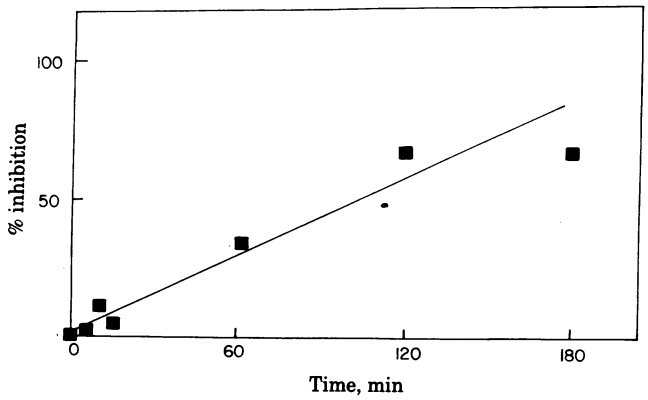

FIG. 2. The inhibition of elastase by reactivated α-1-PI (10.2 μg) is shown as a function of time of incubation with 3.2 μg of Met(O)-peptide reductase.

dimensional electrophoresis (isoelectric focusing followed by crossed immunoelectrophoresis in a second dimension) was used to confirm the ability of oxidized α-1-PI to be reduced by Met(O)-peptide reductase and, thus, regain the ability to form a complex with elastase. Only when the oxidized α-1-PI was incubated with the reductase was a complex formed (Fig. 3 *Upper*). The complex was detected on a second "rocket" in the pH region of 5.9–6.4 that is characteristic of the α-1-PI–neutrophil elastase complex. In the absence of the Met(O)-peptide reductase, oxidized α-1-PI was not reduced, and no complex was seen (Fig. 3 *Lower*).

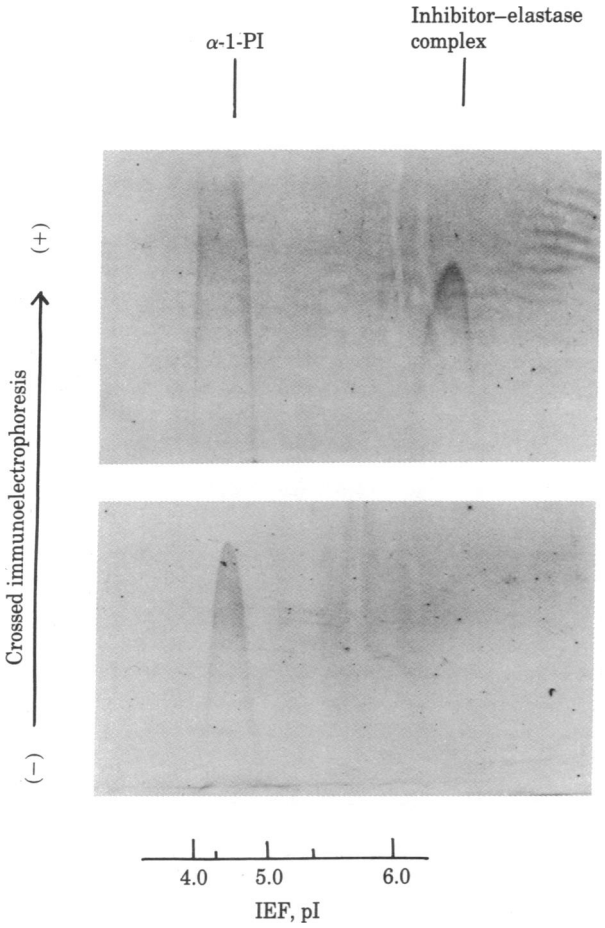

FIG. 3. Isoelectric focusing (IEF; first dimension) and crossed immunoelectrophoresis of enzymatically reduced oxidized α-1-PI. Oxidized α-1-PI was incubated in the presence or absence of Met(O)-peptide reductase as described in the text. After 3 hr at 37°C, elastase was added and the reaction mixture was incubated for 2 min at 37°C and subjected to isoelectric focusing and crossed immunoelectrophoresis. (*Upper*) Complete system. (*Lower*) Met(O)-peptide reductase was omitted.

DISCUSSION

The removal of biological oxidizing agents, such as peroxides, superoxides, hydroxyl radicals, and hypochlorites that are continuously formed in cells is important to prevent oxidation of susceptible essential groups in proteins. Although there are a number of enzymes that destroy these oxidizing agents, under certain conditions it can be assumed that they do not provide complete protection. The presence of an enzyme in cells that can reduce Met(O) residues in proteins to methionine provides a mechanism for restoring biological activity to a protein that has been inactivated because of oxidation of an essential methionine residue. Despite the widespread occurrence of Met(O)-peptide reductase activity (1), its physiological role has remained obscure. The present study was initiated because of the mounting evidence that (*i*) increased elastase activity may be responsible for the loss of lung function in emphysema, which may be associated with a history of cigarette smoking; (*ii*) the increased elastase activity results from inactivation of the serum proteinase inhibitor α-1-PI; and (*iii*) the inactivation of α-1-PI is due to oxidation of an essential methionine residue(s) in α-1-PI to Met(O). In accord with this hypothesis is the recent finding that oxidants in cigarette smoke can inactivate α-1-PI, perhaps by oxidation of methionine residues (4, 10, 11, 13). Because of the possible role of oxidized α-1-PI in the etiology of emphysema, it is significant that the Met(O)-peptide reductase can restore inhibitory activity to oxidized α-1-PI. Although it is felt that the chemically oxidized α-1-PI used in these studies is similar to what may be formed *in vivo* under pathological conditions (15), it will be necessary to demonstrate the reactivation of inactive α-1-PI isolated from the tissues in which inactive α-1-PI is known to occur (13–15). The present results support the concept that Met(O)-peptide reductase activity in tissues may be involved in reversing damage to proteins that have undergone oxidation. It is known that rat lung extracts have Met(O)-peptide reductase activity (1), and preliminary results on canine neutrophils indicate the presence of a similar reductase activity in these cells (data not shown). The latter observation is meaningful because damage to tissues is often associated with neutrophil invasion.

It also should be noted that oxidation of methionine residues in protein may be involved directly or indirectly in other physiological processes. As an example, one of the striking differences between normal and cataractous lens protein is the high level of Met(O) residues in the latter (20). It is obvious that more extensive investigation is needed on the role of oxidized methionine residues in proteins in a variety of physiological processes as well as the possible protective function of Met(O)-peptide reductase.

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1. Brot, N., Weissbach, L., Werth, J. & Weissbach, H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2155–2158.
2. Black, S., Harte, E. M., Hudson, B. & Wartofsky, L. (1960) *J. Biol. Chem.* **235**, 2910–2916.
3. Ejiri, S. I., Weissbach, H. & Brot, N. (1980) *Anal. Biochem.* **102**, 393–398.

4. Abrams, W. R., Eliraz, A., Kimbel, P. & Weinbaum, G. (1980) *Exp. Lung Res.* **1**, 211–223.
5. Cohen, A. B. (1979) *Am. Rev. Resp. Dis.* **119**, 953–960.
6. Satoh, S., Kurecki, T., Kress, L. F. & Laskowski, M., Sr. (1979) *Biochem. Biophys. Res. Commun.* **86**, 130–137.
7. Johnson, D. & Travis, J. (1978) *J. Biol. Chem.* **253**, 7142–7144.
8. Johnson, D. & Travis, J. (1979) *J. Biol. Chem.* **254**, 4022–4026.
9. Travis, J., Garner, D. & Bowen, J. (1978) *Biochemistry* **17**, 5647–5656.
10. Janoff, A. & Carp, H. (1977) *Am. Rev. Resp. Dis.* **116**, 65–72.
11. Carp, H. & Janoff, A. (1978) *Am. Rev. Resp. Dis.* **118**, 617–621.
12. DelMar, E. G., Brodrick, J. W., Geokas, M. C. & Largman, C. (1979) *Biochem. Biophys. Res. Commun.* **88**, 346–350.
13. Gadek, J. E., Fells, G. A. & Crystal, R. G. (1979) *Science* **206**, 1315–1316.
14. Janoff, A., Carp, H. & Lee, D. K. (1979) *Science* **206**, 1313–1314.
15. Wong, P. S. & Travis, J. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1449–1454.
16. Abrams, W. R., Kimbel, P. & Weinbaum, G. (1978) *Biochemistry* **17**, 3556–3561.
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
18. Bieth, J., Spiess, B. & Wermuth, C. G. (1974) *Biochem. Med.* **11**, 350–357.
19. Saravis, C. & Zamcheck, N. (1979) *J. Immunol. Methods* **29**, 91–96.
20. Truscott, R. J. W. & Augusteyn, R. C. (1977) *Biochim. Biophys. Acta* **492**, 43–52.