Role of Disulfide Exchange in α1-Protease Inhibitor†

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ABSTRACT: The major endogenous inhibitor of neutrophil elastase in the plasma, α1-protease inhibitor (α1-PI), has a single cysteine residue which has been shown to form mixed disulfides with a number of thiols in vitro. Under normal physiological conditions, the plasma concentrations of reduced and oxidized thiols are such that a major fraction of α1-PI in the circulation in vivo is in the form of mixed disulfides [Laurell, C.-B. (1979) in *The Chemistry and Physiology of Human Plasma Proteins* (Bing, D. H., Ed.) pp 329–341, Pergamon, New York]. We show here that the mixed disulfide between glutathione or cysteine and α1-PI (α1-PI-SSG or α1-PI-SScys) has an intrinsic fluorescence which distinguishes it from the reduced form of α1-PI. By employing the fluorescence difference, we have measured the ratio of α1-PI-SH to mixed difulfide α1-PI in redox buffers of different ratios of reduced to oxidized glutathione (GSH to GSSG) or reduced to oxidized cysteine (cys to cysSScys) and have calculated an equilibrium constant and redox potential 0f 0.74 ± 0.08 and 8 ± 2 mV, respectively, for the α1-PI-SH/α1-PI-SSG couple and of 0.32 ± 0.02 and 29 ± 2 mV, respectively, for the α1-PI-SH/α1-PI-SScys couple. We are unable to detect any change in Trp fluorescence in the complex of α1-PI and elastase when the preformed complex is added to the same GSH/GSSG or cys/cysSScys redox buffers. In the complete absence of reduced thiols, the mixed disulfide, α1-PI-SSG or α1-PISScys, does not form a stable complex with neutrophil elastase but instead acts as a reversible competitive inhibitor of amidolysis [Tyagi, S. C. (1991) *J. Biol. Chem. 266*, 5279–5285]. However, in the presence of 0.015 mM GSH and 0.2 mM GSSG, under conditions in which only 10% of the inhibitor is reduced at equilibrium, all the α1-PI-SSG can be converted to an irreversible complex with neutrophil elastase by participating in disulfide exchange with the glutathione. This disulfide exchange, which can regenerate reduced α1-PI-SH from α1-PI-SSG and GSH with a second-order rate constant of 100 M^{-1} s^{-1}, ensures that all the α1-PI in the plasma may be available for irreversible inhibition of neutrophil elastase, even though only a small fraction may be reduced at equilibrium.

Several proteolytic cascades of physiologic importance are regulated by endogenous antiproteases, notably the family of inhibitors of serine proteases known collectively as serpins (Carrell & Travis, 1985). The most abundant serpin in the circulation is α1-protease inhibitor (α1-PI),[1] which forms quasi-irreversible complexes with a number of serine proteases but which shows greatest selectivity for neutrophil elastase (Carrell et al., 1982; Travis & Salvesen, 1983). The balance between levels of neutrophil elastase and α1-PI is probably pivotal in determining the extent of proteolytic damage to tissues which may accompany the response to inflammatory stimuli (Janoff, 1985; Bieth, 1986; Travis, 1988; Sandborg & Smolen, 1988; Crystal, 1990).

Specificity of serpins for their target proteases is thought to depend on the amino acid sequence within a conformationally constrained loop (residues 350–359), especially the residue occupying the P_1 position (Met^{358} in α1-PI). Modification to the loop sequence due to species difference, genetic variation (Hill et al., 1984), or chemical alteration, as in oxidation of the thioether in Met^{358}, results in changes in specificity and association rate constant (Padrines et al., 1989). The stability of the resulting protease–inhibitor complexes, however, remains remarkably high (Padrines et al., 1989). It has recently been proposed that the expression of inhibitory activity of serpins is a function of the mobility of the extended α-helical reactive center loop (Stein et al., 1990; Carrell et al., 1991). This conformation has been described in hen ovalbumin, but as α-helices are inactive as inhibitors it is unlikely that such a conformation occurs in active inhibitors in vivo. However, more recently it has been concluded, on the basis of a ^1H NMR study, that the extended α-helical loop of serpins is not unusually mobile (Hood & Gettins, 1991). These observations suggest that residues outside the loop may also contribute significantly to the thermodynamics of the stable complex formation.

The single cysteine at position 232 in α1-PI has a thiol group which has been shown to participate in mixed disulfide formation with such plasma constituents as cysteine, glutathione, and other plasma proteins with free thiols, including IgA and myeloma immunoglobulin light chains (Laurell, 1970, 1974; Laurell & Thulin, 1975; Muriani et al., 1978). We have shown that mixed disulfides of α1-PI which have been separated from any free thiols no longer form irreversible complexes with elastase but instead function as reversible competitive inhibitors of the amidolytic activity of elastase (Tyagi, 1991). In vivo, on the other hand, circulating α1-PI would be present in the plasma along with signifiant levels of mixed disulfides between glutathione, cysteine, and other proteins, as well as concentrations of free glutathione and cysteine which have been estimated to be in the range of 10^{-5}

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[1] Abbreviations: α1-PI, α1-protease inhibitor; serpins, serine protease inhibitors; NE, neutrophil elastase; GSH, reduced glutathione (L-glutamyl-L-cyteinylglycine); GSSG, oxidized glutathione; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; Trp, tryptophan; CD, circular dichroism; Cys, reduced cysteine; cysSScys, oxidized cystine; ANM, *N*-[4-(1-anilinonaphthyl)]maleimide.

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M (Laurell et al., 1976; Laurell, 1979; Ellman & Lysko, 1979; Chawla et al., 1984). This mixture of free thiols and mixed disulfides should facilitate disulfide exchange reactions, so that both reduced and mixed disulfide forms of α1-PI would exist in a dynamic equilibrium. Our goal in this study was to determine if such disulfide exchange reactions would affect the apparent inhibitory properties of α1-PI, even when the major portion of the protein was in the form of a mixed disulfide with cysteine or glutathione. We have employed the endogenous fluorescence of α1-PI as a probe to measure the ratio of reduced inhibitor to mixed disulfide in the presence of different ratios of reduced to oxidized thiols. We present evidence in this report that disulfide exchange reactions facilitate complete formation of irreversible complexes between α1-PI and its targeted proteases, even under conditions when as much as 90% of the inhibitor is in the form of mixed disulfide.

MATERIALS AND METHODS

Materials. α1-Protease inhibitor (α1-PI) was obtained from Calbiochem (San Diego, CA). Human neutrophil elastase was obtained from Elastin Products Co. (St. Louis, MO). The purity of both the elastase and α1-PI were checked on SDS–PAGE by silver staining. Trypsin, methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeOSucAAPVpNa), glutathione (GSH), and glutathione disulfide (GSSG) were from Sigma and were used without further purification. The specific activity of elastase was 13 500–15 000 units/mg of protein on the substrate MeOSucAAPVpNa. The specific activity of α1-PI, to inhibit elastase, was measured against MeOSucAAPVpNa as substrate.

Preparation of Mixed Disulfide α1-PI. α1-PI (5 mg/mL) was mixed with 0.01 mM GSH and 10 mM GSSG, or 0.01 mM cys and 10 mM cysSScys, for 12 h at 4 °C in 0.3 M phosphate-buffered saline (PBS), pH 7.4. Excess low molecular weight reagents were removed from the modified protein by microcentrifugation through 0.8- × 10-cm columns of Sephadex G-25 (Penefsky, 1979) previously equilibrated with 0.3 M PBS.

Amidolytic Assays. Amidolytic activity of neutrophil elastase and trypsin was assayed with MeOSucAAPVpNa (Nakajima et al., 1979). Release of *p*-nitroaniline (pNa) was monitored by recording absorbance at 405 nm in a ThermoMax multiwell microplate reader (Molecular Devices, Palo Alto, CA) operating in the kinetic mode. The substrate concentration was 0.6 mM. Neutrophil elastase and trypsin concentrations were 45 nM. All measurements were carried out in 0.3 M PBS buffer, pH 7.4, containing 0.01% Triton X-100 at 22 ± 1 °C.

SDS–PAGE. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on vertical slabs according to a modification of the procedure of Laemmli (1970) using 10% acrylamide/0.3% methylenebis[acrylamide] at pH 8.8 in the absence of any thiol-reducing agents. All solutions contained 0.1% SDS.

Elastase–α1-PI and Trypsin–α1-PI Complexes. The elastase–α1-PI and trypsin–α1-PI complexes were isolated according to a modification of the method of Banda et al. (1988). Reaction mixtures containing protease and α1-PI (1:1.1 mol/mol) were incubated at 37 °C in PBS (pH 7.4) for 15 min for elastase and 1 h for trypsin. The protease and α1-PI ratios were total protein concentrations. The reaction was stopped by addition of PMSF (1 mM). Samples were loaded onto an anion-exchange column (DEAE-50), 2 × 5 cm, equilibrated with 5 mM Tris-HCl (pH 8), and developed with a salt gradient to 0.8 M NaCl. The eluted fractions were recorded for their absorbance at 280 nm. The peaks for complex, free α1-PI, and protease were characterized by SDS–PAGE.

Spectroscopic Methods. Absorption spectra were recorded with a Hewlett-Packard 8452A diode array spectrophotometer. The concentrations of GSH, cys, cysSScys, and GSSG were determined by the method of Ellman and Lysko (1979). The concentrations of a set of native α1-PI standards were determined by absorbance, using a value of $\epsilon^{1\%}_{280nm} = 5.0$ (Laurell et al., 1975) and these samples were then used to generate a standard curve employing the protein assay procedure of Bradford (1976). This standard curve was also used to determine the concentrations of α1-PI-SSG and α1-PI-SScys. Trypsin concentrations were determined by absorbance, using a value of $\epsilon^{1\%}_{280nm} = 14.3$ (Worthington, 1988). Neutrophil elastase concentrations were determined by absorbance, using a value of $\epsilon^{1\%}_{280nm} = 9.85$ (Baugh & Travis, 1976). The concentrations of α1-PI–elastase and α1-PI–trypsin complexes were determined by adding the $\epsilon^{1\%}_{280nm}$ values of α1-PI and elastase and of α1-PI and trypsin, respectively.

Fluorescence spectra were recorded on a computer-controlled Spex Datamate spectrophotometer. The excitation and emission slits were adjusted for 2.5- and 5.0-nm bandpass width, respectively. Spectra were recorded at 1-nm intervals and were corrected for base line and instrument response. Samples were prepared and incubated for appropriate times prior to measurements at 25 °C in 0.3- × 0.3-cm microcells.

Treatment of Fluorescence Data. Intrinsic tryptophan fluorescence in α1-PI, α1-PI-SScys, and α1-PI-SSG was recorded with excitation at 295 nm. Protein concentration was 8.5 μM and the ratio of [GSH]/[GSSG] or [cys]/[cysSScys] was varied in redox titration experiments. The inner filter effects due to protein, GSH, GSSG, cys, and cysSScys were corrected by

$$F = F_{obs}\ \text{antilog}\ [(A_{ex} + A_{em})/2] \quad (1)$$

where F is the corrected fluorescence intensity, F_{obs} is the observed intensity, A_{ex} is the absorbance of the solution at the wavelength of excitation, and A_{em} is the absorbance of the solution at the wavelength of emission (Lakowicz, 1983).

Intrinsic tryptophan fluorescence of α1-PI-SSG and α1-PI-SScys was also recorded as a function of [GSH]/[GSSG] and [cys]/[cysSScys] concentrations, respectively. Since α1-PI and α1-PI-SSG or α1-PI-SScys have emission maxima at 328 and 340 nm, respectively (Tyagi, 1991), we can employ this difference to calculate the concentrations of reduced and mixed disulfide form of α1-PI in GSH/GSSG or cys/cysSScys buffers of different redox potentials by measuring the fluorescence intensity at 328 nm according to

$$[\alpha1\text{-PI-SH}] = \frac{F - F_{ox}}{F_{red} - F_{ox}}[\alpha1\text{-PI}]_{total} \quad (2)$$

where [α1-PI-SH] and $[\alpha1\text{-PI}]_{total}$ are the concentration of the free thiol form of α1-PI at a specific [GSH]/[GSSG] or [cys]/[cysSScys] ratio and the total α1-PI concentration, respectively. F is the corrected fluorescence intensity at λ_{em} = 328 nm of the mixture of reduced and mixed disulfide forms of α1-PI at each specific ratio of [GSH]/[GSSG] or [cys]/[cysSScys], F_{ox} is the intensity of α1-PI-SSG or α1-PI-SScys in the absence of any free thiols, and F_{red} is the intensity of α1-PI in the presence of a large excess of GSH or cys. Each experimental point (F) is the average of at least three independent observations.

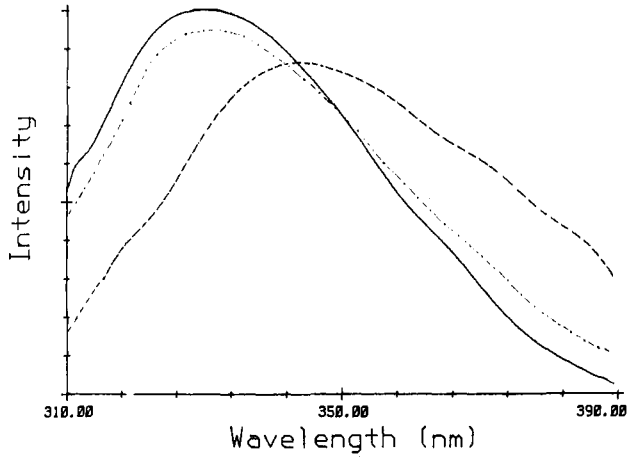


FIGURE 1: Endogenous fluorescence spectra of α1-PI-SH (—), α1-PI-SSG in the absence of low molecular weight thiols (- - -), and α1-PI-SSG which has been partially reduced by incubation for 30 min with a mixture of 0.4 mM GSH and 0.2 mM GSSG [– · –]. The protein concentration was 8.5 μM in 0.3 M PBS at 25 °C. All spectra were obtained with excitation at 295 nm and were corrected for wavelength dependence of excitation energy and for contributions from the buffer and the added glutathione.

RESULTS

Mixed Disulfide Forms of α1-PI. The mixed disulfide forms of α1-PI in which Cys-232 is linked to glutathione (α1-PI-SSG) or to cysteine (α1-PI-SScys) were prepared and freed of low molecular weight thiols as previously described (Tyagi, 1991). The stoichiometry of -SG or -Scys incorporation in α1-PI was determined by titrating isolated α1-PI-SSG or α1-PI-SScys with known concentrations of 5-nitrothiobenzoate [$\epsilon_{412nm} = 13\,600\ M^{-1}\ cm^{-1}$ (Silverstein, 1975)]. The decrease in absorbance of 412 nm was used to calculate the number of -SG or -Scys molecules linked to α1-PI. Our results indicate that 0.87 ± 0.05 and 0.92 ± 0.07 mol of -SG or -Scys, respectively, were incorporated per mole of α1-PI.

Equilibrium between Reduced and Mixed Disulfide Forms of α1-PI. The endogenous fluorescence from the two tryptophan residues (Trp-194 and Trp-238) in α1-PI-SH and α1-PI-SSG is shown in Figure 1. The emission maximum of the reduced inhibitor is at 328 nm, while the emission maximum of the mixed disulfide, α1-PI-SSG or α1-PI-SScys, is shifted about 12 nm to the red, to 340 nm. This shift suggests that at least one of the two tryptophan indole rings is more accessible to the aqueous solvent in the mixed disulfide. The red shift could be reversed in the presence of excess reduced glutathione. We have previously observed similar red shifts in tryptophan fluorescence of α1-PI when the cysteine thiol has been modified by alkylation (Tyagi, 1991). In Figures 2 and 3, we have employed this shift in fluorescence to monitor the ratio of reduced to mixed disulfide forms of α1-PI as a function of the ratio of reduced to oxidized free thiols (GSH to GSSG or cys to cysSScys) in a series of redox buffers. This indicates that α1-PI undergoes reversible conformational changes at physiological concentrations of reduced and oxidized free thiols (Cantin, et al., 1987). A plot of the corrected relative fluorescence is displayed as a function of the ratio of [GSH]/[GSSG] in Figure 2 and of [cys]/[cysSScys] in Figure 3. If the ratio of reduced to oxidized free thiols is regulating the ratio of reduced to mixed disulfide forms of α1-PI by a simple disulfide exchange reaction, then we would expect that the fraction of free α1-PI would be related to the [GSH]/[GSSG] ratio by

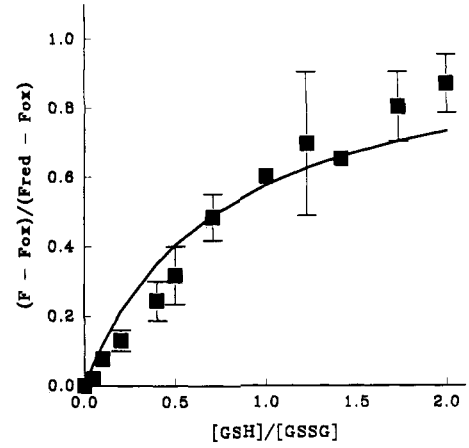


FIGURE 2: Fractional change in emission at 328 nm from tryptophans in mixtures of α1-PI-SH and α1-PI-SSG produced by incubation with redox buffers containing 0.2 mM GSSG and increasing concentrations of GSH, presented as a function of the ratio of GSH to GSSG in the buffers. All measurements were obtained under conditions as in Figure 1. Each data point is the average of at least three independent experiments. The error bars indicate the deviations in the average value. The solid line is the nonlinear least-squares fit of all data points to the equation $(F - F_{ox})/(F_{red} - F_{ox}) = ([GSH]/[GSSG])/\{K_{eq} + ([GSH]/[GSSG])\}$.

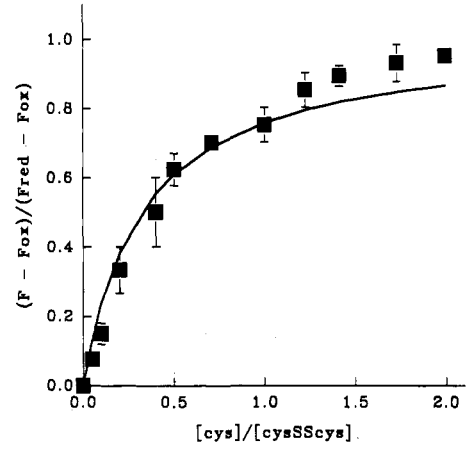


FIGURE 3: Fractional change in emission at 328 nm from tryptophans in mixtures of α1-PI-SH and α1-PI-SScys produced by incubation with redox buffers containing 0.2 mM cystine and increasing concentrations of cystine, presented as a function of the ratio of cys to cysSScys in the buffers. All measurements were obtained under conditions as in Figure 1. Each data point is the average of at least three independent experiments. The error bars indicate the deviations in the average value. The solid line is the nonlinear least-squares fit of all data points to the equation $(F - F_{ox})/(F_{red} - F_{ox}) = ([cys]/[cysSScys])/\{K_{eq} + ([cys]/[cysSScys])\}$.

$$\alpha1\text{-PI-SH} + GSH \rightleftharpoons \alpha1\text{-PI-SSG} + 2H^+ + 2e^-$$

$$GSSG + 2H^+ + 2e^- \rightleftharpoons 2GSH$$

which combine to give

$$\underset{\lambda_{em} = 328\ nm}{\alpha1\text{-PI-SH}} + GSSG \overset{K_{eq}}{\rightleftharpoons} \underset{\lambda_{em} = 340\ nm}{\alpha1\text{-PI-SSG}} + GSH$$

$$\frac{[\alpha1\text{-PI-SH}]}{[\alpha1\text{-PI}]_{tot}} = \frac{[GSH/GSSG]}{K_{eq} + ([GSH]/[GSSG])} \quad (3)$$

The lines in Figures 2 and 3 represent the fits of the data to eq 3 or its counterpart with cys/cysSScys. Fits of the data to expressions involving [GSH] alone, [GSSG] alone, or

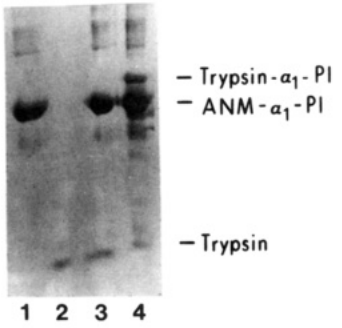


FIGURE 4: SDS–PAGE of mixtures of trypsin with reduced and mixed disulfide forms of α1-PI. Lane 1, ANM-α1-PI; lane 2, trypsin; lane 3, 2 μM trypsin + 6 μM ANM-α1-PI incubated for 5 min at 37 °C before loading on the gel; lane 4, complex between 6 μM α1-PI and 4 μM trypsin after 12-h subsequent reaction with 10 μM ANM.

[GSH][2] are not as good as those to eq 3. From the best fit of the data to eq 3, we have calculated a value of $K_{eq} = 0.74 \pm 0.08$ for glutathione and 0.32 ± 0.02 for cysteine (Segal, 1976).[2] This indicates that the value of E'_0 for the α1-PI-SSG/α1-PI-SH couple is only 8 mV more positive than the value of –0.24 V reported for the GSSG/GSH couple (Scott et al., 1963; Rest & Rappaport, 1964). Similarly, the value of E'_0 for the α1-PI-SScys/α1-PI-SH couple is only 29 mV more positive than the value of –0.22 V reported for the cys/cysSScys couple (Jocelyn, 1967).

Previously, we have shown that the preformed complex of elastase and α1-PI retains a reactive thiol and modification of this thiol does not dissociate the complex (Tyagi, 1991). In Figure 4 we show similar results for the complex of trypsin and α1-PI. It is clear from Figure 4 that ANM-α_1-PI (thiol-modified α1-PI) does not form a complex with trypsin which is stable under conditions of SDS–PAGE. The preformed trypsin–α1-PI complex, however, retains a reactive thiol and subsequent modification of this thiol in trypsin–α1-PI does not dissociate this complex (Figure 4). Also, in Figure 4 we observed a major single band at 53 kDa for α1-PI. The high molecular weight bands in lanes 1, 3, and 4 are probably due to higher polymers of α1-PI. At present we do not know the nature of these polymers. The extra band in lane 4 is due to the irreversible complex formation between α1-PI and trypsin. The bands lower than 53 kDa in lanes 1, 3, and 4 are due to the fact that a small amount of α1-PI can be cleaved by free trypsin, leaving a 48-kDa inactive α1-PI fragment. The extra low molecular bands in lane 4 are probably due to the slow degradation of ANM-α1-PI by trypsin, since ANM-α_1-PI becomes an inhibitor to a competitive substrate. In order to measure the reduction potential of the thiol of α1-PI in the presence of elastase, we have measured the fluorescence arising from tryptophan residues in the α1-PI–elastase complex (5 μM) as a function of the ratio of [GSH]/GSSG], which was varied from 0.001 to 5 under conditions similar to those shown in Figure 2. In spite of the retention of the reactive thiol in the enzyme–inhibitor complex, we observed no significant change in the fluorescence (data not shown). This may indicate that disulfide exchange in the elastase–α1-PI complex does not induce conformational changes in the complex.

Effect of [GSH]/[GSSG] on the Inhibition of Amidolytic Activity of Neutrophil Elastase by Mixed Disulfide Forms of α1-PI. In order to study the effect of disulfide exchange between α1-PI-SH and α1-PI-SSG on the functional properties of the inhibitor, we first examined the effect of addition of a glutathione redox buffer to α1-PI-SSG on the kinetics of inhibition of the amidolytic activity of human neutrophil elastase. In Figure 5, the amidolytic activity of 45 nM neutrophil elastase after addition to a mixture of 0.6 mM MeOSucAAPVpNA and 270 nM α1-PI is illustrated. Under these conditions in which inhibitor and substrate compete for the enzyme, if the α1-PI is fully reduced, the amidolytic activity is irreversibly inhibited with a $t_{0.5}$ of about 3 min. On the other hand, if the inhibitor is stoichiometrically converted to the mixed disulfide, α1-PI-SSG, in the absence of free thiols, the amidolytic activity is diminished in a fashion which we have previously shown to be consistent with reversible competitive inhibition (Tyagi, 1991). If a mixture of 15 μM GSH and 200 μM GSSG is added to the ongoing competitively inhibited amidolytic reaction mix of elastase, substrate, and α1-PI-SSG, the inhibition kinetics are changed dramatically as shown in Figure 5. The curve in the control line in Figure 5 resulted from substrate consumption and does not reflect enzyme instability under the assay conditions we have employed. On the basis of the redox equilibrium for α1-PI and glutathione presented above, we can calculate that this ratio of GSH to GSSG will convert no more than 10% of the α1-PI-SSG to its reduced form at equilibrium, yet we observe that all the elastase is irreversibly inactivated by the α1-PI in the presence of this redox buffer. Under these conditions, the $t_{0.5}$ for irreversible inhibition is extended to 10–15 min. The most straightforward explanation for the results presented in Figure 5 is that only the reduced form of α1-PI can form an irreversible complex with neutrophil elastase, but in the presence of the redox buffer, all the α1-PI can be shuttled through disulfide exchange to the reduced form which will then be complexed with the enzyme.

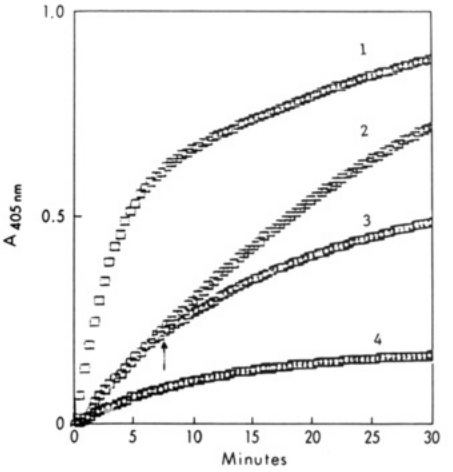


FIGURE 5: Inhibition of neutrophil elastase-catalyzed amidolysis by α1-PI-SH, α1-PI-SSG freed of low molecular weight thiols, and α1-PI-SSG to which a redox buffer containing GSH and GSSG has been added. (1) 45 nM neutrophil elastase was added to 0.6 mM MeOSucAAPVpNA containing a redox buffer of 0.015 mM GSH and 0.2 mM GSSG; (2) 45 nM elastase was added to 0.6 mM MeOSucAAPVpNA plus 270 nM α1-PI-SSG in the absence of low molecular weight thiols; (3) 45 nM elastase was added to 0.6 mM MeOSucAAPVpNA plus 270 nM α1-PI-SSG in the absence of low molecular weight thiols as in (2) and after 7.5 min, as indicated by the arrow, the redox buffer of 0.015 mM GSH and 0.2 mM GSSG was added; (4) 45 nM elastase was added to 0.6 mM MeOSuc-AAPVpNA plus 270 nM α1-PI-SH. All reactions were carried out in 0.3 M PBS, pH 7.4, containing 0.01% Triton X-100 at 25 °C, and release of *p*-nitroaniline was followed by monitoring absorbance at 405 nm.

Kinetics of Disulfide Exchange in α1-PI in the Presence of Neutrophil Elastase. As an alternative approach to demonstrating the role of disulfide exchange in maintaining

[2] The value of $\Delta E_0'$ is calculated according to the relationship $\Delta E_0' = 0.059/n \log (1/K_{eq})$, where n is the number of electrons transferred.

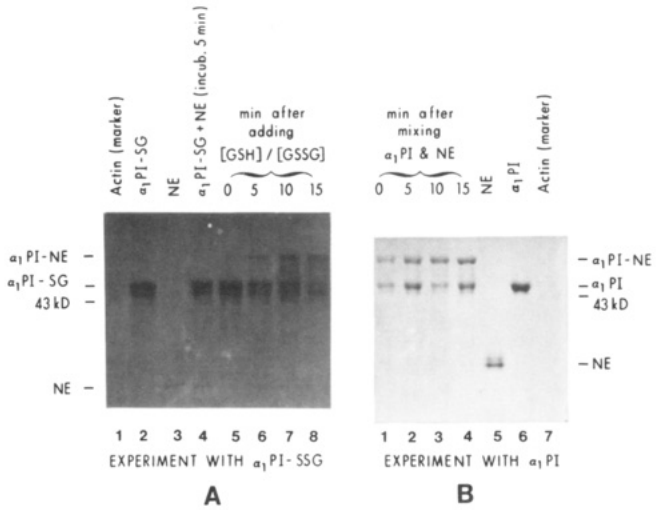


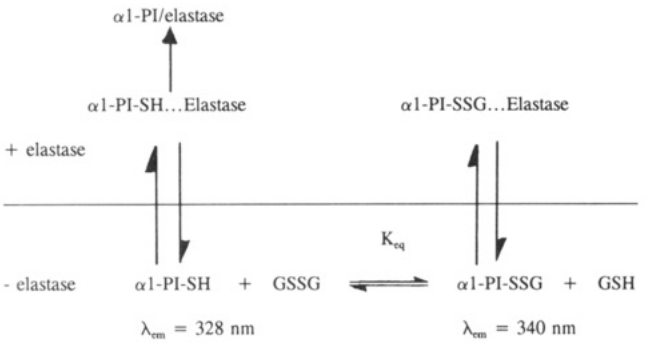
FIGURE 6: (Panel A) SDS–PAGE of mixtures of α1-PI-SSG and neutrophil elastase in the absence and presence of low molecular weight thiols. Lane 1 shows the position of a marker of 43 kDa (actin). The position of the α1-PI–elastase complex at 78 kDa is indicated by α1-PI-NE. The positions at 53 and 29 kDa are indicated by α1-PI and NE, respectively. Lane 2 contained 2.21 μg of α1-PI-SSG. Lane 3 contained 1.02 μg of neutrophil elastase. Lane 4 contained 2.04 μg of elastase and 4.42 μg of α1-PI-SSG in the absence of low molecular weight thiols. Lanes 5–8 contained 10-μL aliquots removed 0, 5, 10, and 15 min after addition of 6.8 μM elastase to 8.5 μM α1-PI-SSG in 0.015 mM GSH plus 0.2 mM GSSG. All mixtures were prepared in 0.3 M PBS, pH 7.4, containing 0.01% Triton X-100 at 37 °C. To prevent degradation of α1-PI by free neutrophil elastase, 1 mM diisopropyl fluorophosphate (DFP) was added to each aliquot prior to addition of SDS–PAGE sample buffer. (Panel B) Results obtained with α1-PI-SH. Lanes 1–4 contained 10-μL aliquots removed 0, 5, 10, and 15 min after addition of 6.8 μM elastase to 8.5 μM α1-PI-SH in the absence of low molecular weight thiols. Lane 5 contained 1.02 μg of elastase. Lane 6 contained 2.21 μg of α1-PI-SH. Lane 7 shows the position of a marker of 43 kDa (actin).

a dynamic equilibrium between α1-PI-SH and α1-PI-SSG, we have added neutrophil elastase to separate preparations of α1-PI-SH and α1-PI-SSG which have been freed of low molecular weight thiols and to α1-PI-SSG in the presence of the same redox buffer of 15 μM GSH and 200 μM GSSG employed in the amidolysis studies. We have then examined the mixtures on SDS–PAGE, as shown in Figure 6. Whereas a 78-kDa complex between reduced α1-PI and elastase is detected on SDS gels even if the mixture of inhibitor and enzyme is transferred to the sample buffer within a few minutes of preparation, no stable complex between α1-PI-SSG and elastase can be seen to form over 15 min of incubation in the absence of free thiols. The formation of a stable complex can be easily detected over time, however, if the enzyme is added to the inhibitor in the redox buffer of GSH and GSSG. The prevention of degradation of α1-PI by DFP treatment suggested that not all the material is in active conformation capable of forming 1:1 enzyme/inhibitor complexes. Some of these conformations can be proteolytically inactivated by free enzyme by cleavage at the Met^{358}–Ser^{359} bond in α1-PI, leaving a 48-kDa inactive fragment. As shown in Figure 6, panel A, α1-PI migrates as two bands reflecting active inhibitor at 53 kDa and inactive inhibitor at 48 kDa. We have obtained a semiquantitative measurement of the kinetics of formation of the stable complex of α1-PI–NE (78 kDa) by densitometry. The densitometric scans of the 78-kDa band corresponding to the α1-PI–elastase complex from Figure 6 (panel A), lanes 5–8, were plotted as a function of time. A nonlinear least-squares fit of the data to a second-order reaction scheme was performed. The $t_{0.5}$ for approach of the 78-kDa band to its maximum density is 8–10 min. According to our hypothesis, the rate of generation of the 78-kDa band is a reflection of the reduction of α1-PI-SSG by GSH in the redox buffer, followed by the formation of a nondissociable α1-PI–elastase complex. Since the formation of the complex is controlled by an extraordinarily rapid second-order rate constant, which has been estimated to be on the order of 10^7 M^{-1} s^{-1} (Travis & Salvesen, 1983; Padrines et al., 1989), we can assume that the rate-limiting step is the reduction by glutathione, for which we compute an apparent second-order rate constant, $k = 0.693/(t_{0.5}[GSH])$, of approximately 10^2 M^{-1} s^{-1}.

DISCUSSION

Examination of the crystallographic structure of α1-PI indicates that the single cysteinyl residue in α1-PI is protected in a crevice (Loeberman et al., 1984). This interpretation is supported by the finding that SS-linked dimers do not occur in α1-PI nor are any complexes formed with albumin, the most abundant free-thiol-containing protein in plasma (Laurell, 1979). However, the terminal cysteines of the k-type light chains of immunoglobulins and the penultimate cysteine of the heavy chains of IgA, which are more fully exposed, can form SS-linked complexes with α1-PI (Laurell, et al., 1976). The exchange of cys between α1-PI and free cystine in dog plasma has been measured in vivo (Block et al., 1969; Pierce et al., 1976; Laurell, 1979). However, no functional studies of the properties of mixed disulfide forms of α1-PI have been previously reported.

We have shown that formation of a mixed disulfide between free thiol and Cys-232 in α1-PI induces conformational changes in the protein as measured by a change in the environment of one or both of the Trp residues. These conformational changes are reversed upon reduction of the disulfide. We have employed the endogenous fluorescence from the tryptophans of α1-PI as a probe to determine the redox equilibrium for disulfide formation, which we have found is close to that for the GSH/GSSG and cys/cysSScys couples. These observations indicate that a dynamic equilibrium between the free thiol and disulfide-bridged forms of α1-PI could exist in the presence of physiological concentrations of reduced and oxidized thiols. The kinetics of disulfide exchange in α1-PI catalyzed by glutathione are in good agreement with rates observed for other plasma proteins (Lash & Jones, 1985) and support the conclusion that formation of irreversible complexes (possibly an acyl-bonded complex; Matheson et al., 1991) between α1-PI and neutrophil elastase could take place through the free thiol form of the inhibitor in vivo as well as in vitro, even in an environment in which the oxidized form of glutathione predominates.



The possiblity that elastase can influence the reduction potential of the thiol in α1-PI by forming a quasi-irreversible complex (SDS–PAGE-stable, tight complex between elastase

and α1-PI), thereby fully or even partially protecting the thiol from oxidation, has been effectively ruled out in our previous study, in which we showed that the elastase–α1-PI complex retains a reactive thiol which can undergo mixed disulfide formation with free thiols (Tyagi, 1991). We have also shown that the mixed disulfide form of α1-PI becomes a reversible inhibitor of neutrophil elastase (Tyagi, 1991). However, we observed no change in the tryptophan fluorescence upon mixed disulfide formation in the preformed α1-PI–elastase complex. This indicates that the quasi-irreversible complex of elastase and α1-PI does not change its conformation as a function of mixed disulfide formation. This distinction between the conformational flexibilities of free α1-PI and its complexes with proteases is consistent with the conclusions of Mast el al. (1991) that different properties of free α1-PI, its complexes with proteases, and its cleaved inactivated form are reflections of three different conformations of the protein. Also, Bruch et al. (1988) have shown by fluorescence and circular dichroism (CD) spectrosopy that intact α1-PI has a more open extended conformation whereas the proteolytically cleaved form of α1-PI (a 48-kDa fragment) as well as the complexes with proteases have more compact conformations.

A study on the distribution of thiols in rat plasma (Lash & Jones, 1985) has demonstrated that approximately 70% of the total plasma glutathione is linked by mixed disulfides with proteins, with another 30% of the nonprotein fraction in the form of GSSG or the mixed disulfide with cysteine. While only 23% of the total plasma cysteine is in the form of mixed disulfides with plasma proteins, only 5% of the nonprotein fraction is in the form of the free thiol, with the bulk in the form of cystine (CysSSCys) or the mixed disulfide with glutathione. Thus, the distribution between reduced and oxidized thiols in the plasma is strongly in favor of the disulfides, in contrast with the preponderance of reduced thiols in the cytosol [estimated at 95% (Tietze, 1969)], which is maintained through the action of glutathione reductase. Upon addition of exogenous GSH to rat plasma, the glutathione equilibrated with plasma proteins through mixed disulfide formation with a $t_{0.5}$ of 4 min (Lash & Jones, 1985).

The total thiol concentration in human plasma has been variously estimated to lie in the range of 400–600 μM (Ellman & Lysko, 1979; Chawala et al., 1984), the bulk of which is also in the form of mixed disulfides with plasma proteins, especially albumin. Mixed disulfide bond formation involving the Cys-34 residue of serum albumin has been shown to alter the ability of this protein to bind metal ions (Inoue, 1989), organic dyes (Inoue et al., 1985), and fatty acids (Takabayashi et al., 1983). It has been proposed that such alterations in function of the plasma proteins may be a reflection of a more comprehensive mechanism of buffering against oxidant stress in which the total thiol levels are maintained by hepatic synthesis, renal excretion, and redistribution within the plasma via disulfide exchange reactions (Inoue, 1989). In this report we provide direct experimental evidence for the induction of a conformational change in a plasma protein by mixed disulfide bond formation. We would expect that similar conformational changes should be detected in the other plasma proteins in which alterations in functional properties have been shown to arise as a result of mixed disulfide bond formation. Such measurements should also facilitate determination of the redox potential for these other proteins. The conformational changes induced by mixed disulfide bond formation will only occur if the proteins have a cysteine residue which is accessible for bond formation. Furthermore, such changes will only be apparent in other serpins if the tryptophan residues are highly conserved.

The results reported here demonstrated that the disulfide exchange in α1-PI under conditions of an overall redox poise which favors the formation of mixed disulfides will still generate sufficient reduced α1-PI so that all the inhibitor will react irreversibly with elastase. Moreover, such disulfide exchange reactions may have other implications for α1-PI function, especially in the interstitium, where local oxidant stress may lead to changes in the overall distribution of oxidized and reduced thiols.

The significance of a high GSH/GSSG ratio in the airway lining fluid of lung (Cantin et. al., 1987) ensures maximum availability of the rapidly reacting form of α1-PI even when elastase levels rise during inflammatory processes. The GSH/GSSG gradient across the interstitium from low in plasma to high in airway suggests that disulfide exchange can occur across the entire interstitial zone (Cantin et al., 1987).

REFERENCES

Banda, M. J., Rice, A. G., Griffin, G. L., & Senior, R. M. (1988) *J. Exp. Med. 167*, 1608–1615.

Baugh, R. J., & Travis, J. (1976) *Biochemistry 15*, 836–841.

Bieth, J. G. (1986) in *Biology of Extracellular Matrix: Regulation of Matrix Accumulation* (Mecham, R. P., Ed.) pp 217–232, Academic Press, Orlando, FL.

Block, W. D., Markovs, M., & Steele, B. (1969) *Am. J. Clin. Nutr. 22*, 33.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bruch, M., Weiss, V., & Engel, J. (1988) *J. Biol. Chem. 263*, 16626–16630.

Cantin, A. M., North, S. L., Hubbard, R. C., & Crystal, R. G. (1987) *J. Appl. Physiol. 63* (1), 152–157.

Carrell, R. W., & Travis, J. (1985) *Trends Biochem. Sci. 10*, 20.

Carrell, R. W., Jeppsson, J.-O., Laurell, C.-B., Brennan, S. O., Owen, M. C., Vaughan, L., & Boswell, D. R. (1982) *Nature 298*, 329–334.

Carrell, R. W., Evans, D. L., & Stein, P. E. (1991) *Nature 353*, 576–578.

Chawla, R. K., Lewis, F. W., Kutner, M. H., & Bate, D. M. (1984) *Gastroenterology 87*, 770–776.

Crystal, R. G. (1990) *J. Clin. Invest. 85*, 1342–1352.

Ellman, G., & Lysko, H. (1979) *Anal. Biochem. 93*, 98–102.

Hood, D. B., & Gettins, P. (1991) *Biochemistry 30*, 9054–9060.

Hill, R. E., Shaw, P. H., Boyd, P. A., Baumann, H., & Hastie, N. D. (1984) *Nature 311*, 175–177.

Inoue, M. (1989) in *Glutathione: Chemical, Biochemical and Medical Aspects* (Dolphin, D., Avramovic, O., & Poulson, R., Eds.) Part B, pp 613–644, Wiley, New York.

Inoue, M., Koyama, H., Nagase, S., & Morino, Y. (1985) *J. Lab. Clin. Med. 105*, 484–488.

Janoff, A. (1985) *Annu. Rev. Med. 36*, 207–216.

Jocelyn, P. C. (1967) *Eur. J. Biochem. 2*, 327–331.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum, New York.

Lash, L. H., & Jones, D. P. (1985) *Arch. Biochem. Biophys. 240*, 583–592.

Laurell, C. B. (1970) *Immunochemistry 7*, 461–465.

Laurell, C. B. (1974) *Immunochemistry 11*, 703–709.

Laurell, C. B. (1979) in *The Chemistry and Physiology of the Plasma Proteins*: Proceedings of Conference (Bing, D. M., Ed.) pp 329–341, Pergamon, New York.

Laurell, C. B., & Thulin, E. (1975) *Scand. J. Immunol. 4* (Suppl. 2), 7–12.

Laurell, C.-B., Pierce, J., Persson, U., & Thulin, E. (1975) *Eur. J. Biochem. 57*, 107–113.

Laurell, C. B., Grubb, A., & Thulin, E. (1976) *La Recerca Clin. Med. 6* (Suppl. 3), 57.

Loeberman, H., Tokuoka, R., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 177*, 531–556.

Mast, A. E., Enghild, J. J., Pizzo, S. V., & Salvesen, G. (1991) *Biochemistry 30*, 1723–1730.

Matheson, N. R., Van Halbeek, H., & Travis, J. (1991) *J. Biol. Chem. 266*, 13489–13491.

Muriani, P., Lauriola, L., & Piantelli, M. (1978) *Clin. Chim. Acta 85*, 61–66.

Nakajima, K., Powers, J. C., Ashe, B., & Zimmerman, M. (1979) *J. Biol. Chem. 254*, 4027–4032.

Padrines, M., Schneider-Pozzer, M., & Bieth, J. G. (1989) *Am. Rev. Respir. Dis. 139*, 783–790.

Penefsky, H. S. (1979) *J. Biol. Chem. 254*, 2891–2899.

Pierce, J. A., Jeppsson, J.-O., & Laurell, C.-B. (1976) *Anal. Biochem. 74*, 227–241.

Rost, J., & Rappaport, S. (1964) *Nature 201*, 185.

Sandborg, R. R., & Smolen, J. E. (1988) *Lab. Invest. 59*, 300–320.

Scott, E. M., Duncan, I. W., & Ekstrand, V. (1963) *J. Biol. Chem. 238*, 3928–3933.

Segel, I. H. (1976) in *Biochemical Calculations*, pp 172–174, Wiley, New York.

Silverstein, R. (1975) *Anal. Biochem. 63*, 281–282.

Stein, P. E., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. J., & Carrell, R. W. (1990) *Nature 347*, 99–102.

Takabayashi, K., Imada, T., Saito, Y., & Inada, Y. (1983) *Eur. J. Biochem. 136*, 291–295.

Tietze, F. (1969) *Anal. Biochem. 27*, 502–522.

Travis, J. (1988) *Am. J. Med. 84* (Suppl. 6A), 37–42.

Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.

Tyagi, S. C. (1991) *J. Biol. Chem. 266*, 5279–5285.

Worthington, C. C. (1988) *Worthington Enzyme Manual*, p 320, Worthington, Freehold, NJ.

Registry No. GSH, 70-18-8; GSSG, 27025-41-8; Cys, 52-90-4; cysSScys, 56-89-3; elastase, 9004-06-2; trypsin, 9002-07-7.