The Reactivity and Oxidation Pathway of Cysteine 232 in Recombinant Human α1-Antitrypsin*

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Abstract

Oxidative damage to the sulfur-containing amino acids, methionine and cysteine, is a major concern in biotechnology and medicine. α1-Antitrypsin, which is a metastable and conformationally flexible protein that belongs to the serpin family of protease inhibitors, contains nine methionines and a single cysteine in its primary sequence. Although it is known that methionine oxidation in the protein active site results in a loss of biological activity, there is little specific knowledge regarding the reactivity of its unpaired thiol, Cys-232. In this study, the thiol-modifying reagent NBD-Cl (7-chloro-4-nitrobenz-2-oxa-1,3-diazole) was used to label peroxide-modified α1-antitrypsin and demonstrate that the Cys-232 in vitro oxidation pathway begins with a stable sulfenic acid intermediate and is followed by the formation of sulfonic and cysteic acid in successive steps. pH-dependent reactivity with hydrogen peroxide showed that Cys-232 has a pKₐ of 6.86 ± 0.05, a value that is more than 1.5 pH units lower than that of a typical protein thiol. pH-induced conformational changes in the region surrounding Cys-232 were also examined and indicate that mildly acidic conditions induce a conformation that enhances Cys-232 reactivity. In summary, this work provides new insights into α1-antitrypsin reactivity in oxidizing environments and shows that a unique structural environment renders its unpaired thiol, Cys-232, its most reactive amino acid.

A common problem in the biotechnology industry is the degradation of protein therapeutics by chemical modification (1). Oxidation is one of the most prevalent forms of chemical modification, and the sulfur-containing amino acids, methionine and cysteine, are susceptible to modification by a wide array of oxidants (1). In vivo, oxidation of these residues can be beneficial for modulation of biological activity. In vitro, however, oxidation is aberrant, and in the case of proteins produced for therapeutic purposes can lead to the degradation of an otherwise highly valuable and useful product. Therefore, it is important to understand amino acid reactivity not only within the context of physiological functioning but also within the context of in vitro stability.

α1-Antitrypsin is the archetypal member of the serine protease inhibitor (serpin) superfamily of plasma protease inhibitors (2). Other members of this medically and biologically important family include antithrombin III, plasminogen activator inhibitor-1, C1-inhibitor, and α1-antichymotrypsin (3). α1-Antitrypsin, which contains nine methionine residues and a single unpaired cysteine, has a primary physiological role of regulating the activity of human neutrophil elastase, a serine protease involved in the degradation of connective tissue components (4). This protease inhibitor is exposed to mildly oxidizing conditions while circulating in the blood plasma and to a greater extent at sites of inflammation where its activity is essential for mitigating the extent of proteolytic tissue damage that may accompany the inflammatory response.

Oxidation of either methionine 351 (Met-351) or methionine 358 (Met-358), the two active-site methionine residues of α1-antitrypsin, results in a significant loss of inhibitory activity against neutrophil elastase (5). This loss of inhibitory activity in the lungs of smokers is thought to be caused by oxidants present in cigarette smoke (6) and contributes to the pathology of pulmonary emphysema (7). Although the unquestionable physiological importance of methionine oxidation in α1-antitrypsin has led to intense study of this particular oxidation reaction (5, 8, 9), the unpaired thiol of the protein, cysteine 232 (Cys-232), is also susceptible to oxidation at neutral pH (10). In addition, this residue is reactive under physiological conditions with proteins and small molecules such as cysteine, glutathione, myeloma immunoglobulin light chains (11, 12), immunoglobulin A (13), and nitric oxide (14). However, neither disulfide-linked α1-antitrypsin dimers nor disulfide-linked complexes between α1-antitrypsin and albumin, the most abundant free-thiol-containing protein in human blood plasma, have been found (15). Based on these observations it is clear that in environments containing oxidants and sterically compatible disulfides, Cys-232 is capable of undergoing many forms of covalent modification, the significance of which is only beginning to be elucidated (14, 16).

Although modification of Cys-232 may have physiological implications and is extremely important with regard to in vitro degradation, there have been no studies aimed at understanding the biochemical basis for the reactivity of this residue. However, there is an existing knowledge base pertaining to cysteine reactivity from which to begin an investigation. Recent work has described the role of sulfenic acid, the initial oxidation product formed when cysteine is exposed to an oxidant, in enzyme catalysis and redox regulation (17–20). These studies have been facilitated by the development of analytical techniques capable of identifying this unstable oxidation intermediate (21). In this report, the Cys-232 oxidation pathway is determined by these and other techniques that exploit the biochemical properties of cysteine oxidation products. After establishing the Cys-232 oxidation pathway, its pH-dependent local structural environment are examined. These studies show that α1-antitrypsin possesses a unique structural environment around Cys-232 that confers a high degree of reactivity across a broad pH range. Furthermore, it is shown that in environments containing oxidants...
that the α1-antitrypsin unpaired thiol is the protein’s most oxidation susceptible residue.

MATERIALS AND METHODS

Expression and Purification—Recombinant human α1-antitrypsin harboring the plasmid pEAT8 (22) was expressed in Escherichia coli BL21(DE3). Following protein expression and cell harvest, the soluble protein fraction was purified as previously described (Griffiths, 2001 492).

Site-directed Mutagenesis—A cysteine 232 to serine mutant (C232S) was constructed as previously described (10).

Isoelectric Focusing—Isoelectric focusing samples were run on Bio-Rad IEF Ready Gels (pH 5–8) using Bio-Rad Ready Gel cell apparatus. A continuous, non-denaturing pH gradient was run in the following manner: 60 min at 100 V, 60 min at 250 V, and 30 min at 500 V. Ampholytes were removed by fixing the gel in 30% methanol, 10% trichloroacetic acid, 3.5% sulfosalicylic acid for 1 h, followed by >2 h in 30% methanol, 12% trichloroacetic acid. Gels were stained using Bio-Rad Silver Stain Plus. Pharmacia Broad pl calibration kit was used for pl estimation.

Buffer Preparation for Oxidation Experiments—All buffers were prepared with 20 mM buffer salt and adjusted to 100 mM ionic strength with NaCl. The following buffering species were used for the pH range of 5–10: pH 5.0 (acetate), pH 5.5–6.5 (MES), pH 7.0–7.5 (phosphate), pH 8.0–8.5 (Tris), pH 9.0–9.5 (TAPS), pH 10.0 (CAPS). Compensation was made for the effect of temperature and ionic strength according to Beynon and Easterby (23).

In Vitro Oxidation for Determination of Thiol pKₐ—Highly purified recombinant α1-antitrypsin was desalted into oxidation buffer and equilibrated at 25 °C. Oxidation reactions were carried out in 30.8% H₂O₂ diluted to 0.2–1.0 mM. A 2 mg/ml bovine catalase stock (Sigma catalog no. C-40) was prepared in 1× phosphate-buffered saline prepared from 10× premixed phosphate-buffered saline (Roche Molecular Biochemicals). At various times after oxidation was initiated, 90 μl of the bovine catalase stock was added to rapidly quench the oxidation reaction. Samples were then desalted into 1× phosphate-buffered saline using PD-10 columns (Amersham Biosciences).

Thiol Modification with NBD-Cl—A 1 mg/ml stock solution of 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) was prepared in dimethyl sulfoxide. A 40-fold molar excess of NBD-Cl (NBD-Cl/α1-antitrypsin) was added to each oxidized and desalted sample. The thiol modification reaction was allowed to proceed at 25 °C for 45 min. The extent of NBD labeling was determined by absorbance using a molar extinction coefficient (ε₉₀) of 26,000 M⁻¹ cm⁻¹ for the IANBD-thiol adduct (25). Fluorescence emission spectra of samples that had been desalted in buffers of various pH values were collected using a Hitachi F-4500 spectrophotometer with excitation and emission slit widths both set to 5 nm and PMT voltage set to 950 V. Samples were excited at 480 nm, and emission spectra were collected from 500 to 600 nm.

RESULTS

Identification of Cys-232 Sulfenic Acid—The pathway for thiol oxidation by hydrogen peroxide begins with the formation of sulfinic acid (Fig. 1). Small molecules with unpaired thiol groups that are oxidized to sulfinic acid rapidly condense to form intermolecular disulfides (26). In proteins, however, steric hindrance may prevent an oxidized thiol from reacting with a second thiol to form a disulfide. In this case, a sulfinic acid intermediate is formed. Because sulfinic acid is reactive as a nucleophile (26), further exposure to hydrogen peroxide leads to the irreversible formation of sulfonic acid (Cys-SO₃H). It was important to identify whether the Cys-232 location within the protein scaffold makes it a potential site for formation of these acid thiold species. Because of inherent instability, sulfinic acid intermediates usually have extremely short half-lives and are therefore difficult to identify (27). However, Ellis and Poole (21) showed that reaction between NBD-Cl and a protein thiol oxidized to sulfinic acid leads to the covalent incorporation of a spectrscopically detectable sulfinic acid conjugate (Cys-S(O)-NBD). Sulfinic acid conjugates have absorption maxima at 347 nm, whereas reduced thiol conjugates (Cys-S-NBD) have absorption maxima at 420 nm (24). This allows one to readily distinguish between the two species. As shown in Fig. 2, reacting α1-antitrypsin with 0.2 mM hydrogen peroxide at 25 °C for 20 min followed by immediate incubation with NBD-Cl shifts the absorption maximum of NBD-labeled α1-antitrypsin from 420 to 347 nm. These spectral data are consistent with Cys-232 oxidation to sulfenic acid.

Identification of Cys-232 Sulfonic Acid— Oxidation of a protein thiol to sulfonic acid is followed by the formation of either a disulfide bond or, upon further exposure to oxidant, sulfonic acid. Previous studies used peptide mapping to show that sulfonic and cysteic acids are Cys-232 oxidation products (10). However, it was not clear whether these species were formed from sulfenic acid in the native state or by air oxidation of sulfenic acid during the peptide mapping procedure. Here we use isoelectric focusing to address this issue. The pKₐ values of sulfinic acid and cysteic acid are both less...
than 2 (28, 29). Therefore, when a surface-exposed protein thiol is oxidized to either of these species, the net surface charge and isoelectric point (pI) of the protein are altered. This makes it possible to use isoelectric focusing to identify the formation of protein sulfenic and cysteic acids. Fig. 3 shows that exposure of α1-antitrypsin to the high concentration of hydrogen peroxide (20 mM) required to generate detectable amounts of methionine oxidation lowers the pI of the protein (5.21 → 5.17). A Cys-232 → Ser α1-antitrypsin analogue (C232S) was oxidized under identical conditions to verify that the observed change in net surface charge was attributable only to Cys-232 oxidation.

Under the same oxidizing conditions that were found to lower the α1-antitrypsin pI, Cys-232 was not reactive with NBD-Cl. This indicates that sulfenic acid is not a populated thiol oxidation state. Therefore, it can be concluded that the sulfenic and cysteic acid species observed during peptide mapping were not generated during sample preparation and that these are the populated Cys-232 oxidation states in α1-antitrypsin exposed to concentrations of hydrogen peroxide that are sufficient to generate substantial amounts of methionine oxidation.

Investigation of Intermolecular Disulfide Formation—As shown by non-reducing SDS-PAGE (Fig. 4), no disulfide-linked α1-antitrypsin dimers (molecular mass of ~90 kDa) formed when 0.1 mg/ml of α1-antitrypsin was exposed to 20 mM hydrogen peroxide for up to 20 min at pH 10. This indicates that under the experimental conditions used to study Cys-232 oxidation, the only thiol oxidation products generated are sulfenic, sulfenic, and cysteic acid. This result is in agreement with the prior observation that intermolecular disulfide bond formation between α1-antitrypsin monomers does not play a significant role in the Cys-232 oxidation pathway (15). Rather, the in vitro oxidation pathway that we have elucidated shows that sulfenic acid is an initial oxidation product that is sufficiently stable to remain populated following a 20-min exposure to 0.2 mM hydrogen peroxide but not sufficiently stable to preclude further oxidation to sulfenic, and eventually cysteic, acid.

Modification Reaction and Spectrophotometric Analysis to Determine the Rate of Cys-232 Oxidation—NBD-Cl modification was used to quantify the rate at which Cys-232 is oxidized following exposure to hydrogen peroxide. At pH 7.4, oxidized Cys-232 either reacts with NBD-Cl as a sulfenic acid to form an NBD adduct or does not react with NBD-Cl at all (sulfenic and cysteic acids). Only reduced Cys-232 reacts with NBD-Cl to incorporate an NBD adduct that absorbs light at 420 nm. Therefore, the loss of absorbance at 420 nm for α1-antitrypsin exposed to oxidant, and subsequently reacted with NBD-Cl, provides a measure of Cys-232 oxidation.

The assay used to quantify Cys-232 oxidation involved reacting α1-antitrypsin with hydrogen peroxide, quenching the oxidation reaction with catalase, and then reacting the α1-antitrypsin/catalase mixture with a 40-fold molar excess of NBD-Cl. Online diode array detection of α1-antitrypsin chromatographically separated from reactants allowed identification of the modified absorbance of the protein at 420 nm (Fig. 2). This technique eliminated the need for removal of either catalase or unreacted NBD-Cl prior to spectral analysis. Peak height and...
integrated peak area at 420 nm were normalized to those of fully reduced and NBD-labeled α1-antitrypsin to obtain a quantitative measure of Cys-232 oxidation.

**Oxidation Kinetics and pKₐ Determination**—The rate of nucleophilic attack by a cysteine residue in either a disulfide exchange or an oxidation reaction is determined by local protein structure (30) and the extent to which side-chain sulfur atom of the residue is ionized (31, 32). Because these properties are pH-dependent we examined the relationship between pH and Cys-232 oxidation. The pH range of 5–10 was chosen for an oxidation kinetic study because it encompasses both the pH limits of the recombinant α1-antitrypsin biological activity (33) and a pH range relevant in bioprocessing. Although aggregation was a concern between pH 5 and 6 and between pH 8 and 10 (34), no aggregation was detected under the conditions in which the oxidation experiments were performed.

As shown in Fig. 5A, reaction between α1-antitrypsin and excess hydrogen peroxide at pH 7 follows pseudo-first-order kinetics. Similar oxidation profiles were found over the entire pH range studied, thus making it possible to calculate observed bimolecular rate constants (kobs) at each pH level. A plot of these rate constants as a function of pH has the sigmoid shape expected of a reaction in which only ionized cysteine is reactive (Fig. 5B).

Based on an equilibrium between thiol and thiolate and an oxidation reaction in which only the thiolate has appreciable reactivity, Equation 1 can be derived (35).

\[
\text{Eq. 1}
\]

\[
k_{\text{obs}} = \frac{k_{\text{t}}}{(1 + 10^{pK_{\alpha} - \text{pH}})}
\]

From Equation 1 we determined that the α1-antitrypsin cysteine 232 has a pKₐ of 6.86 ± 0.05 and a pH-independent oxidation rate constant (kₜ) of 7.0 ± 0.1 M⁻¹ s⁻¹.

**Cys-232 Disulfide Exchange Rate**—To extend the work presented here to Cys-232 reactivity in glycosylated human α1-antitrypsin it was necessary to establish that Cys-232 is equally reactive in both forms of the inhibitor. Because the reactivity of Cys-232 in human α1-antitrypsin has been investigated via disulfide exchange (15, 36), the disulfide exchange rate of Cys-232 with oxidized glutathione (GSSG) was determined using the same analytical method that was applied in the analysis of Cys-232 modification by hydrogen peroxide. (The only difference was that reactions were quenched by desalting rather than catalase.) The measured exchange rate, 1.19 ± 0.02 M⁻¹ s⁻¹, is nearly identical to the 1.29 ± 0.1 M⁻¹ s⁻¹ exchange rate determined for the reaction between glycosylated human α1-antitrypsin and GSSG (36). This suggests similar structural and electrostatic environments surrounding Cys-232 in both human and recombinant α1-antitrypsin.

**Effect of pH on the Environment Surrounding Cys-232**—At high pH levels, the upper limit of the Cys-232 reactivity with hydrogen peroxide, kₜ, is 7.0 ± 0.1 M⁻¹ s⁻¹, a value that is significantly less than that of a small molecule thiol (kₜ = 12.6 M⁻¹ s⁻¹ at 25°C) (37, 38). Between pH 5 and 6, the Cys-232 reactivity with hydrogen peroxide is 2–3 times greater than that which would be predicted solely on the basis of side-chain ionization (Fig. 5B). This observation suggested that the structural factors that influence the reactivity of Cys-232 are pH-dependent.

Structural changes were investigated by labeling Cys-232 with a fluorescent probe, IANBD. IANBD exhibits appreciable fluorescence only after reaction with thiols that are buried or unsolvated, and this fluorescence is highly sensitive to changes in the solvent level of the NBD fluorophore. Therefore, α1-antitrypsin was modified with IANBD at pH 7 and then exchanged into buffers ranging from pH 5 to 9 to detect pH-induced conformational changes in the region surrounding Cys-232. Cys-S-ANBD conjugates in pH 6–9 buffers each showed reduced quenching and had a 3-nm blue-shifted fluorescence spectrum relative to that of fully denatured and IANBD-labeled α1-antitrypsin (Fig. 6). The fluorescence spectrum of the Cys-S-ANBD conjugate exchanged into pH 5 buffer was blue-shifted 7 nm relative to fully denatured IANBD-labeled α1-antitrypsin and had a fluorescence quantum yield ~40% greater than those of the higher pH conjugates. These observations are consistent with conformational changes in the region surrounding Cys-232 induced by acidic, but not alkaline, pH.
Sulfenic acids are highly reactive not only as nucleophiles but also as electrophiles (18). Therefore, a cysteine that has been oxidized to sulfenic acid under highly oxidizing conditions can be maintained in a reversible oxidation state by subsequent reaction with a reduced thiol. Although the possibility of finding reduced thiols under oxidizing conditions seems unlikely, this is the case in the airway lining fluid of the lungs where α1-antitrypsin is known to incur extensive methionine oxidation (8). The concentration of reduced GSH in the lungs of smokers is maintained at a concentration of ~775 μM, more than twice the concentration for non-smokers and more than 250 times the concentration of GSH found in blood plasma (40). Because GSH is known to react with sulfenic acids extremely quickly, oxidation of Cy232 to sulfenic acid in the lungs is likely to be followed by immediate reaction with GSH to yield a stable glutathione adduct (Cys–S–SG). Glutathiolation via a sulfenic acid intermediate in stress and signaling pathways is well established (20) and, although not applicable to the in vitro studies presented here, may indeed be relevant to lung physiology.

Regardless of whether in vivo or in vitro oxidation is considered, thiolate stabilization and local protein structure are the factors that determine the reactivity of Cy232. We evaluated these parameters by measuring the rate at which Cy232 is oxidized by hydrogen peroxide between pH 5 and 10. Kinetics of the reaction between Cy232 and hydrogen peroxide were used to determine the pKs of Cy232 and the intrinsic bimolecular rate constant for its reaction with hydrogen peroxide (kR.). The pKs of 6.86 ± 0.05, is ~1.5 pH units lower than that of a typical protein thiol (~8.5) (41). Because this depressed pKs is clearly the major determinant of the reactivity of Cy232, it is important to identify the means by which the thiolate is stabilized.

There are a number of structural interactions capable of lowering the pKa of a thiol. One is ion pairing with a histidine residue. This type of interaction lowers the pKa values of vapain (42), thiopeptilisin (43), and glutathione S-transferase (44) by up to 5 pH units. His-231 is next to Cy232 in the linear sequence of α1-antitrypsin, but its side chain is not within 5 Å of the Cy232 side chain in any x-ray structures. The thioredoxin fold has been shown to use elements of protein secondary structure to provide a decrease in thiol pKa (45). For this to occur, however, the cysteine residue must be located at or near a helical dipole (45), and this is not the case for Cy232.

Hydrogen bond formation, such as that observed for Cy282 in muscle creatine kinase (46), can also lower the pKa of a thiol, but once again, this does not appear to be possible for Cy232. With these possibilities ruled out we considered electrostatic effects brought about by the presence of nearby positive charges.

Analysis of the crystal structure of α1-antitrypsin shows that lysine residues 233, 234, and 274 are proximal to Cy232 (Fig. 7B) and clearly provide a positively charged electrostatic environment. Thus, we suggest that it is the presence of these positively charged residues, and the absence of any nearby negatively charged residues, that provides Cy232 with the thiolate stabilization required for a high degree of reactivity across the neutral pH range.

Because of thiolate stabilization, Cy232 is expected to be more reactive at low pH than most protein thiols. However, Fig. 5B shows that the residue is 2–3 times more reactive between pH 5 and 6 than would be expected solely on the basis of thiol ionization (see Equation 1), and this deviation is not within the limits of experimental error. Therefore, Equation 1 provides a reasonable estimation for pKa determination, but does not fully
describe the pH-dependent reactivity of Cys-232 with hydrogen peroxide.

Cysteine labeling with the fluorescent probe IANBD showed no sign of pH-dependent conformational changes that might affect reactivity under neutral or alkaline conditions (Fig. 6). This suggests that the structure around Cys-232 is not significantly altered by high pH. In contrast, IANBD labeling clearly indicated that the local environment of Cys-232 is altered at slightly acidic pH. At pH 5, the IANBD probe attached to Cys-232 is positioned in a more hydrophobic, less solvent accessible region than it is between pH 6 and 9.

The extent to which IANBD is able to interact with the hydrophobic interior of a protein is assessed by changes in its fluorescence. The blue shift and reduced fluorescence quenching observed for IANBD-labeled α1-antitrypsin exchanged into pH 5 buffer are indicative of increased accessibility of the NBD fluorophore to the protein interior, suggesting a more open conformation in the immediate proximity of Cys-232 at slightly acidic pH (Fig. 7). This suggests that the reactivity of Cys-232 at acidic pH is enhanced by increased side-chain solvent accessibility. Between pH 5 and 5.5, however, Cys-232 is more reactive than even a completely solvent-exposed thiol with a $pK_a$ of 6.86. Therefore, it seems that conformational changes at acidic pH may affect the local electrostatic environment of Cys-232 as well. It was shown that such a structurally mediated change in local electrostatic environment is the basis of the pH-dependent ionization and reactivity of cysteine residues 31 and 32 in monomeric seminal ribonuclease (31). In the case of Cys-232, acid-induced reorientation of the residues that determine its observed $pK_a$, which we believe to be Lys-233, Lys-234, and Lys-274, alter its local electrostatic environment in a manner that further stabilizes the thiolate.

Combined with our studies of methionine oxidation in α1-antitrypsin (47), the work presented here allows us to conclude that Cys-232 is α1-antitrypsin’s most oxidation-susceptible amino acid between pH 5 and 10, which is the pH range compatible with the global structural stability of the protein (48). Even when thiolate neutralization is maximized at pH 5, Cys-232 remains ~20 times more reactive with hydrogen peroxide than the most reactive methionine, Met-358. Although the previously discussed mechanism for sulfenic acid reduction by small molecule thiols may aid in preventing irreversible oxidation under physiological conditions, the inability to suppress ionization by low pH makes Cys-232 a significant liability with regard to in vitro stabilization. Indeed, it has been necessary to use low temperature in conjunction with sub-neutral pH to prevent Cys-232 oxidation during bioprocessing studies.

In summary, we present here a quantitative analysis of cysteine oxidation in α1-antitrypsin that provides new insights into the reactivity of the protein’s unpaired thiol, Cys-232. These insights have been applied to the crystal structure of α1-antitrypsin in an effort to understand the structural factors that influence reactivity and to benefit future studies regarding the role that this residue may play in physiological functioning. We have also investigated the relationship between pH and Cys-232 oxidation and found that an unusually low $pK_a$ of 6.86 and altered conformation at acidic pH makes cysteine the most reactive of α1-antitrypsin’s sulfur-containing amino acids. Therefore, even though the protein contains exposed and reactive methionine residues, cysteine oxidation is far more likely to occur in oxidizing environments. Because the results presented here are all directly related to aspects of the structure of α1-antitrypsin, this study should be useful not only to future work with α1-antitrypsin, but also to the consideration of cysteine oxidation in other medically important proteins with complex structural biochemistries.

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