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

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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 sulfinic and cysteic acid in successive steps. pH-dependent reactivity with hydrogen peroxide showed that Cys-232 has a pK_a 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 immunglobulin 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 pK_a and 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

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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).

Isoelectic 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 pI 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 pI calibration kit was used for pI estimation.

Buffer Preparation for Oxidation Experiments—All buffers were prepared with 10 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),[1] 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 pK_a—Highly purified recombinant α1-antitrypsin was desalted into oxidation buffer and equilibrated at 25 °C. Oxidation reactions were carried out in 30.8% H_2O_2 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 (ϵ_{420}) of 13,000 M^{-1} cm^{-1} for the NBD-thiol product (24). Following reaction, thiol-modified samples were stored at −20 °C until HPLC analysis.

Analytical HPLC—Following reaction with NBD-Cl, 25 μg of recombinant α1-antitrypsin was injected onto a Vydac 214TP54 3.2 × 250 mM reversed-phase C_4 column using a Hitachi model AS-4000 autosampler. The column was equilibrated with 40% acetonitrile, and a gradient from Buffer A (0.1% trifluoroacetic acid) to B (80% acetonitrile, 0.09% trifluoroacetic acid) was run as follows: 50% from 0–6 min, 50–81.3% from 6–18.5 min, 81.3% hold from 18.5–23.5 min, and 81.3–100% B from 23.5–28.5 min. The flow rate was 0.4 ml/min, detection was from 260–450 nm, and the column temperature was set at 50 °C using an Eppendorf TC-45 column heater. Peak absorbance was determined using Beckman Coulter System Gold Nouveau software with data collected from a Beckman Coulter HPLC system consisting of a model 168 diode array detector and model 126 pumps. All runs were performed with HPLC grade water and acetonitrile. For a given set of oxidation kinetic experiments, each oxidation time point sample was analyzed in duplicate.

Fluorescent Labeling with IANBD—A 10 mM stock solution of IANBD amide (*N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazole-y-yl) ethylenediamine) purchased from Molecular Probes, Inc. was prepared in dimethyl sulfoxide. A 20-fold molar excess of IANBD (IANBD/α1-antitrypsin) was used for thiol modification of recombinant α1-antitrypsin that had been desalted into 10 mM phosphate, pH 7.0, 100 mM ionic strength (adjusted with NaCl). The reaction was allowed to proceed at 25 °C in the dark for 2 h, at which time labeled samples were desalted into buffers ranging from pH 5 to pH 9.

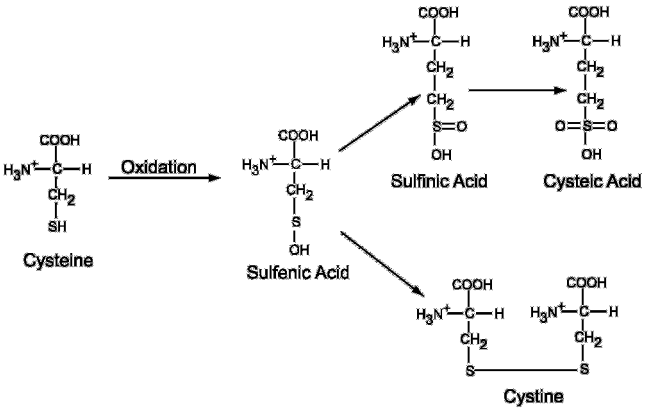

FIG. 1. **Pathways for cysteine oxidation by hydrogen peroxide.**

Characterization of IANBD-labeled α1-Antitrypsin—The extent of IANBD labeling was determined by spectroscopic analysis using a molar extinction coefficient (ϵ_{497}) 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 spectrofluorometer 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 sulfenic acid (Fig. 1). Small molecules with unpaired thiols that are oxidized to sulfenic 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 sulfenic acid intermediate is formed. Because sulfenic acid is reactive as a nucleophile (26), further exposure to hydrogen peroxide leads to the irreversible formation of sulfinic acid (Cys-SO_2H). It was important to identify whether the Cys-232 location within the protein scaffold makes it a potential site for formation of these acid thiol species.

Because of inherent instability, sulfenic 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 sulfenic acid leads to the covalent incorporation of a spectroscopically detectable sulfenic acid conjugate (Cys-S(O)-NBD). Sulfenic 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 Sulfinic and Cysteic Acid Species—Oxidation of a protein thiol to sulfenic acid is followed by the formation of either a disulfide bond or, upon further exposure to oxidant, sulfinic acid. Previous studies used peptide mapping to show that sulfinic 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 pK_a values of sulfinic acid and cysteic acid are both less

[1] The abbreviations used are: MES, 4-morpholineethanesulfonic acid; TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; IANBD, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazole-y-yl) ethylenediamine; HPLC, high pressure liquid chromatography.

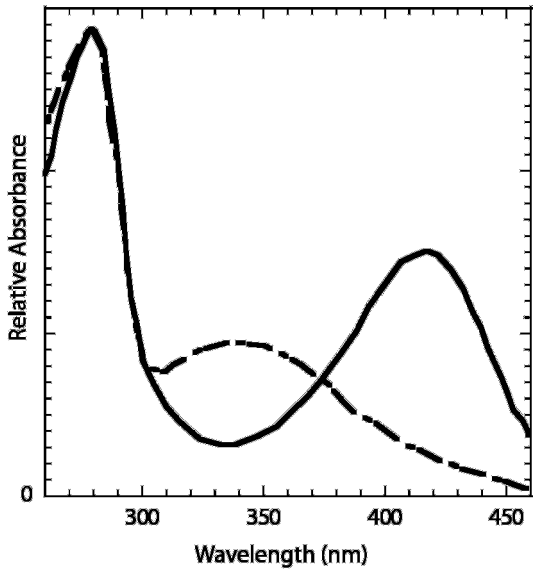

FIG. 2. **UV-vis spectra of NBD-modified recombinant α1-antitrypsin.** Cys-232 sulfenic acid (Cys-S(O)-NBD conjugate (*dotted line*)) was generated by incubating 2 μM α1-antitrypsin with a 100-fold molar excess of hydrogen peroxide (0.2 mM) for 20 min at 25 °C in phosphate buffer pH 7, quenching the oxidation reaction with catalase, and then immediately incubating the oxidized protein with a 40-fold molar excess of NBD-Cl for 45 min. Modified protein was directly injected onto a Vydac 214TP54 reversed-phase HPLC column. The spectral data were taken at the absorption maximum of α1-antitrypin as it eluted from the reversed-phase column (diode array detection from 260 to 450 nm). Non-oxidized Cys-232 (Cys-S-NBD conjugate (*solid line*)) was labeled and analyzed in the same manner as the Cys-S(O)-NBD conjugate. The spectral change corresponding to NBD modification of either Cys or Cys-S(O) was complete within 15 min.

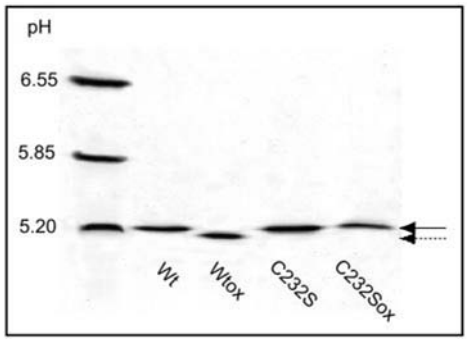

FIG. 3. **Isoelectric focusing analysis of oxidized α1-antitrypsin.** Recombinant α1-antitrypsin (*Wt*, *Wtox*) and a C232S mutant (*C232S*, *C232Sox*) were oxidized with 20 mM H_2O_2 at 25 °C for 30 min in 10 mM phosphate, pH 7. Oxidation was stopped by desalting, and both the oxidized (*Wtox*, *C232Sox*) and reduced samples (*Wt*, *C232S*) were immediately loaded onto an isoelectric focusing gel. The *solid arrow* indicates a pI of 5.21 for non-oxidized α1-antitrypsin (*Wt*), C232S, and oxidized C232S. The *dashed arrow* indicates a pI of 5.17 for oxidized α1-antitrypin (*Wtox*).

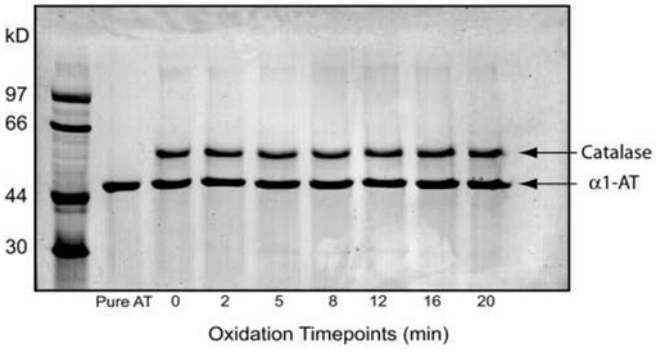

FIG. 4. **Non-reducing SDS-PAGE analysis of intermolecular disulfide bond formation.** 0.1 mg/ml of recombinant α1-antitrypsin was oxidized with 0.2 mM H_2O_2 at 25 °C in 10 mM CAPS buffer, pH 10. At various times after oxidation was initiated, 180 μg of bovine catalase was added to quench the oxidation reaction, and a 40-fold molar excess of NBD-Cl was added to modify Cys-232. The modification reaction was allowed to proceed for 45 min prior to non-reducing SDS-PAGE analysis. The bands corresponding to α1-antitrypsin (*α1-AT*) and bovine catalase are indicated.

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 sulfinic 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 sulfinic 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-antitrypin dimers (molecular mass of ~90 kDa) formed when 0.1 mg/ml of α1-antitrypsin is exposed to 0.2 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, sulfinic, 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* oxidiation 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 sulfinic, 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 with an absorbance at 347 nm or does not react with NBD-Cl at all (sulfinic 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 pK_a 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. 5*A*, 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 (k_{obs}) 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. 5*B*)

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).

$$k_{obs}=\frac{k_s}{(1+10^{(pK_a-pH)})} \quad \text{(Eq. 1)}$$

From Equation 1 we determined that the α1-antitrypsin cysteine 232 has a pK_a of 6.86 ± 0.05 and a pH-independent oxidation rate constant (k_{S-}) 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, k_{S-}, is 7.0 ± 0.1 M^{-1} s^{-1}, a value that is significantly less than that of a small molecule thiol (k_{S-} ~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. 5*B*). 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 solvation 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.

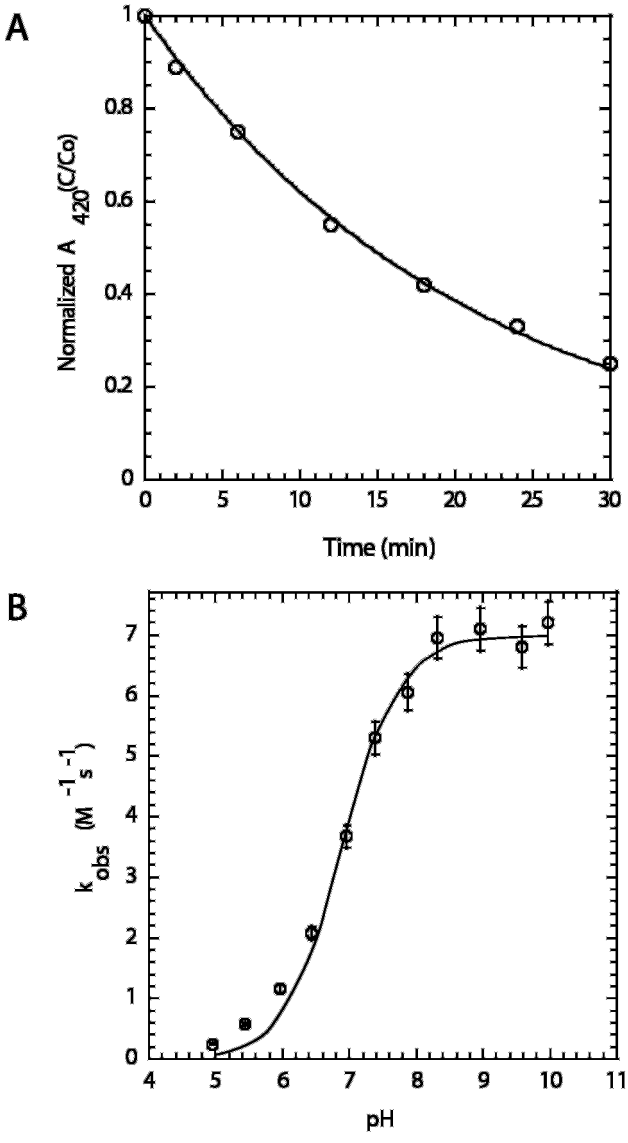

FIG. 5. **Cys-232 pH-dependent oxidation by hydrogen peroxide.** *A*, 2 μM recombinant α1-antitrypsin was incubated with 0.2 mM H_2O_2 at 25 °C in 10 mM phosphate, pH 7.0. The pseudo-first-order oxidation kinetic profile was obtained by quenching the oxidation reaction with catalase, modifying Cys-232 with NBD-Cl, and using reversed-phase chromatography coupled with diode array detection to measure the absorbance of the Cys-232-NBD conjugate at 420 nm. The ratio of the oxidized conjugate's absorbance relative to that of a non-oxidized sample is plotted as a function of time. Pseudo-first-order rate constants were calculated by fitting the experimental data (*open circles*) to a first-order kinetic model (*solid line*). *B*, the same oxidation kinetic experiment described in *A* was performed at various pH between 5 and 10 to obtain a plot of the observed bimolecular oxidation rate constant, k_{obs}, as a function of pH. *Error bars* represent the S.D. of three separate oxidation kinetic experiments. The experimental data were fit to Equation 1 (*solid line*).

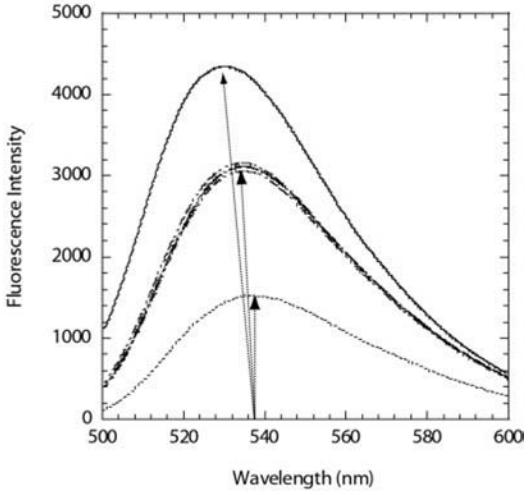

FIG. 6. **pH-dependent fluorescence spectra of IANBD-labeled recombinant α1-antitrypsin.** 0.1 mg/ml of recombinant α1-antitrypsin was incubated in the dark for 2 h with a 20-fold molar excess of IANBD (25 °C, 10 mM phosphate, pH 7.0). The labeling reaction was stopped by desalting into pH 5, 6, 7, 8, and 9 buffers. The concentration of each sample was adjusted to 0.065 mg/ml, and fluorescence spectra were recorded. Spectra were recorded every 60 min for 6 h to ensure that conformational equilibrium had been reached. The Cys-S-ANBD conjugate in pH 5 buffer (*solid line*) had an emission maximum at 531 nm and the highest fluorescence intensity. Cys-S-NBD conjugates in pH 6, 7, 8, and 9 buffers (*dashed lines*) had emission maxima at 535 nm and approximately the same fluorescence intensity. Fully denatured and IANBD-labeled α1-antitrypsin (*dotted line*) had an emission maximum at 538 nm and the lowest fluorescence intensity. *Arrows* indicate the emission maxima shift for Cys-S-ANBD conjugates in folded α1-antitrypsin relative to a Cys-S-ANBD conjugate in α1-antitrypsin that has been denatured in 6 M guanidine hydrochloride at pH 7.

DISCUSSION

A challenge in the biotechnology industry is the development of strategies for the stabilization of therapeutic proteins against chemical modification. However, a detailed understanding of these reactions is important not only in biotechnology but also in biology and medicine. A relevant example is the oxidation of α1-antitrypsin. Although it is established that methionine oxidation is the modification reaction of greatest significance within the context of the physiological functioning of α1-antitrypsin**,** the protein also contains a highly reactive cysteine residue, Cys-232. A detailed understanding of the reactivity of this residue is necessary to fully understand the oxidation susceptibility of α1-antitrypsin in oxidizing environments. In this study we employed a number of techniques that exploit the biochemical properties of Cys-232 oxidation products to achieve this goal.

In the presence of hydrogen peroxide, the first step in the Cys-232 oxidation pathway is the reversible formation of sulfenic acid. Reaction of oxidized α1-antitrypsin with NBD-Cl was used to determine that sulfenic acid is a relatively stable Cys-232 oxidation state. This sulfenic acid intermediate was not stable, however, in the presence of the high peroxide concentrations required to generate significant amounts of methionine oxidation in α1-antitrypsin (10). Rather, it was quickly oxidized to sulfinic and cysteic acid, both of which are irreversible thiol oxidation products that alter the net surface charge of the protein (Fig. 3). This observation is significant because it is active site methionine oxidation and not cysteine oxidation that has been found in α1-antitrypsin recovered from inflammatory effusions (39) and lung secretions of smokers (8). This raises the question of why *in vitro* experiments under conditions that are sufficient to oxidize methionine yield cysteine oxidation products that have not been observed in α1-antitrypsin recovered from highly oxidizing physiological environments. Discussion of this question requires consideration of reversible oxidation mechanisms found in physiological settings.

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 Cys-232. We evaluated these parameters by measuring the rate at which Cys-232 is oxidized by hydrogen peroxide between pH 5 and 10. Kinetics of the reaction between Cys-232 and hydrogen peroxide were used to determine the pK_a of Cys-232 and the intrinsic bimolecular rate constant for its reaction with hydrogen peroxide (k_{S^-}). The pK_a, 6.86 ± 0.05, is ~1.5 pH units lower than that of a typical protein thiol (~8.5) (41). Because this depressed pK_a is clearly the major determinant of the reactivity of Cys-232, it is important to identify the means by which the thiolate is stabilized.

There are a number of structural interactions capable of lowering the pK_a of a thiol. One is ion pairing with a histidine residue. This type of interaction lowers the pK_a values of papain (42), thiosubtilisin (43), and glutathionine *S*-transferase (44) by up to 5 pH units. His-231 is next to Cys-232 in the linear sequence of α1-antitrypsin, but its side chain is not within 5 Å of the Cys-232 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 pK_a (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 Cys-232. Hydrogen bond formation, such as that observed for Cys-282 in muscle creatine kinase (46), can also lower the pK_a of a thiol, but once again, this does not appear to be possible for Cys-232. 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 Cys-232 (Fig. 7*B*) 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 Cys-232 with the thiolate stabilization required for a high degree of reactivity across the neutral pH range.

Because of thiolate stabilization, Cys-232 is expected to be more reactive at low pH than most protein thiols. However, Fig. 5*B* 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 pK_a determination, but does not fully

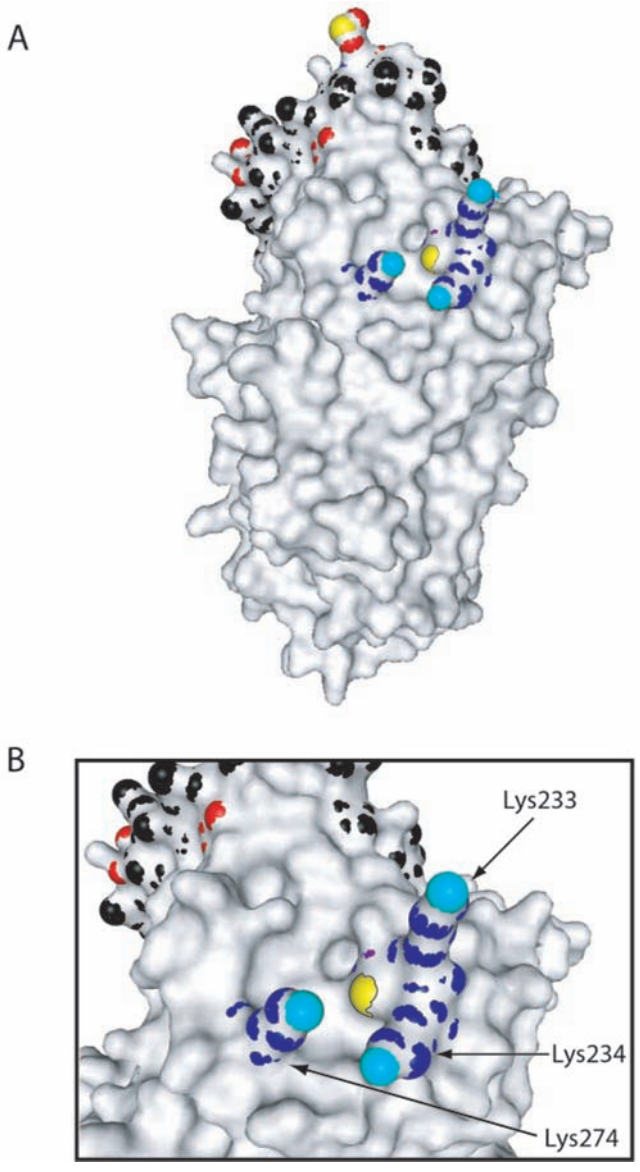

FIG. 7. **Crystal structure of recombinant human α1-antitrypsin (PDB code: 1QLP).** The solvent accessible surface as seen by a molecular probe with the radius of water (1.4 Å) is shown. Methionine carbon is in *red*, cysteine carbon is in *purple*, lysine carbon is in *dark blue*, nitrogen atoms are in *light blue*, and sulfur atoms are in *yellow*. Active-site residues (344–365) other than Met-351 and Met-358 are shown in *black*. *A*, location of Cys-232 within recombinant α1-antitrypsin's tertiary structure. *B*, the expanded structural region in the vicinity of Cys-232. Molecular surfaces were generated using WebLab ViewerPro (Accelrys).

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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