Relationship between Protein Structure and Methionine Oxidation in Recombinant Human α1-Antitrypsin

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ABSTRACT: α1-Antitrypsin is a metastable and conformationally flexible protein that belongs to the serpin family of protease inhibitors. Although it is known that methionine oxidation in the protein's active site results in a loss of biological activity, there is little specific knowledge regarding the reactivity of each of the protein's methionine residues. In this study, we have used peptide mapping to study the oxidation kinetics of each of α1-antitrypsin's methionines in α1-AT$_{(C232S)}$ as well as M351L and M358V mutants. These kinetic studies establish that Met1, Met226, Met242, Met351, and Met358 are reactive with hydrogen peroxide at neutral pH and that each reactive methionine is oxidized in a bimolecular, rather than coupled, mechanism. Analysis of Met226, Met351, and Met358 oxidation provides insights regarding the structure of α1-antitrypsin's active site that allow us to relate conformation to experimentally observed reactivity. The relationship between solution pH and methionine oxidation was also examined to evaluate methionine reactivity under conditions that perturb the native structure. Methionine oxidation data show that at pH 5, global conformational changes occur that alter the oxidation susceptibility of each of α1-antitrypsin's 10 methionine residues. Between pH 6 and 9, however, more localized conformational changes occur that affect primarily the reactivity of Met242. In sum, this work provides a detailed analysis of methionine oxidation in α1-antitrypsin and offers new insights into the protein's solution structure.

A common problem in the biotechnology industry is degradation of protein therapeutics by aggregation and chemical modification (*1*). In the development of formulation strategies for preventing such degradation reactions, it is typical to assume that a protein will adopt its most thermodynamically stable state in a given solvent environment. This is not the case, however, for a metastable protein with biological activity that depends on conformational strain. Proteins that are metastable include α-lytic protease, surface proteins of human influenza virus and human immunodeficiency virus, and members of the serine protease inhibitor (serpin) superfamily of plasma protease inhibitors (*2*). The serpins are a medically and biologically important family of proteins, which includes α1-antitrypin, antithrombin III, plasminogen activator inhibitor-1, C1-inhibitor, α1-antichymotrypsin, and many others (*3*).

The archetypical serpin is α1-antitrypsin (*4*). Like all other members of the serpin family, this protein has a structure consisting of three β-sheets, nine α-helices, and a reactive center loop (RCL), which in inhibitory serpins contains the residues that directly interact with protease substrates (Figure 1) (*5*). An important aspect of α1-antitrypsin's physiology is that the most thermodynamically stable form of the protein is not achieved until the reactive center loop (RCL) of the "stressed" native state is inserted as an additional strand (s4A) into the A β-sheet (*6*). This can occur by proteolytic cleavage (*6*) or, in the case of the latent form of the protein, by direct insertion (*7*). As a requirement of this loop−sheet insertion

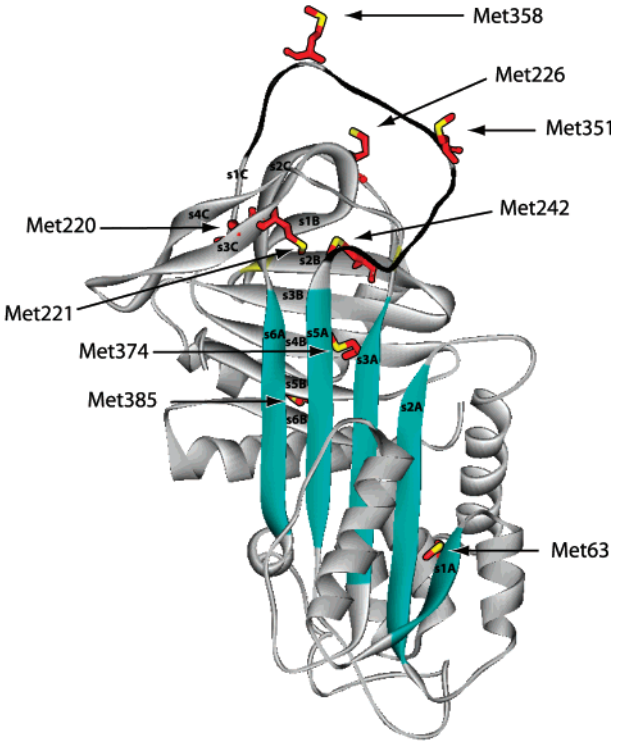

FIGURE 1: Crystal structure of recombinant human α1-antitrypsin [PDB entry 1QLP (*12*)]. The ribbon diagram shows α1-antitryspin's secondary structure. The A β-sheet is shown in light blue; the active site loop (residues 342−362) is shown in black, and strands of the A, B, and C β-sheets are labeled. Nine of the protein's 10 methionines are shown in red (Met1 is not defined by X-ray data), with each residue's side chain sulfur atom in yellow.

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mechanism, certain regions of the protein have a low activation barrier for conformational change (*8*). This predisposes the protein to structure-related degradation mechanisms such as aggregation and chemical modification. Although studies regarding the relationship between α1-antitrypsin's structure and aggregation propensity (*9*−*11*) have led to useful aggregation models, studies regarding the relationship between structure and chemical modification have been limited.

We are interested in the oxidation of methionine and cysteine in α1-antitrypsin. Methionine oxidation is significantly important both to the biological functioning of proteins (*13*, *14*) and to the stability of proteins produced for therapeutic purposes (*1*, *15*). In α1-antitrypsin, methionine oxidation in the RCL results in significant loss of inhibitory activity against human neutrophil elastase (*16*). This contributes to the pathology of pulmonary emphysema in the lungs of smokers (*17*) and may be a primary mechanism for physiological regulation of α1-antitrypsin activity (*13*, *16*). Although it is accepted that inhibitory activity against elastase is only affected by oxidation in the RCL, reports pertaining to the total number of methionines that are susceptible to oxidation have been contradictory. It has been stated that only two of α1-antitrypsin's 10 methionines are susceptible to oxidation when the protein is exposed to *N*-chlorosuccinimide (*18*). This is in contrast to the four oxidized methionines found in α1-antitrypsin oxidized by cigarette smoke (*19*, *20*), and the three oxidized methionines reported for α1-antitrypsin exposed to hydrogen peroxide at pH 5.0 (*16*). We have found that five of recombinant α1-antitrypsin's 10 methionine residues (Met1, Met226, Met242, Met351, and Met358) are susceptible to oxidation by hydrogen peroxide at neutral pH (*21*).

There is clearly a need to develop a detailed understanding of methionine oxidation in α1-antitrypsin and to understand why structural models have been unable to conclusively show which methionines are susceptible to oxidation. Therefore, in this study, we investigate the mechanism by which α1-antitrypsin's reactive methionines are oxidized at neutral pH and also examine pH-dependent methionine oxidation.

MATERIALS AND METHODS

Expression and Purification. Recombinant human α1-antitrypsin [α1-$AT_{(C232S)}$] was expressed in *Escherichia coli* BL21(DE3) from the plasmid pEAT8 (*22*). Following protein expression and cell harvest, the soluble protein fraction was purified as previously described (*21*). Briefly, soluble α1-antitrypsin (>98% pure) was obtained from a bacterial cell extract using two consecutive anion exchange steps, one performed at pH 8.5 and the other at approximately pH 6.

Site-Directed Mutagenesis. A Cys232 → Ser [α1-$AT_{(C232S)}$] mutant of α1-antitrypsin was constructed as previously described (*21*). This mutant was used in oxidation studies to limit the scope of the present work to methionine oxidation. In agreement with prior work (*23*), we found that this mutation does not affect protein conformation or stability. Met351 → Leu (M351L) and Met358 → Val (M358V) mutants harboring the C232S mutation were also constructed.

Buffer Preparation for Oxidation Experiments. All buffers were prepared with 10 mM buffer salt and adjusted to an ionic strength of 100 mM with NaCl. The following buffering species were used for the pH range of 5−9: acetate for pH 5.0, MES for pH 6.0, phosphate for pH 7.0, Tris for pH 8.0, and TAPS for pH 9.0. Compensation was made for the effect of solution temperature on ionic strength according to the methods of Beynon and Easterby (*24*).

In Vitro Oxidation. Purified α1-$AT_{(C232S)}$ was exchanged into buffers prepared at the indicated pH (ionic strength adjusted to 0.1 M with NaCl) using pre-equilibrated PD-10 columns (Amersham Biosciences), and incubated at 25 °C (unless otherwise noted). Oxidation reactions were performed with various concentrations of hydrogen peroxide by addition of a diluted 30% H_2O_2 (w/w) stock solution (Sigma). At various times after oxidation was initiated, aliquots were removed and separated from H_2O_2 on PD-10 columns. Desalting columns were equilibrated with endoproteinase Lys-C digestion buffer [25 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 10% (v/v) HPLC grade acetonitrile].

Peptide Mapping. Endoproteinase Lys-C digestion of recombinant α1-antitrypsin and analyses of the resultant peptides by reversed-phase HPLC were performed following the procedure outlined by Griffiths and Cooney (*21*).

Elastase Inhibitory Capacity (EIC). The elastase inhibitory capacity (EIC) of α1-antitrypsin was determined using a method previously described (*17*) with slight modifications (*25*). The difference in reaction rates between an elastase-catalyzed reaction in the presence and absence of α1-antitrypsin is equal to the amount of elastase inhibited by α1-antitrypsin, or the elastase inhibitory capacity (EIC). A 0.05% (w/v) elastase stock solution was prepared just before the assays by adding porcine pancreatic elastase (crystalline suspension, Sigma E-1250) to 100 mM NaCl. A substrate working stock solution of 2 mg/mL *N*-succinyl-$(Ala)_3$-nitroanilide (Sigma S-4760) in 100 mM Tris (pH 8) was prepared from a stock of 40 mg/mL *N*-succinyl-$(Ala)_3$-nitroanilide in dimethyl sulfoxide (stored at −20 °C). α1-Antitrypsin was mixed with 50 μL of the elastase working stock and Tris buffer (100 mM, pH 8) to a final volume of 1.05 mL and incubated at room temperature for 30 min. The amount of residual free elastase was measured by adding 2 mL of Tris buffer (100 mM, pH 8), 100 μL of a substrate working stock solution, and the incubation mixture to a quartz cuvette. Following rapid mixing, the appearance of the *N*-succinyl-$(Ala)_3$-nitroanilide cleavage product was assessed at 410 nm for 30−60 s with a Hewlett-Packard 8450 spectrophotometer. The absorbance data were fit to a straight line, and the slope was recorded in milli-absorbance units per second. The EIC was calculated as the difference in slope between the α1-antitrypsin-containing sample and the α1-antitrypsin-free controls. The amount of elastase that was added was chosen such that the slope of the blank samples (free of α1-antitrypsin) was approximately 1 mAU/s. Samples and blanks were incubated and assayed in triplicate.

Solvent Accessible Surface Area Calculations. The solvent accessible surface areas of methionine side chain sulfur atoms were calculated from PDB entries 1QLP (*12*) and 1HP7 (*26*) using GETAREA 1.1 (*27*).

RESULTS

Methionine Oxidation Kinetics at pH 7. We previously developed a peptide mapping procedure to study methionine oxidation in recombinant α1-antitrypsin, and found that five

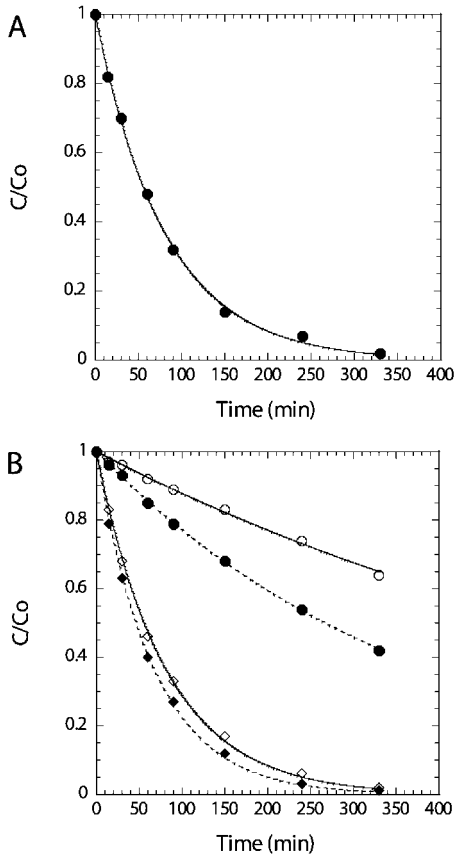

FIGURE 2: Pseudo-first-order methionine oxidation kinetic profiles at pH 7. α1-AT$_{(C232S)}$ was incubated with 21 mM hydrogen peroxide at 25 °C and pH 7.0 for 5.5 h. At various times after oxidation was initiated, samples were removed, desalted, and digested with endoproteinase Lys-C. Digest peptides were separated by reversed-phase chromatography, and the fractional oxidation of each methionine was calculated by dividing the area of the peptide containing the oxidized form of a particular methionine residue by the combined areas of the peptides containing the reduced and oxidized forms of that residue: (A) Met1 oxidation profile (●) and (B) Met242 (○), Met226 (●), Met351 (◇), and Met358 (◆) oxidation profiles.

of the protein's 10 methionine residues (Met1, Met226, Met242, Met351, and Met358) are susceptible to oxidation by hydrogen peroxide at pH 7 (*21*). With the exception of Met1, each of these residues is located in or near the protein's active site loop (Figure 1). Given their close spatial orientation, it was possible that the oxidation of one or more of these methionines might be influenced by the oxidation of another. If these residues were to follow a coupled oxidation mechanism, exposure of α1-antitrypsin to oxidant would lead to the formation of protein subpopulations with differing numbers of methionine residues that are susceptible to oxidation. Those residues that depend on the oxidation of others for oxidation susceptibility would not display bimolecular reaction kinetics when exposed to hydrogen peroxide, and hence could be detected by kinetic analysis (*28*, *29*).

The oxidation kinetics of α1-antitrypsin's reactive methionine residues were determined by in vitro oxidation followed by peptide mapping. As shown in Figure 2, when α1-antitrypsin was exposed to excess hydrogen peroxide at pH 7, Met1, Met226, Met242, Met351, and Met358 exhibited first-order kinetic profiles. The rate constants obtained from these profiles were directly proportional to the hydrogen peroxide concentration. This provided strong evidence that each reactive residue is oxidized in a bimolecular, rather than coupled, oxidation mechanism. Second-order rate oxidation constants for the oxidation reactions are presented in Table 1.

Table 1: Bimolecular Rate Constants for the Oxidation of α1-AT$_{(C232S)}$'s Reactive Methionines with H_2O_2 at pH 7

	k_{ox} ($\times 10^2$ M^{-1} s^{-1})		k_{ox} ($\times 10^2$ M^{-1} s^{-1})
Met1	0.95 ± 0.06	Met351	0.95 ± 0.03
Met226	0.20 ± 0.04	Met358	1.15 ± 0.03
Met242	0.10 ± 0.005	Met[a]	0.93−1.07

[a] Rate constant for the reaction between H_2O_2 and methionine that is either free in solution or part of a linear peptide sequence (*30*).

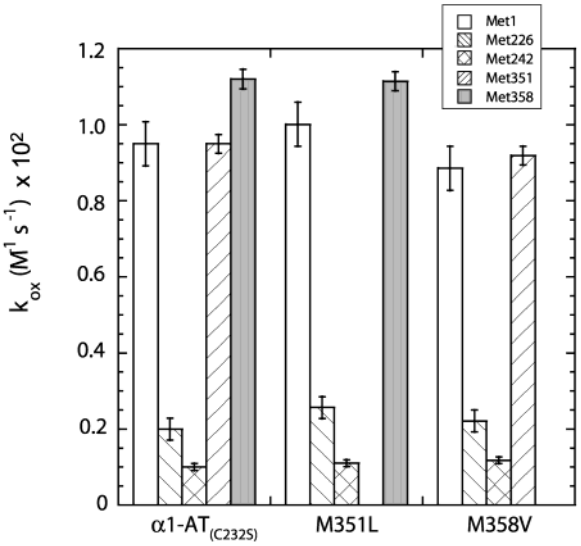

FIGURE 3: Bimolecular rate constants for oxidation of α1-AT$_{(C232S)}$, M351L, and M358V by H_2O_2 at pH 7. α1-AT$_{(C232S)}$, M351L, and M358V were incubated with 21 mM H_2O_2 at 25 °C and pH 7.0 for 5.5 h to obtain pseudo-first-order oxidation kinetic profiles for reactive methionines. Pseudo-first-order rate constants were a linear function of H_2O_2 concentration, which provided a basis for calculation of bimolecular rate constants. Error bars represent the standard deviation of two separate oxidation kinetic experiments with each protein.

The lack of involvement of either Met351 or Met358 in a cooperative oxidation mechanism was confirmed by analysis of methionine oxidation kinetics in Met351 → Leu (M351L) and Met358 → Val (M358V) mutants. As shown in Figure 3, conservative substitutions for the active site methionines do not influence the reactivity of other residues.

Effect of Methionine Oxidation on Biological Activity. Taggart et al. (*16*) have shown that the oxidation of either Met351 or Met358 results in a loss of biological activity against human neutrophil elastase (HNE). Although previous work had shown that mutants with oxidation resistance at position 358 are resistant to oxidative inactivation against HNE (*31*), the use of a Met351 → Val mutant apparently allowed for the discovery of Met351's involvement. Therefore, we investigated the role of Met351 in the oxidative inactivation of α1-antitrypsin against porcine pancreatic elastase (PPE) using α1-AT$_{(C232S)}$, M351L, and M358V (Figure 4). Our results were consistent with previous work showing that only the oxidation of Met358 leads to a loss of inhibitory activity against PPE (*32*).

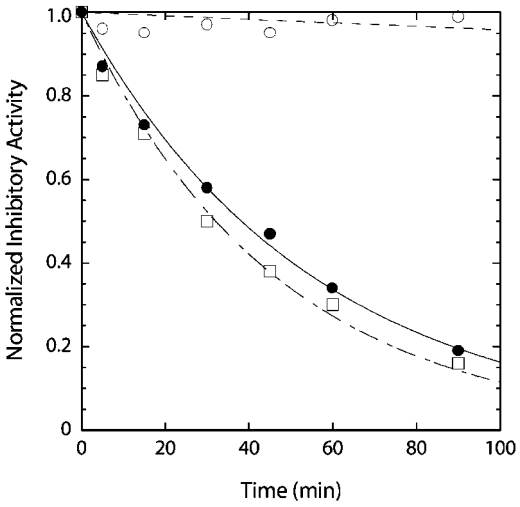

FIGURE 4: Inhibitory activity of α1-antitrypsin following exposure to H_2O_2 at pH 7. α1-$AT_{(C232S)}$ (□), M351L (●), and M358V (○) were incubated with 30 mM H_2O_2 at 25 °C and pH 7.0 for 90 min. Oxidation reactions were quenched by the addition of bovine catalase, and samples were assayed for inhibitory activity against porcine pancreatic elastase (PPE). The data were fit to a first-order kinetic model (solid and broken lines).

pH-Dependent Methionine Oxidation. The mechanism by which α1-antitrypsin's reactive methionines are oxidized was studied at pH 7 because this is approximately the pH at which the protein normally functions in human blood plasma. However, greater insight into the relationship between protein structure and amino acid reactivity can be obtained under varied environmental conditions. Therefore, the extent to which methionine reactivity is affected by solution pH was investigated.

Peptide mapping was used to assay methionine oxidation in α1-antitrypsin that had been exchanged into buffers ranging from pH 5 to 9 and exposed to 21 mM hydrogen peroxide for 2.5 h (Figure 5). Between pH 6 and 9, the extent to which Met1 and Met242 were oxidized increased with pH, while the extent to which Met351 and Met358 were oxidized was not statistically different (Figure 6). At pH 5, all of the protein's methionine residues were susceptible to oxidation, with Met220 and Met221 exhibiting the sharpest increase in reactivity. Because Met220 and Met221 are adjacent to each other on the same peptide generated from endoproteinase Lys-C cleavage of α1-antitrypsin (Figure 6), the extent to which each individual residue was oxidized could not be conclusively determined. However, there was no accumulation of the mono-oxidized form of the peptide (L17ox, Figure 6B), thus indicating that both residues were oxidized to nearly the same extent at pH 5.

DISCUSSION

A challenge in the biotechnology industry is the development of strategies for the stabilization of therapeutic proteins against structure-related forms of degradation such as aggregation and chemical modification. However, a detailed understanding of these reactions is important not only in biotechnology but also in biology and medicine. An important example is the oxidative inactivation of α1-antitrypsin, which has consequences for the use of the protein as a therapeutic, and for its role in the pathogenesis of pulmonary emphysema (*33*) and rheumatoid arthritis (*34*). In this study, we examined the oxidation of α1-antitrypsin's methionine residues between pH 5 and 9 to obtain insights into the relationship between this protein's structure and its oxidation susceptibility.

Previous studies have shown that two to four of α1-antitrypsin's methionines are susceptible to oxidation (*35*). Four oxidized residues were identified in α1-antitrypsin recovered from inflammatory synovial effusions (*34*) and lung secretions of smokers (*19*), but their identities were uncertain. We previously developed a peptide mapping method that allowed us to determine that five of recombinant α1-antitrypsin's 10 methionine residues (Met1, Met226, Met242, Met351, and Met358) are susceptible to oxidation by hydrogen peroxide at pH 7 (*21*). Met1, which is not part of human α1-antitrypsin's primary sequence, is located at the protein's N-terminus and is apparently retained during bacterial expression as a result of inefficient intracellular processing of the Met-Asp N-terminal sequence (*36*, *37*). This means that Met226, Met242, Met351, and Met358, which are all part of human α1-antitrypsin's primary sequence and located in or near the reactive center loop (RCL) (Figure 1), are the methionines oxidized in the lungs and at sites of inflammation.

Met358 and Met351 assume the P1[1] and P8 positions in the RCL, respectively. Because of their solvent-exposed structural locations, these two residues are reactive with most oxidants and have well-established oxidation susceptibility (*35*). This is not true of Met226 and Met242, however. Taggart et al. (*16*) only recently reported the reactivity of Met226, and we were the first to identify the reactivity of Met242 (*21*). Both of these residues are located near the RCL, but only Met226 makes direct contact; its backbone amide hydrogen bonds with the side chain of Glu354 to help stabilize an extended β-strand conformation in the crystal structure of wild-type α1-antitrypsin [PDB entry 1QLP (*38*)]. We examined α1-antitrypsin's two most recently published high-resolution crystal structures, that of the wild-type protein (1QLP) and that of an Ala70 → Gly (A70G) mutant (*26*) (PDB entry 1HP7), to determine whether the reactivity of these methionines could be determined on the basis of solvent exposure. The A70G and 1QLP structures, however, indicated different degrees of methionine side chain solvent exposure (Figure 7 and Table 2), especially for Met226. Therefore, a relationship between methionine reactivity and solvent exposure could not be established from crystal structure analysis, and other modes of oxidation needed to be investigated.

The proximity of Met226 to the RCL suggested that this residue's oxidation susceptibility might result from structural changes that accompany the oxidation of either Met351 or Met358. This type of coupled oxidation mechanism has been identified in both recombinant G-CSF (*29*) and recombinant Factor VIIa (*28*). The relevance of this mechanism to methionine oxidation in α1-antitrypsin was investigated by oxidation kinetic analysis of α1-$AT_{(C232S)}$ as well as M351L and M358V mutants. As shown in Figure 2, all reactive methionines in α1-$AT_{(C232S)}$ were oxidized in a bimolecular,

[1] Using the nomenclature of Shechter and Berger (*38*), the P1−P1′ peptide bond is that which is cleaved by the protease. Residues N-terminal to this bond are designated P2, P3, and so on, and those C-terminal to this bond are designated P2′, P3′, and so on.

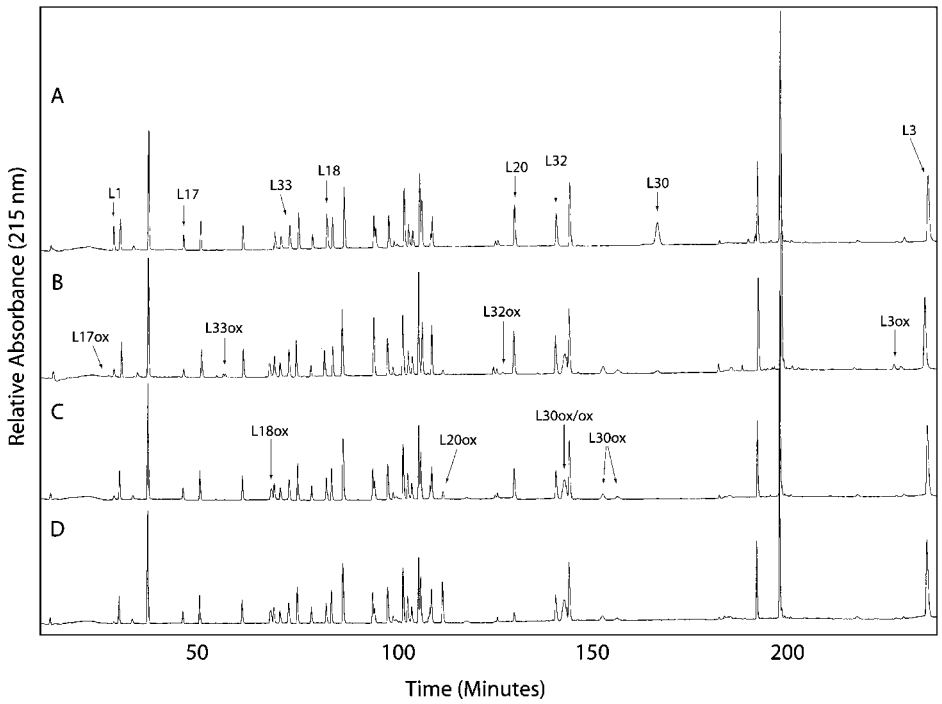

FIGURE 5: Peptide maps α1-AT$_{(C232S)}$ oxidized at various pHs (25 °C). (A) Nonoxidized α1-AT$_{(C232S)}$, (B) α1-AT$_{(C232S)}$ oxidized at pH 5.0, (C) α1-AT$_{(C232S)}$ oxidized at pH 7.0, and (D) α1-AT$_{(C232S)}$ oxidized at pH 9.0. Peptides are labeled according to the methionine(s) that they contain: L1, Met1; L3, Met63; L17, Met220 and Met221; L18, Met226; L20, Met242; L30, Met351 and Met358; L32, Met374; and L33, Met385. Peptides labeled with ox contain a single oxidized methionine. L30ox/ox contains both oxidized Met351 and oxidized Met358. The chromatographic conditions, as well as the identity of each mono-oxidized form of L30, are described elsewhere (*21*).

rather than coupled, mechanism. Similarly, methionine reactivity in M351L and M358V mutants was bimolecular and unaffected by oxidation resistance at residues 351 and 358 (Figure 3). These results show that oxidation in the active site region is determined by the extent to which each reactive methionine's side chain sulfur atom is exposed to solvent.

In many cases, the reactivity of a methionine that is not oxidized in a coupled mechanism is intuitive from crystal structure analysis (*39*−*41*). As already discussed, however, this is not the case for α1-antitrypsin. A large part of the reason that methionine oxidation in α1-antitrypsin cannot be adequately predicted from crystal structure analysis seems to be that serpin crystal structures differ in their depiction of the active site region due to specific crystal contacts made by the highly flexible RCL (*42*, *43*). It has been shown that crystal contacts made by α1-antitrypsin's RCL in the A70G structure differ from those of the 1QLP structure, thus producing the very different RCL conformations shown in Figure 9 (*26*). This is the reason that the reactivity of Met226 and the difference in reactivity between Met351 and Met358 (Table 1) could not be predicted a priori from analysis of the 1QLP and A70G structures. Nonetheless, the results of this work indicate that the average solution structure at neutral pH necessarily has a conformation in which methionine solvent exposure is directly related to methionine reactivity. Therefore, evaluation of both crystal structures within the context of the experimental data can provide important insights into the RCL's conformation.

The simplest means of relating reactivity to solvent exposure is to plot the solvent-exposed surface area of a particular methionine's sulfur against the bimolecular rate constant for the residue's reaction with hydrogen peroxide. This was done for both the 1QLP and A70G structures, but only the A70G structure provided the correct qualitative relationship (Figure 8).

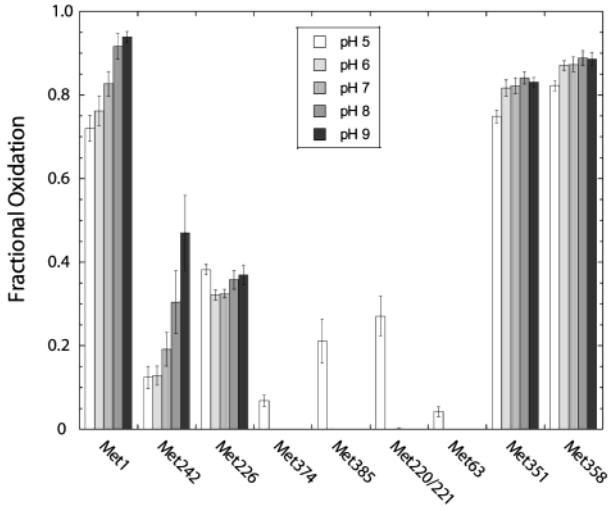

FIGURE 6: Methionine oxidation as a function of pH. α1-AT$_{(C232S)}$ was exchanged into buffers ranging from pH 5 to 9 and then oxidized with 21 mM H_2O_2 at 25 °C. After 2.5 h, samples were removed, desalted, and digested with endoproteinase Lys-C. Digest peptides were separated by reversed-phase chromatography, and the fractional oxidation of each methionine was calculated by dividing the area of the peptide containing the oxidized form of a particular methionine residue by the combined areas of the peptides containing the reduced and oxidized forms of that residue. For methionine residues in which corresponding oxidized peptides were not recovered by reversed-phase chromatography [Met1 (L1) and Met220/221 (L17)], fractional oxidation was determined by dividing the area of the peptide corresponding to the reduced form of the residue by the area of the same peptide obtained from a nonoxidized α1-antitrypsin sample. Error bars represent the standard deviation of six experiments.

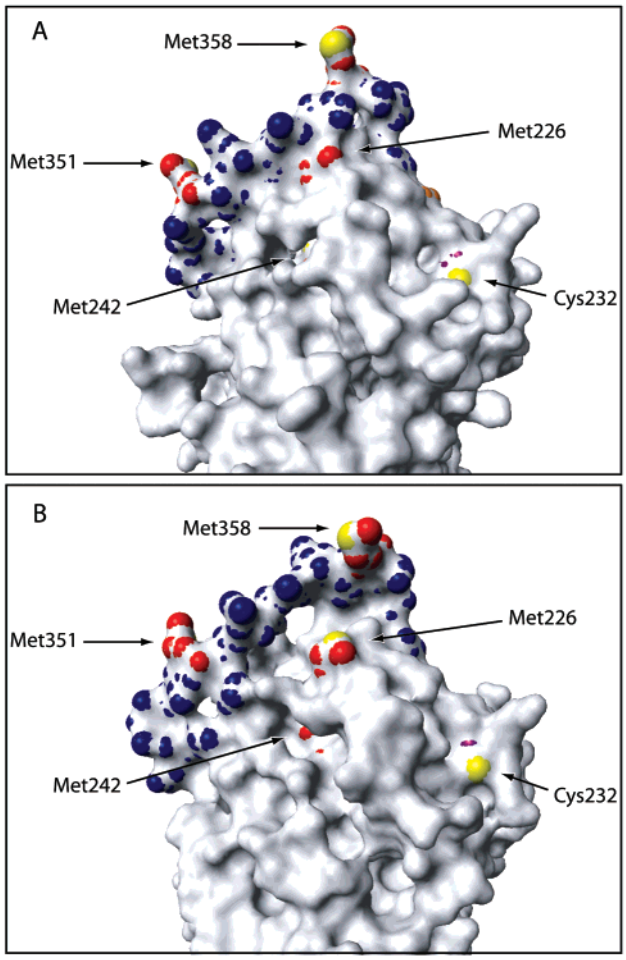

FIGURE 7: Recombinant human α1-antitrypsin's solvent accessible surface. The solvent accessible surface as seen by a molecular probe with the radius of water (1.4 Å) is shown for PDB entries (A) 1QLP (*12*) and (B) 1HP7 (*26*). Methionine (Met) carbon is in red; cysteine (Cys) carbon is in purple, and sulfur atoms are in yellow. RCL residues (344−365), other than Met351 and Met358, are shown in blue. The crystal structures are slightly out of alignment to more clearly show side chain sulfur atoms. Molecular surfaces were generated using WebLab ViewerPro (Accelrys).

Table 2: Methionine Side Chain Sulfur Atom Solvent Accessible Surface Areas (SASAs) Calculated from Two Different Crystal Structures (PDB entries 1QLP and 1HP7)

	SASA (Å^2)	
	1QLP	1HP7
Met1	NA[a]	NA
Met226	0.0	14.8
Met242	1.3	0.0
Met351	41.7	29.9
Met358	41.5	37.7

[a] Met1 is not defined by X-ray data.

Analysis of both crystal structures suggests that the A70G structure, but not the 1QLP structure, provides the correct relationship between the reactivity of Met351 and that of Met358 due to the conformation of the RCL's proximal hinge region, which consists of residues 342−350 (Figure 9). In the A70G structure, the hinge region makes tight contacts with the body of the molecule until a sharp turn occurs (Figure 9B). Because this conformation seems to render Met351 slightly less solvent accessible than does the hinge conformation of the 1QLP structure (Figure 9A), it would

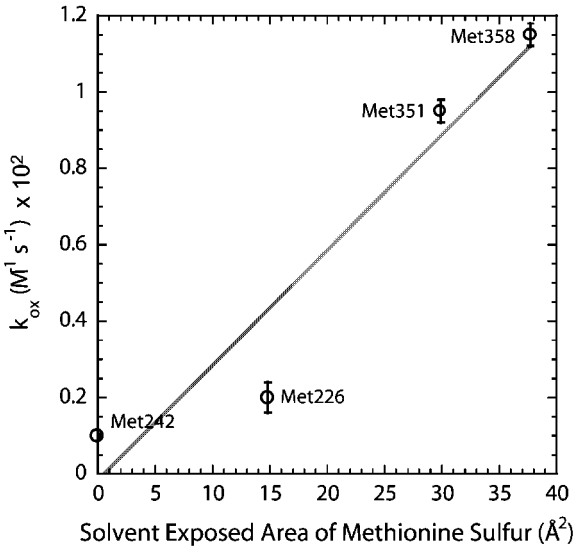

FIGURE 8: Methionine reactivity as a function of solvent-exposed sulfur surface area. Solvent accessible surface areas for Met226, Met242, Met351, and Met358 sulfur atoms were calculated as described in Materials and Methods. Experimental bimolecular rate constants for the reaction between each methionine residue and hydrogen peroxide are shown in Table 1. The line represents the relationship between solvent accessibility and oxidation rate constant obtained from a linear regression [$F(x) = mx + B$] in which the sum of the squared deviations between the data and the minimized fit provides an R^2 value of 0.91. For reference, the solvent-exposed surface area of free methionine's sulfur atom is 43 Å^2 (*44*).

be possible to suggest that the A70G structure better reflects the hinge region's solution conformation solely on the basis of our experimental data. However, Kim et al. (*26*) made the same suggestion on the basis of the fact that residues 342−349 are not stabilized by specific crystal contacts in the A70G structure.

The solvent exposure of Met226 in the 1QLP structure differs from that of the A70G structure as a result of Met226 solvent shielding by RCL residues 355−357. The proximity of these residues to Met226 is largely determined by the side chain interactions of Glu354. In the 1QLP structure, Glu354 appears to form salt bridges with Arg196, Arg223, and Arg281 (Figure 9A), while in the A70G structure, it forms contacts with α1-antitrypsin's main structure via water-mediated interactions with Glu199 and Asp202 (Figure 9B, water interactions not shown). The electrostatic interactions of Glu354 in the 1QLP structure stabilize an extended *β*-strand conformation, which has been proposed to be the RCL's conformation under physiological conditions (*38*, *45*). The reactivity of Met226, however, is more consistent with the RCL conformation depicted by the A70G structure. In this regard, it is also noteworthy that oxidation of Met226 to a highly polar sulfoxide does not alter the RCL conformation to the extent that inhibitory activity is affected (Figure 4). Like the reactivity of Met226, this result is more intuitive from the separation between Met226 and the RCL in the A70G structure than it is from the ∼4 Å separation between Met226 and the RCL in the 1QLP structure.

On the basis of the reactivities of Met226 and Met351, we suggest that the A70G crystal structure may provide an accurate representation of both the RCL's hinge conformation and the side chain interactions of Glu354 at neutral pH. This hypothesis, and the experimental methionine oxidation data

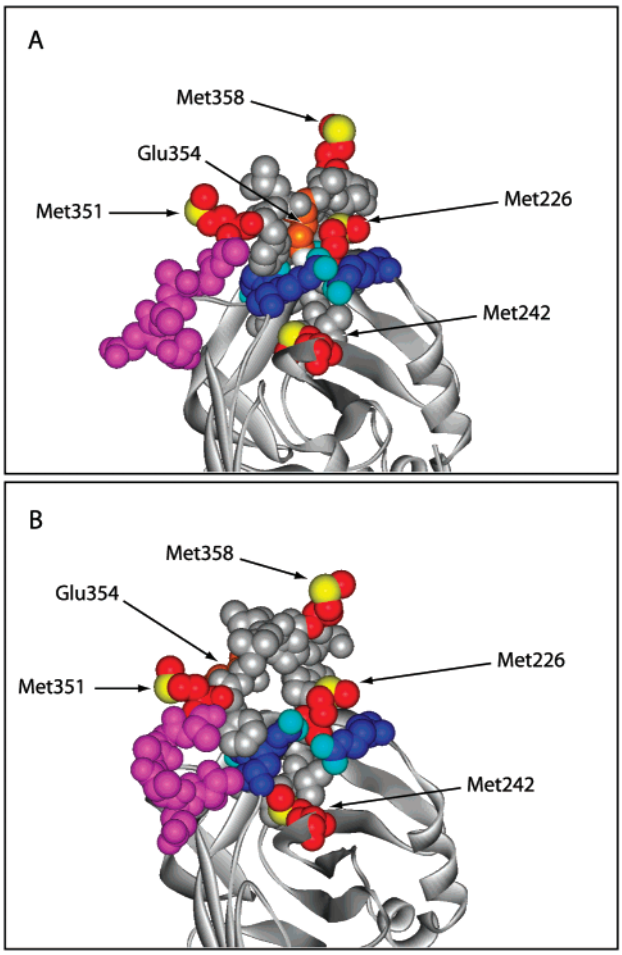

FIGURE 9: Crystal structures of recombinant human α1-antitrypsin's RCL. Ribbon diagrams of PDB entries (A) 1QLP (*12*) and (B) 1HP7 (*26*) are shown with a space-filling representation of RCL residues (342−365), Glu354, Arg196, Arg223, Arg281, and Met242. Methionine (Met) carbon is in red. Arginine (Arg) carbon is in dark blue. Glutamate (Glu) carbon is in orange. Sulfur atoms are in yellow. Nitrogen atoms are in light blue. Glu354 oxygen is in white. The proximal hinge residues (342−350) are colored lavender.

presented, should be of use in further studies regarding the structure of α1-antitrypsin's active site.

Met242, which is the least reactive of α1-antitrypsin's oxidation susceptible methionines, has approximately the same amount of solvent exposure (∼10 Å^2) in both the 1QLP and 1HP7 structures, although only the 1QLP structure indicates exposure of the sulfur atom, and this exposure is quite minimal (Table 2). Nonetheless, we have found that this residue is reactive with hydrogen peroxide. The most likely reason Met242 was not identified as being susceptible to oxidation in other in vitro oxidation studies is that this residue has limited solvent accessibility, and many studies have used *N*-chlorosuccinimide (NCS, MW = 134 Da) as an oxidant. Because NCS is a relatively large molecule, it is not as likely as hydrogen peroxide (MW = 34 Da) to react with methionines that are not highly solvent exposed.

The oxidation kinetics determined at pH 7 provided insights into methionine reactivity when α1-antitrypsin's structure is reflective of that found under physiological conditions. However, a salient feature of the serpin family is that each member has an inherently low barrier to conformational change. Therefore, environmental conditions such as pH (*11*), ionic strength (*46*), and temperature (*47*) can result in conformational changes. Previous work used near-UV circular dichroism to show that pH does not affect α1-antitrypsin's tertiary structure between pH 5 and 10.5 (*48*), which is consistent with the fact that the protein maintains full biological activity in this pH range (*48*, *49*). We, however, found that local conformational changes do occur at acidic and alkaline pH, and these changes affect the reactivities of certain methionine residues.

Between pH 6 and 9, Met1, which is located at the solvent-exposed N-terminus, and Met242 exhibited significant pH-dependent reactivity. Although the reactivity of Met226 does increase somewhat, the increase is not substantial. From these data, it is clear that the structural environment around Met242 readily undergoes a conformational change at alkaline pH, which allows this residue to become highly reactive with hydrogen peroxide.

At pH 5, conformational changes occur that alter the reactivity of many of α1-antitrypsin's methionines, especially Met220, Met221, and Met385. Met220 and Met221 are both located on strand s3C of the C β-sheet (Figure 1). This strand is located in the gate region of the protein that undergoes conformational change when the RCL inserts into the A β-sheet without prior cleavage (*50*). Met385 is located on strand s5B of the B β-sheet (Figure 1), which is part of the shutter region of the protein that is important in facilitating opening of the A β-sheet (*51*). The reactivity of these residues indicates a significant conformational change that involves the RCL and A β-sheet.

These data show that methionine reactivity in α1-antitrypsin is largely dictated by subtle changes in the protein's environment. Of particular importance is the effect of alkaline pH on the reactivity of Met242. If other environmental factors are capable of eliciting a similar increase in reactivity, then oxidation under physiological conditions may occur much more readily than indicated by our work at pH 7 and 25 °C. This may be significant if Met242 oxidation does in some way play a role in one of α1-antitrypsin's diverse biological functions.

In summary, we present here a quantitative analysis of methionine oxidation in α1-antitrypsin that provides new insights into the reactivities of Met226, Met242, Met351, and Met358. These insights have been applied to α1-antitrypsin's crystal structure in an effort to understand the structural factors that affect methionine oxidation and to benefit future studies regarding the protein's RCL conformation. We have also investigated the relationship between pH and methionine oxidation and found that pH significantly affects methionine reactivity in regions of the molecule that are subject to conformational change. This may have consequences for physiological oxidation if other environmental stresses are capable of perturbing the protein's structure in a similar manner. Because all of these results are directly related to aspects of α1-antitrypsin's structure, this study should be useful to the consideration of methionine oxidation in other metastable and conformationally flexible proteins that are important in both biotechnology and medicine.

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