Oxidation of Methionine Residues in Antithrombin

EFFECTS ON BIOLOGICAL ACTIVITY AND HEPARIN BINDING

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Commercially available human plasma-derived preparations of the serine protease inhibitor antithrombin (AT) were shown to contain low levels of oxidation, and we sought to determine whether oxidation might be a means of regulating the protein’s inhibitory activity. A recombinant form of AT, with similarly low levels of oxidation as purified, was treated with hydrogen peroxide in order to study the effect of oxidation, specifically methionine oxidation, on the biochemical properties of this protein. AT contains two adjacent methionine residues near the reactive site loop cleaved by thrombin (Met314 and Met315) and two exposed methionines that border on the heparin binding region of AT (Met17 and Met20). In forced oxidations with hydrogen peroxide, the methionines at 314 and 315 were found to be the most susceptible to oxidation, but their oxidation did not affect either thrombin-inhibitory activity or heparin binding. Methionines at positions 17 and 20 were significantly oxidized only at higher concentrations of peroxide, at which point heparin affinity was decreased. However at saturating heparin concentrations, activity was only marginally decreased for these highly oxidized samples of AT. Structural studies indicate that highly oxidized AT is less able to undergo the complete conformational change induced by heparin, most probably due to oxidation of Met17. Since this does not occur in less oxidized, and presumably more physiologically relevant, forms of AT such as those found in plasma preparations, oxidation does not appear to be a means of controlling AT activity.

More recently, it was shown that oxidation of human growth hormone had no effect on binding to lactogenic receptors, whereas oxidation of the closely related human chionic somatomammotropin on nonhomologous methionines caused a loss of binding to these receptors (6). Methionine oxidation may also lead to an increase in biological activity, as in the case of the C5 component of complement (7), or to no apparent change in structure or activity, such as with α2-plasmin inhibitor (8).

One of the most thoroughly studied examples of methionine oxidation and its effect on activity involves the α1-protease inhibitor (α1-PI). This protein can be oxidized on two of its eight methionine residues, and oxidation of one of these residues (Met358) causes an almost complete loss of inhibitory activity of α1-PI toward its primary biological target, elastase (9). As in the case of chymotrypsin, the relevant methionine in α1-PI is proximal to the active site for binding of the target protease, in this case the PI residue within the reactive loop of the protease cleavage site. Oxidation of this methionine in vivo has been suggested to contribute to the development of emphysema in cigarette smokers through a change in the equilibrium between lung elastase and its inhibitor, ultimately resulting in increased elastase activity (10, 11). Such an imbalance is known to promote emphysema in cases of inherited α1-PI deficiency, particularly among those who smoke (12, 13). It has been proposed that methionine oxidation may actually be a general means of physiological inactivation of plasma protease inhibitors (1).

Here we have examined the susceptibility to oxidation of a plasma protease inhibitor closely related to α1-PI, namely antithrombin (AT). Like α1-PI and other serpins, AT has a reactive loop structure that binds to and then is cleaved by the target protease (for a review, see Ref. 14). In the case of AT, however, the reactive loop is partially inserted into the protein, based on x-ray crystal structure (15, 16). It has been proposed that the binding of heparin causes a conformational change that results in activation of AT as an inhibitor (17). This conformational shift also changes the environment of tryptophan residues within AT, causing an increase in fluorescence (18, 19).

There has been one report of oxidative inactivation of AT (20); however, the sites(s) and extent of oxidation were not characterized in this study. AT contains 12 methionines, five of which are relatively exposed on the surface of the protein. Within the three-dimensional structure, two of these methionine residues (Met314 and Met315) are positioned adjacent to the reactive loop of AT, which is cleaved by thrombin between Arg393 and Ser394. Another two of the exposed methionines (Met17 and Met20) border on the heparin binding site of AT. The

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The abbreviations used are: PI, protease inhibitor; AT, antithrombin; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry.

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three-dimensional structure of AT is quite similar to α1-PI, which, as discussed above, is inactivated by oxidation of a methionine within its reactive loop. If, as suggested by Swaim and Pizzo (1), methionine oxidation serves as a general means of plasma protease inhibitor inactivation, it might be expected that oxidation of Met314 and/or Met315 in AT could be a means of physiologically down-regulating its activity. We report here on the relative sensitivity of these methionine residues to oxidation and the resulting effects on AT in terms of both thrombin-inhibitory activity and heparin binding.

**EXPERIMENTAL PROCEDURES**

Antithrombin—Antithrombin used in the forced oxidation experiments was recombinant human AT purified from the milk of transgenic goats as described in Ref. 21. Commercially available preparations of human plasma derived AT were obtained from Behringwerke (Kybernin) and Miles (Thrombate III). Nonoxidized AT refers to samples that have not been treated with hydrogen peroxide but may contain some oxidized material. The term “unoxidized” is used here to refer to protein (or peptide) that has been shown to contain no oxidation, for example the A form in Fig. 1.

Hydrogen Peroxide-Induced Oxidation of AT—Purified recombinant AT was oxidized to varying degrees using hydrogen peroxide (Sigma catalog no. H-1009) essentially as described by Teh et al. (6). Each reaction contained 200 μg of AT in a total volume of 150 μl (23 μM AT) in 100 mM NH4HCO3, 0.5 mM EDTA, pH 8.0, with varying concentrations of hydrogen peroxide as indicated. All reactions were carried out for 30 min on ice with two exceptions. The samples referred to as 400/60/ice and 400/60/RT were incubated for 60 min in 400 mM peroxide either on ice or at room temperature, respectively. The reaction was stopped by the addition of immobilized catalase (Sigma catalog no. C-9208), which was subsequently removed by filtration (Rainin Microfilterno. 7016–022).

Separation of Oxidized Forms by High Performance Liquid Chromatography (HPLC) —Samples of AT oxidation reactions were applied to a C4 HPLC column (2.1 × 250 mm; 5 μm; narrow bore; Vydac catalog no. 214TP5251) equilibrated in 75% solvent A (0.1% trifluoroacetic acid), 25% solvent B (acetonitrile in 0.08% trifluoroacetic acid) at a flow rate of 0.3 ml/min and eluted with solvent B using a series of linear gradients as follows: 0–5 min 25–40% solvent B, 5–20 min 40–45% solvent B, 20–25 min 45–80% solvent B.

The eluted protein was monitored by absorbance at 215 and 275 nm. For preparative HPLC, for elutions involving isolation of the A, B, and C forms of oxidized AT, solvent B was held constant at 45% for 5 min (20–25 min) followed by the 45–80% increase. Fractions were collected automatically on a Gilson FC203B fraction collector.

Endoproteinase Lys-C Mapping of Oxidized Forms of Recombinant AT—Samples of oxidized AT (50 μg) were reduced and pyridylethylated in denaturing buffer (0.25 M Tris, 6.0 M guanidine HCl, pH 8.6) containing 1% sodium dodecyl sulfate (SDS) at a ratio of 1000:1 (SDS/lysozyme) and 4-5% acrylamide and were loaded onto a 4% polyacrylamide gel. The gel was stained and destained and then scanned by autoradiography. The eluted protein was monitored by absorbance at 215 nm.

LC/MS Analysis of Proteolytic Digests—LC/MS was carried out using a Finnigan TSQ 700 triple quadrupole mass spectrometer (San Jose, CA) interfaced through a Finnigan electrospray ionization source with a Microm Mass Resources UMA HPLC system (Auburn, CA). Peptide mapping was carried out on 0.5–1.0 mmol of AT Lys-C digests, which were separated using a Microm column (1.0 × 100 mm) packed with Vydac C8 resin. The flow rate was 50 μl/min, and peptides were eluted with a gradient of 0–46% acetonitrile in 0.1% trifluoroacetic acid. HPLC eluant from the UV detector was mixed with methoxyethanol (Pierce/methanol/Burdick & Jackson, Muskegon, MI) (3:1) at 25 μl/min through a “T” union before entering the electrospray interface. Centroid mass spectra were acquired over an m/z range of 200–3000 at a 3-s scan rate.

Analysis of Oxidation Sites on the K30 Lys-C Peptide—Unoxidized, singly oxidized, and doubly oxidized forms of the K30 Lys-C peptide were collected from Lys-C maps of AT that had been partially oxidized with hydrogen peroxide, and the resultant material was analyzed using an abbreviated gradient similar to that described above but with the second and third segments shortened to 12 and 8 min, respectively. The isolated peptides (1–2 nmol) were dried, reconstituted in 70% formic acid, and digested with CNBr (CNBr/Met molar ratio of 200:1) for 18 h in the dark at room temperature with a nitrogen overlay. Reacted peptides were then dried under nitrogen, reconstituted in 1% trifluoroacetic acid, and analyzed by LC/MS as described above except that a Vydac C18 column (1.0 × 100 mm) was used with a gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid over 30 min.

AT Activity Assay (Thrombin Inhibition)—AT activity was assayed as described previously (21) by measuring inhibition of thrombin (Calbiochem catalog no. 605180; 243 milliunits/ml in the assay)-catalyzed cleavage of the thrombin-specific chromogenic substrate S2238 (Kabi; 337 μM in the assay). A saturating concentration of heparin (55 nM; porcine; Sigma catalog no. H-3393) was used except where noted. In calculations of the molar concentration of heparin, the molecular mass was assumed to be 13,500 daltons for this heparin preparation.

**RESULTS**

Shift in Reverse Phase Mobility of AT with Oxidation—Incubation of AT with hydrogen peroxide resulted in a concentration-dependent shift in the reverse phase HPLC mobility of the protein to shorter retention times. We used this forced oxidation as a means of characterizing the sites on AT that are most sensitive to oxidation and to study the effect of oxidation on protein function. We refer here to the predominant peak prior to oxidation as the A form of the protein and to the two earlier eluting peaks that increase with oxidation as the B and C forms, as shown in Fig. 1. The use of the term “unoxidized” for protein forms with increasing oxidation suggests that each peak represents a distinct oxidized form of the protein. Quantitation of the peaks in Fig. 1 by integration of absorbance (inset) illustrates the transition from the A to the B to the C forms with increasing peroxide concentration. Other relatively minor peaks eluting even earlier than the C form were...
methionine residues (Met$^{314}$ and Met$^{315}$) are exposed at the surface of the protein and are in fact adjacent to the active site loop that is cleaved during serpin inhibition, based on the three-dimensional structure of AT (Fig. 3). The tryptophan residue in K30 (Trp$^{307}$) is partially exposed in the intact protein (not seen in Fig. 3, since it is on the opposite side of the protein) and therefore could be oxidized; however, tryptophan oxidation is less likely under the conditions used here. The K2 peptide contains two methionines (Met$^{17}$ and Met$^{20}$), which are both exposed at the surface of the intact protein and are at one end of the binding site for heparin, which stretches along helix D (Fig. 3).

To determine the specific sites of oxidation on K30, the three singly oxidized peaks and one doubly oxidized peak of the K30 peptide were collected along with unoxidized K30 from Lys-C peptide maps of partially oxidized AT as shown in Fig. 4A. The three singly oxidized peaks were designated x, y, and z for the purposes of this analysis. Each of the isolated peptides was subjected to CNBr digestion, which would be expected to result in four peptide fragments in the case of unoxidized K30 (M1–M4; Fig. 4B). These digests were analyzed by LC/MS using a short reverse phase HPLC gradient, and the observed masses for each digest are listed in Table I. Oxidation of a given methionine residue should not only result in an increase of 16 mass units for peptides containing that methionine, it should also prevent cleavage at that site and therefore result in the absence of specific peptides in the digest (23). CNBr digestion was not complete in all cases as evidenced by the presence of M(2–3), M(3–4), and M(2–4) in the digest of unoxidized K30. Also, M2 is a single amino acid and is therefore too small to be detectable by this analysis. Nevertheless, the absence of M3 in the singly oxidized (x) digest, along with the fact that M1 and M4 were present, indicates that cleavage did not occur at Met$^{315}$ and therefore that this methionine was oxidized. This is supported by the fact that M(2–3) and M(2–4) displayed an increase of 16 mass units in this digest. Similarly, M1 was absent in the singly oxidized y and z digests, while M3 and M4 were present, indicating that oxidation of Met$^{314}$ blocked cleavage between M1 and M2. Once again this was supported by a 16-mass unit increase in M(1–2) and M(1–3). The digest of the doubly oxidized K30 peptide contained only M(1–3) (plus 32 mass units) and M4, indicating that both Met$^{314}$ and Met$^{315}$ are oxidized in this peptide. The absence of an M1 + 16 mass units peptide in any of the oxidized K30 peaks demonstrates that Trp$^{307}$ is not oxidized under these conditions.

As shown in Table I, the y and z forms of K30 resulted in identical sets of peptides in the CNBr digest but were clearly separated on the HPLC of the original peptide map (Fig. 4A). The most likely explanation for this is that these two peptides are stereoisomers with respect to the added oxygen atom on the chiral sulfur of Met$^{314}$. Such separation of stereoisomers of oxidized peptides by HPLC has been reported previously (24). In some maps, peak splitting was also seen for oxidation at Met$^{315}$ (peak x), although this was not as distinct as for the peptide oxidized at Met$^{314}$. Peak splitting of doubly oxidized K30 was consistently observed in the maps (see Figs. 2 and 4A), presumably for the same reason.

**Presence of Oxidized Forms in Commercial Preparations of AT**—Two different commercially available preparations of AT (Kybernin from Behringwerke and Thrombate III from Miles) as well as transgenic recombinant AT (Genzyme Transgenics Corp.) were tested for the presence of oxidized forms (Fig. 5) employing the reverse phase HPLC method used in Fig. 1. Each of these preparations contained the B, and to a lesser extent C, oxidized forms of AT. The A, B, and C forms of AT were isolated in this manner from both a peroxide-oxidized apparent following treatment with the highest concentrations of peroxide.

**Identification of Oxidation Sites on AT**—Samples of oxidized AT from the reactions represented in Fig. 1 were reduced, alkylated, and digested with Lys-C endopeptidase. The resulting digests were fractionated by HPLC, and these peptide maps are shown in Fig. 2A. The only two peptides significantly affected by oxidation were the K2 peptide (residues Pro$^{12}$–Lys$^{28}$ in AT) and the K30 peptide (residues Glu$^{298}$–Lys$^{332}$ in AT). The peaks corresponding to these peptides and their oxidized counterparts were identified by LC/MS analysis of the digests as shown for the K30 peptide in Fig. 2B. There was a substantial decrease in the amount of unoxidized K30 peptide in the digest as the peroxide concentration was increased, and there was a corresponding increase in peaks having somewhat shorter retention times. LC/MS analysis of the digests (Fig. 2B) revealed that the three peaks eluting at 94–96 min in the oxidized samples correspond to K30 plus 16 mass units (singly oxidized K30), and the overlapping peaks at ~88 min correspond to K30 plus 32 mass units (doubly oxidized K30). There is a similar change in the K2 peptide, although at higher peroxide concentrations, and peaks corresponding to singly and doubly oxidized K2 were again shown to be present using mass spectrometry. From these experiments, it appears that the K30 region of AT is the most sensitive to oxidation, followed by the K2 region.

The K30 peptide from AT contains three methionines and one tryptophan as possible sites of oxidation. One of the methionines (Met$^{120}$) is buried within the core of the intact protein and is therefore an unlikely target of oxidation. The other two
sample of recombinant AT (50 mM peroxide) and one that had not been treated with peroxide. They were each then Lys-C-mapped as described above. The peptide maps for the three isolated forms from the untreated sample (Fig. 6) were comparable with those for the three forms isolated from the peroxide-treated sample (data not shown), with oxidation again occurring primarily in the K2 and K30 regions of the protein. The fact that oxidation was consistently seen in AT isolated from both plasma and milk suggested that oxidation may occur in vivo as a means of controlling the activity of this protein.

Peaks corresponding to unoxidized, singly oxidized, and doubly oxidized K2 and K30 in Fig. 6 were integrated for each of the three isolated forms of intact AT, and the results are shown in the bar graphs to the right. The amount of each oxidized form for each peptide is plotted relative to the total amount of that peptide. These data clearly show that the B and C forms correlate well with singly and doubly oxidized K30, respectively, indicating that it is oxidation of K30 that is primarily responsible for the shift in retention time seen for intact AT with oxidation (Fig. 1).

Effect of Oxidation on Antithrombin Activity—As mentioned above, the K30 region of AT contains two adjacent methionines on the surface of the protein (Fig. 3), which are in close proximity to the reactive loop of AT that binds to thrombin and is subsequently cleaved. It is, therefore, reasonable to expect that oxidation of one or both of these residues might affect the ability of AT to bind and inhibit thrombin. Samples of untreated (nonoxidized) antithrombin and AT oxidized to various levels were assayed as described under “Experimental Procedures” at multiple concentrations of AT in the presence of saturating concentrations of heparin (Fig. 7A). In the sample of AT that was oxidized using 50 mM peroxide for 30 min on ice, ~80% of the protein was oxidized to at least some degree, based on the data of Fig. 1, yet there was no difference in its ability to inhibit thrombin in this assay. The sample of AT that was oxidized with 400 mM peroxide for 60 min on ice (400/60/ice) was >95% oxidized, but even this preparation was able to inhibit thrombin nearly as effectively as nonoxidized AT in this assay.

The activity assays in Fig. 7A were done in the presence of excess heparin. At less than saturating concentrations of heparin, however, high levels of oxidation on AT were found to result in decreased thrombin-inhibitory activity relative to the
Nevertheless, the preparation of AT that was oxidized (50 mM peroxide) exhibited little if any difference in its requirement for heparin in this assay. At the higher levels of oxidation, there was a clear effect on the requirement for heparin that increased with increasing oxidation. The half-maximal heparin concentrations for thrombin inhibition are 0.49 nM for highly oxidized AT (400/60/RT) and 0.034 nM for the control, based on this assay.

**Effect of Oxidation on Heparin Affinity**—The data of Fig. 7B suggested that the binding of heparin to AT might be affected by oxidation. We therefore studied this more directly using the heparin binding assay of AT based on fluorescence. The inherent fluorescence of AT at 340 nm increases with increasing heparin concentration due to a conformational change in the protein that occurs with the binding of heparin and alters the exposure of one or more buried tryptophan residues (19). This property can be exploited to measure the binding affinity of AT for heparin (22). Less oxidized samples of AT (peroxide concentration ≤50 mM) displayed no significant change in heparin binding relative to the nonoxidized material (Fig. 8A). Since the AT in the 50 mM oxidation was ~80% oxidized within the K30 peptide (Fig. 1; inset), oxidation of the K30 methionines does not appear to affect heparin binding. This is consistent with the fact that the K30 methionines are far removed from the heparin binding site. At higher levels of oxidation (peroxide concentration >50 mM), where increasing oxidation of K2 methionines is observed, there was a shift in the binding curve toward lower maximal fluorescence, and this shift increased with increasing oxidation. This suggests that a high level of oxidation either results in a difference in the heparin-induced conformational change in AT or that it generates a subpopulation of AT that does not bind heparin and, therefore, lowers the overall measured change. The fact that thrombin-inhibitory activity (in the presence of heparin) is relatively unaffected by even high levels of K2 and K30 oxidation indicates that the first alternative is most likely the case.

**FIG. 4. Identification of specific sites of oxidation on K30.** A, reverse phase HPLC of AT that had been oxidized with 50 mM peroxide and then reduced, alkylated, and digested with Lys-C. Peaks corresponding to unoxidized K30 as well as its singly oxidized (K30 + 16; peaks x, y, and z) and doubly oxidized (K30 + 32) forms were collected and subjected to CNBr digestion, which would be expected to result in three peptides and a methionine residue for the unoxidized peptide (B). Cleavage would be blocked at oxidized methionine residues. Digests were analyzed by LC/MS in order to determine sites of oxidation (Table I).

**FIG. 5. Oxidized forms are present in commercial preparations of AT.** C4 reverse phase HPLC analysis of recombinant human AT derived from transgenic goats and two commercially available AT preparations derived from human plasma (Behringwerke Kybernin and Miles Thrombate III). Peak A is unoxidized AT, while peaks B and C represent oxidized forms. AU, absorbance units.
To further assess changes in heparin binding in these samples of AT, a heparin affinity column was used. Nonoxidized and oxidized samples were bound to the column and then eluted using a linear salt gradient (Fig. 8). Both preparations of AT eluted from the column as multiple peaks due to heterogeneity both in the AT itself and in the heparin on the column. However, there was a significant shift in the binding of the oxidized preparation toward lower affinity (elution at decreased salt concentration). It is clear that high levels of oxidation caused a decrease in the ability of AT to bind to heparin. As discussed previously, the K2 region of AT contains two exposed methionine residues that can become oxidized and are near the binding site for heparin on AT (Fig. 3). It is, therefore, likely that oxidation of one or both of these methionines was responsible for this decrease in heparin affinity. The K2 region of AT is considerably more difficult to oxidize than is the K30 region of AT.
In the sample treated with 400 mM peroxide for 60 min on ice while the positive band at 196 nm shows a marginal decrease. Samples of oxidized AT, including 50 mM peroxide (inset at 210 and 220 nm) show an increase in intensity (see Fig. 9A). For the 50 mM peroxide sample, the spectra (Fig. 9B) shows the far UV CD spectrum for AT in its native form and in three different oxidized forms. For AT treated with 50 mM peroxide, the negative bands at 210 and 220 nm show an increase in intensity (see inset), while the positive band at 196 nm shows a marginal decrease. In the sample treated with 400 mM peroxide for 60 min on ice (400/60/ice), the band at 220 nm becomes less intense when compared with the 50 mM oxidized form. For the sample treated with 400 mM peroxide for 60 min at room temperature (400/60/RT), there is a further reduction in the magnitude of the positive band at 196 nm. The spectra also indicate that the positive to negative cross-over point is right-shifted upon oxidation of AT.

Changes are even more pronounced in the near UV region of the spectra (Fig. 9B). For the 50 mM peroxide sample, the changes in the spectra are marginal except for a minor variation in the peak observed at 275 nm. However, for the more oxidized 400/60/ice sample, there is a significant upward shift for all peaks and valleys between 255 and 280 nm, indicating a change in the environment around phenylalanine and/or tyrosine residues. It is also interesting to note that the peaks at 284, 288, 292, and 300 nm are not affected in this sample. Since the peaks for these latter wavelengths usually arise from tryptophan residues, these data would suggest that methionine oxidation does not perturb tryptophan even at relatively high levels of oxidation. In the most highly oxidized sample, 400/60/RT, all peaks are increased somewhat, including those at 292 and 300 nm.

Changes in heparin binding affinity as a result of oxidation were also monitored by both near and far UV spectroscopy. As described under “Experimental Procedures,” a saturating concentration of heparin was added to preparations of AT for these experiments. Changes in the far UV spectra of highly oxidized AT (400/60/RT) with the addition of a saturated level of heparin are virtually the same as those for the nonoxidized control, with only marginal variation except a left shift of the positive peak (around 196 nm) by 1.5 nm when compared with that in the absence of heparin (data not shown).

Near UV spectra of AT-heparin complex on the other hand shows a dramatic upward shift for all bands upon adding heparin to AT. The spectra depicted in Fig. 10 represent the difference in CD between AT alone and AT-heparin complex for the nonoxidized (left panel), moderately oxidized (50 mM peroxide; center panel), and highly oxidized (400/60/RT; right panel) forms. It is clear from these spectra that the upward shift is not affected in the moderately oxidized form but is substantially less in the highly oxidized form, particularly in the 280–300 nm region, which reflects changes in the environment of tryptophan residues. This inverse relationship between the level of oxidation and the relative change in tryptophan band intensity upon binding of heparin is consistent with the fluorescence data presented in Fig. 8A.

To determine which oxidation site(s) might be responsible for the reduced response to heparin binding, the crystal structure of AT (15) was analyzed using INSIGHT graphics software (Molecular Simulations) with particular attention to the residues surrounding the potential oxidized methionine sites. Methionines located at 17, 20, 314, and 315 have been shown here to be the primary sites of oxidation in the protein. Since treatment with 50 mM hydrogen peroxide oxidizes primarily Met314 and Met315 and there is very little change in the heparin response for this sample of oxidized AT, it is unlikely that oxidation of these sites affects the heparin response. Methionines at 17 and 20 become much more oxidized at higher hydrogen peroxide concentrations, at which point the reduced response to heparin is seen in the CD and fluorescence data. However, these two residues are not very close to any aromatic residues; hence, oxidation of these two methionines may not have any direct influence on changes in the spectra due to aromatics. Nevertheless, any change in the conformation of the segment that contains these two methionines could affect the orientation of neighboring aromatic residues. Oxidation of Met30 is unlikely to cause such confomational changes, since its sulfur is well exposed and pointing away from the peptide backbone (from examination of the three-dimensional structure; not shown). The sulfur of Met17, on the other hand, is close to the backbone; thus, oxidation could change the backbone orientation. Since the N-terminal segment containing Met17 borders on the heparin binding site of AT, our analysis strongly suggests that oxidation of Met17 is the major cause for the change in response to heparin binding.

**DISCUSSION**

It is now well established that oxidation of methionine at the elastase cleavage site serves as a means of down-regulating the activity of the serpin $\alpha_1$-PI in the lung. Antithrombin is a serpin closely related in structure to $\alpha_1$-PI but with an Arg-Ser bond at the P1-P1' cleavage site rather than a Met-Ser bond as in the case of $\alpha_1$-PI. Although AT does not contain an oxidizable methionine at this site, it does contain a pair of methionine residues (Met314 and Met315) that are quite close to the Arg-Ser...
cleavage site in the three-dimensional structure of the protein. Also, AT and other serpins have been reported to be inactivated by oxidation (20), suggesting that oxidation might be a general mechanism for down-regulating serpin activity.

Here we have shown that the two methionines at positions 314 and 315 are the most sensitive to oxidation in AT. Oxidation of either methionine alone causes a discrete shift in retention time on reverse phase HPLC to a form that is easily resolved from the unoxidized protein. Oxidation of the second of these two residues causes a further shift to lower retention time, again to a clearly resolved form. Oxidation of other methionines in AT does occur, particularly in the K2 region of the protein, however less readily than on the K30 methionines.

Based on the data presented here for AT incubated with 50 mM peroxide, oxidation of Met\textsuperscript{314} and Met\textsuperscript{315} does not appear to affect either the ability of AT to bind heparin or to inhibit thrombin. However, further oxidation of AT does affect heparin binding to some degree, as evidenced by the heparin affinity column data presented in Fig. SB. Nevertheless, even at very high levels of oxidation, thrombin-inhibitory activity is unaffected in the presence of high heparin concentrations. This would appear to conflict with the earlier study in which AT was inactivated by oxidation (20); however, in this study, the oxidation was carried out with chloramine T in the presence of detergent, and the extent of oxidation was not characterized.

The results of the fluorescence assay presented here (Fig. 8A), in which the maximal change in fluorescence was decreased, suggest that oxidation interferes with the ability of the protein to undergo the conformational change that occurs as a result of heparin binding. This is supported by structural data from CD, which also shows a reduced response to saturating heparin in the highly oxidized sample, particularly in the portion of the spectrum representing changes in tryptophan. Other factors have been shown to influence the ability of AT to bind heparin. In particular, the presence of an oligosaccharide chain on Asn\textsuperscript{135} decreases heparin affinity significantly (25). The form lacking glycosylation at this site (known as the \( \beta \) form) and having a higher heparin affinity represents ~10% of total AT in plasma. Recently, it was proposed that glycosylation of this site decreases heparin affinity by interfering with the conformational change in AT that occurs as a consequence of the initial binding of heparin (26). It is thought that this conformational change results in a much tighter association between heparin and AT. It is possible that the decreased heparin affinity seen here with relatively high levels of oxidation also occurs as a result of interference with this conformational shift.

The two methionines within the K2 peptide (Met\textsuperscript{17} and Met\textsuperscript{20}) border on the binding site for heparin (Fig. 3) but are at the opposite end of the D helix from the Asn\textsuperscript{135} glycosylation site. This helix contains multiple basic residues that participate in the binding of heparin and is thought to be involved in the conformational change that leads to tighter binding. It is likely that the oxidation of one or both of these residues, which occurs only at higher levels of oxidation, interferes with the conformational change and, therefore, the ability of AT to adopt the high affinity conformation. Based on the structure of AT, neither of these two residues is close enough to any tryptophan residue to be directly responsible for the reduced response to heparin resulting from oxidation. Changes in the conformation of the segment containing these two methionines could affect the orientation of neighboring tryptophan residues. However, oxidation of Met\textsuperscript{20} is unlikely to cause such conformational changes, since its sulfur is directed away from the peptide backbone. The sulfur of Met\textsuperscript{17}, on the other hand, is close to the backbone, and thus oxidation could change the backbone orientation. It is therefore likely that oxidation of Met\textsuperscript{17} is the major cause for the change in response to heparin binding.

As mentioned above, it has been proposed that methionine oxidation might be a general means for regulating the activity of proteins. It has clearly been demonstrated that oxidation of an active site methionine inactivates \( \alpha \)-PI and that this is probably a physiologically important means of regulating this inhibitor. However, we have shown here that for antithrombin, oxidation of the two most susceptible methionines, those on K30 near the active site, does not inactivate the protein, nor does it affect the ability of AT to bind heparin. Higher levels of oxidation do affect heparin binding. However, it is unlikely that such high levels would occur physiologically, as indicated by the low level of oxidation seen in preparations of AT from plasma in this study. We conclude that oxidation is not a critical factor in the regulation of AT activity.

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