On the Domain Structure of Antithrombin III. Tentative Localization of the Heparin Binding Region Using ^{1}H NMR Spectroscopy†

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ABSTRACT: The denaturation of human and bovine antithrombin III by guanidine hydrochloride has been followed by ^{1}H NMR spectroscopy. The same unfolding transition seen previously from circular dichroism studies [Villanueva, G. B., & Allen, N. (1983) *J. Biol. Chem. 258*, 14048–14053] at low denaturant concentration was detected here by discontinuous changes in the chemical shifts of the C(2) protons of two of the five histidines in human antithrombin III and of three of the six histidines in bovine antithrombin III. These two histidines in human antithrombin III are assigned to residue 1 and, more tentatively, to residue 65. Two of the three histidines similarly affected in the bovine protein appear to be homologous to residues in the human protein. This supports the proposal of similar structures for the two proteins. In the presence of heparin, the discontinuous titration behavior of these histidine resonances is shifted to higher denaturant concentration, reflecting the stabilization of the easily unfolded first domain of the protein by bound heparin. From the tentative assignment of one of these resonances to histidine-1, it is proposed that the heparin binding site of antithrombin III is located in the N-terminal region and that this region forms a separate domain from the rest of the protein. The pattern of disulfide linkages is such that this domain may well extend from residue 1 to at least residue 128. Thermal denaturation also leads to major perturbation of these two histidine resonances in human antithrombin III, though stable intermediates in the unfolding were not detected.

In the absence of a crystal structure for antithrombin III, attempts to delineate the heparin binding site and more generally to determine details of the three-dimensional structure of the protein have relied on chemical and spectroscopic means. Thus, chemical modifications of arginine and lysine residues have demonstrated that these residues are necessary for the acceleratory effect of heparin on antithrombin III (ATIII)[1] (Rosenberg & Damus, 1973; Pecan & Blackburn, 1984; Jorgensen et al., 1985). There is also a single tryptophan residue that can be modified (out of a total of four) with the result that heparin binding is blocked, whereas the antiprotease activity of ATIII is unaffected (Blackburn & Sibley, 1980; Blackburn et al., 1981). This tryptophan was recently identified as residue 49 (Blackburn et al., 1984). The sequence characterization of two abnormal ATIII molecules that possess normal antiprotease activity but lack heparin cofactor activity, one with an arginine-cysteine substitution at position 47 (Koide et al., 1984) and one with a proline-leucine substitution at position 41 (Chang & Tran, 1986), supports the importance of this region of the polypeptide in heparin binding. In contrast, an antithrombin III fragment lacking this region has been isolated following cyanogen bromide cleavage and found to bind to heparin as well as to possess a site for thrombin binding (Rosenfeld & Danishefsky, 1986).

A rather different approach to investigating the location of the heparin binding site has been taken by Villanueva and Allen (1983a,b). These authors followed the unfolding of human ATIII by guanidine hydrochloride and showed that the process occurs in discrete steps, with formation of stable intermediates (Villanueva & Allen, 1983a). Furthermore, they showed that the less stable domain is made more resistant to unfolding by the presence of heparin, suggesting that this domain may be important in mediating the anticoagulant activity of heparin (Villanueva & Allen, 1983b).

It has recently been shown that the ^{1}H NMR spectra of both human and bovine ATIII contain resonances identified as arising from the C(2) and C(4) protons of histidine residues and that these histidine residues are solvent accessible and are perturbed by heparin binding (Gettins, 1987). These histidine residues are potentially very useful probes for local ATIII conformation and for perturbations resulting from denaturation or ligand binding. We have investigated the effects of guanidine hydrochloride denaturation on the ^{1}H NMR spectra of human and bovine ATIII, both in the presence and in the absence of bound heparin. The results of these studies are presented here, together with the perturbations to the ^{1}H NMR spectrum of human ATIII caused by thermal denaturation, both with and without heparin bound to the protein.

Materials and Methods

Materials. Antithrombin III was isolated from plasma by a modification of the method of Thaler and Schmer (1975) and is described in detail elsewhere (Gettins, 1987). Human ATIII was prepared from outdated plasma obtained from the Vanderbilt Hospital blood bank, and bovine ATIII was prepared from freshly collected citrated blood obtained from a local slaughterhouse.

Ultrapure ammonium sulfate and guanidine hydrochloride were purchased from Schwarz/Mann. Other reagents were obtained from Sigma. The protons of guanidine hydrochloride were exchanged for deuterium by two cycles of freeze-drying

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[1] Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill; Gdn-DCl (GdDCl in figures), deuteriated guanidine hydrochloride; ATIII, antithrombin III; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; SDS, sodium dodecyl sulfate.

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concentrated solutions from D_2O.

Methods. Antithrombin samples for NMR analysis were prepared by dissolving the freeze-dried protein in D_2O. The protein had previously been freeze-dried at a concentration of 10 mg/mL from a 30 mM NaCl/4 mM phosphate buffer so that the NMR samples, at approximately 50 mg/mL protein, were in 150 mM NaCl/20 mM phosphate buffer. Protein concentrations were determined spectrophotometrically using $A^{0.1\%}_{280nm} = 0.65$ (Nordenstrom et al., 1977) and a molecular weight of 58 000 (Petersen et al., 1979; Chandra et al., 1983) for human ATIII and $A^{0.1\%}_{280nm} = 0.60$ and a molecular weight of 56 000 for bovine ATIII (Kurachi et al., 1976). SDS–polyacrylamide gel electrophoresis, to check the purity of ATIII preparations, was carried out in 7% slab gels according to Laemmli (1970).

^1H NMR spectra were recorded on a Bruker AM 400 narrow-bore spectrometer. A Carr–Purcell–Meiboom–Gill pulse train $[90°_x-(\tau-180°_y-\tau)_n]$ was used for the guanidine hydrochloride denaturation studies, with $\tau = 1$ ms and $n = 6$. The probe temperature was maintained at 298 ± 1 K. A sweep width of 6000 Hz and a data block size of 8K points zero filled to 16K points prior to Fourier transformation were employed. pH* values are reported as pH meter readings uncorrected for deuterium isotope effects and were measured in the NMR tube using an Ingold 3-mm diameter combination electrode and an Orion 501 pH meter. Chemical shifts are given relative to external DSS at 0 ppm.

Results

Denaturation of Human Antithrombin III by Guanidine Deuteriochloride. At pH 6.10, the five histidine residues in human ATIII give C(2) proton resonances at 8.43, 8.12, 8.08, 8.05, and 7.83 ppm (Gettins, 1987). These resonances are from histidines with pK_a's of 6.90, 6.00, 5.93, 5.75, and 5.13, respectively (Gettins, 1987), and will be referred to as histidine-1 through histidine-5, respectively, for convenience of reference and to correspond to the numbering of Gettins (1987).

Upon addition of Gdn-DCl to human ATIII, to a denaturant concentration of 0.2 M, the C(2) proton resonance from histidine-1 moves upfield by 0.01 ppm while the resonances from the remaining four histidines titrate downfield by about 0.06 ppm each. An increase in Gdn-DCl concentration to 0.4 M and then to 0.6 M results in similar very small upfield shifts on the resonance from histidine-1 and larger downfield shifts for the other four resonances. A further increase in Gdn-DCl concentration to 0.8 M causes a much more dramatic change. Whereas the resonances from residues 2, 3, and 4 continue to titrate downfield, the resonance from histidine-5 disappears from the region around 7.98 ppm and a new resonance appears at 8.36 ppm. Also, the resonance from histidine-1, which titrated very slightly upfield as a function of Gdn-DCl concentration at low Gdn-DCl concentrations, now titrates downfield at a similar rate to those of the resonances from histidines-2, -3, and -4. At higher Gdn-DCl concentrations, all the histidine C(2) resonances titrate smoothly downfield. The chemical shifts of these resonances are plotted as a function of Gdn-DCl concentration in Figure 1. Figure 2 shows the aromatic region of the ^1H NMR spectra of human ATIII at Gdn-DCl concentrations above and below the discontinuity.

The experiment was repeated with human ATIII to which 1 equiv of porcine heparin had been added. Heparin itself causes perturbation of the histidine resonances upon binding, as has been reported previously (Gettins, 1987). At low Gdn-DCl concentrations, the behavior of each of the histidine

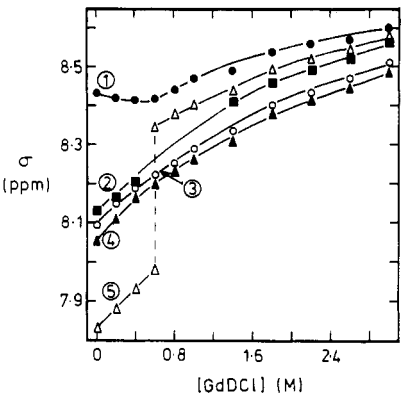

FIGURE 1: ^1H chemical shifts of histidine C(2) protons of human antithrombin III as a function of added guanidine deuteriochloride. The labels 1–5 correspond to the numbering of resonances used in the text. The protein sample was 1 mM in 20 mM phosphate/150 mM NaCl at pH 6.1. The temperature was 298 K.

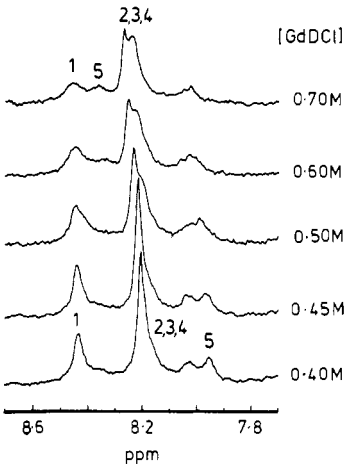

FIGURE 2: Low-field region of the ^1H NMR spectra of human antithrombin III in the presence of the indicated concentrations of guanidine deuteriochloride. Only the portion of the spectrum showing the histidine C(2) protons is given in each case. Numbering of the resonances corresponds to that in the text.

C(2) resonances is analogous to the behavior in the absence of heparin. Thus, the resonance from histidine-1 moves slightly upfield while the remaining resonances titrate downfield. However, between 0.6 and 0.8 M Gdn-DCl, there is no longer any discontinuity. Instead, resonance 1 continues to titrate upfield, and resonance 5 titrates further downfield. It is not until the Gdn-DCl concentration exceeds 0.8 M that resonance 5 disappears from its upfield position and a new resonance appears at 8.50 ppm. Also, resonance 1 reverses its direction of titration and now titrates downfield with increasing Gdn-DCl concentration. The titration behavior of these two protons as well as their behavior in the absence of heparin is summarized in Figure 3. The resonances from histidines-2, -3, and -4 are more poorly resolved than in the absence of heparin and give only a single resonance. This resonance titrates smoothly downfield as the Gdn-DCl concentration is increased as was seen for the individual resonances 2, 3, and 4 in the absence of heparin.

Denaturation of Bovine Antithrombin III by Guanidine Deuteriochloride. According to the numbering of Gettins (1987), the six histidine residues in bovine ATIII, 1–6, have pK_a's of 7.25, 7.15, 6.10, 5.80, 5.60, and 5.35 and chemical shifts for the C(2) proton resonance at pH 6.15 of 8.63, 8.54, 8.17, 8.05, 7.92, and 7.87 ppm, respectively.

Guanidine deuteriochloride was titrated into this sample, and ^1H NMR spectra were recorded for each 0.2 M increase

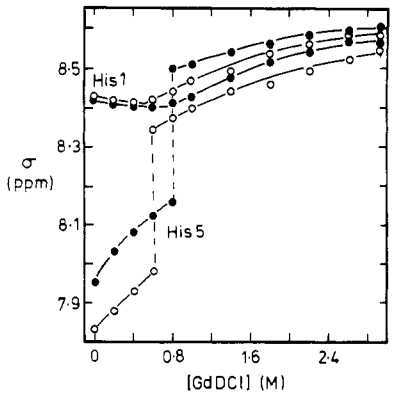

FIGURE 3: Chemical shift titration behavior of histidine C(2) protons 1 and 5 from human antithrombin III as a function of added guanidine deuteriochloride. (○) No heparin present; (●) in the presence of 10 mg/mL heparin. The solid lines are for visual aid. The dashed lines connect the resonance that disappears from the spectrum with the resonance that appears downfield at higher denaturant concentration.

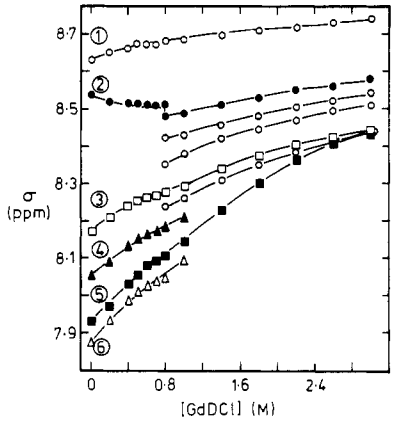

FIGURE 4: ^1H chemical shifts of the six histidine C(2) protons of bovine antithrombin III as a function of added guanidine deuteriochloride. The labels 1–6 correspond to the numbering of resonances used in the text. No lines are shown connecting the resonances of two of the histidines at the unfolding transition, since it is not possible to correlate the histidines in the folded and unfolded states. The protein concentration was 1 mM in 20 mM phosphate/150 mM NaCl at pH 6.15. Spectra were recorded at 298 K.

in denaturant concentration. The chemical shifts of the histidine C(2) resonances are given in Figure 4. As was found with human ATIII, most of the histidine resonances titrate downfield as the Gdn-DCl concentration is increased. The exception is the resonance from histidine-2, which titrates slightly upfield. With bovine ATIII, the discontinuity in titration behavior that was seen with human ATIII between 0.6 and 0.8 M denaturant occurs between 0.7 and 0.8 M Gdn-DCl. Spectra above and below the transition are shown in Figure 5. In the regions between 0.7 and 1.0 M Gdn-DCl, resonances 2, 4, and 6 diminish in intensity and finally disappear above 1.0 M denaturant. At the same time, new resonances appear at 8.48, 8.42, 8.35, and 8.24 ppm. This is somewhat different behavior from human ATIII, where resonance 2 was clearly identifiable throughout the titration, even though it changed direction at the transition, and only a single resonance disappeared, to be replaced by a new resonance. In bovine ATIII, it is no longer possible to identify the histidine C(2) protons above the unfolding transition, though it seems likely that histidine-2 gives rise to the new resonance at 8.48 ppm. At high Gdn-DCl concentrations, all of the resonances titrate smoothly downfield.

Binding of heparin perturbs all of the histidine C(2) protons. The resonances are no longer all resolvable, and because of

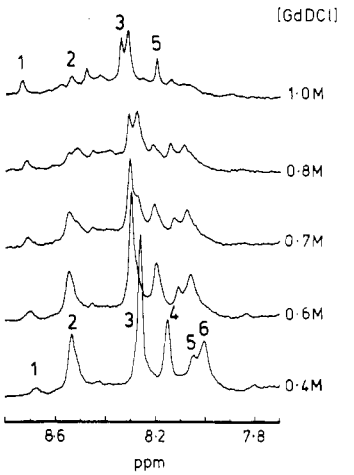

FIGURE 5: Histidine C(2) proton resonances of bovine ATIII at Gdn-DCl concentrations above and below the critical value for unfolding of the first domain. Spectra were recorded at 298 K. Numbering of the resonances corresponds to that in the text.

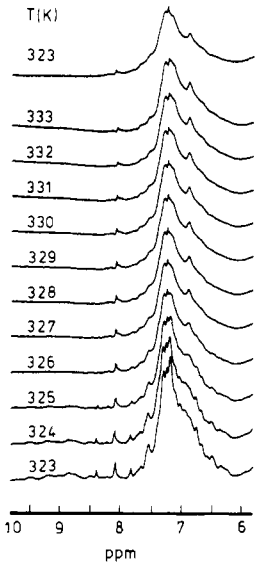

FIGURE 6: Aromatic region ^1H NMR spectra of human antithrombin III as a function of temperature. The temperature is indicted for each spectrum. The top spectrum was recorded after the sample, originally at 333 K, was cooled to 323 K. The ATIII concentration was 1 mM.

changes in chemical shift upon heparin binding, there is uncertainty in the assignment of histidines-5 and -6. Nevertheless, histidines-1 and -2 can be clearly identified throughout the titration with Gdn-DCl. Histidine-1 titrates very slightly downfield as the concentration of denaturant is raised. Histidine-2 titrates upfield until 1 M Gdn-DCl is reached and then disappears. Even though there are uncertainties in resonance assignment, it is clear from the dramatic change in the appearance of the spectra that the unfolding transition occurs between 0.8 and 1.0 M Gdn-DCl.

Thermal Denaturation of Human Antithrombin III. A sample of antithrombin III at approximately 50 mg/mL was heated in 5 K steps, and its ^1H NMR spectrum was recorded at each temperature. At a temperature of 323 K or below, the spectra were very similar and showed a number of resonances in the aromatic region characteristic of an intact globular protein, i.e., resonances shifted from the absorption frequencies of the free amino acids by proximity to other perturbing residues. Also, there are broad resonances to lower field than 8 ppm arising from unexchanged amide NH protons. At a temperature of 328 K and above, the spectra are quite different and are characteristic of an unfolded protein. To

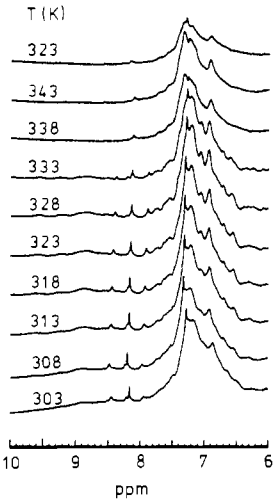

FIGURE 7: Effect of heparin on the temperature dependence of human antithrombin III ^{1}H NMR spectra. The temperature is indicated for each spectrum. The top spectrum was recorded after the sample, originally at 343 K, was cooled to 323 K. The ATIII concentration was 1 mM, pH 6.3, and heparin was present at a stoichiometry of 1:1 assuming an average molecular weight of 12 000.

follow the changes in more detail, the experiment was repeated, but with spectra recorded at 1 K intervals from 323 to 333 K. The aromatic and amide regions of these spectra are shown in Figure 6. From these spectra, it is clear that the thermal denaturation occurs over a narrow temperature range, beginning at 324 K and being complete by 326 K. Between these temperatures, the histidine resonances at 8.4 and 7.8 ppm and the broad amide resonances at 8.5 and 8.8 ppm diminish in intensity and disappear. Also, the distinct resonances at high field between 6.2 and 6.8 ppm and the large resonance at 7.5 ppm are lost. At the higher temperatures, there is no further change. Upon cooling the sample from 333 to 323 K, the spectrum does not show recovery of the lost histidine or other aromatic resonances, thus indicating that part, at least, of the protein does not regain its original folded conformation.

The thermal denaturation was repeated on human ATIII in the presence of 10 mg/mL heparin. Spectra were recorded from 303 K through 343 K in 5 K increments. These are shown in Figure 7. The denaturation transition occurs between 333 and 338 K, i.e., at least 9 K higher than in the absence of heparin. As with ATIII in the absence of heparin, there are amide and high-field aromatic resonances indicative of the folded state that are lost upon denaturation. Also, the lowest and highest field histidine C(2) resonances, from histidines-1 and -5, respectively, are the two most strongly affected by the denaturation step. Cooling the sample from 343 K back to 323 K (Figure 7) fails to result in regeneration of the original conformation; the spectrum is similar to that at 343 K, i.e., above the denaturation transition.

DISCUSSION

Domain Structure of Antithrombin III. The titration behavior of two of the histidine C(2) protons in the NMR spectra of human ATIII upon addition of guanidine deuteriochloride (Figures 1 and 2) clearly shows the occurrence of a conformation transition at a denaturant concentration between 0.6 and 0.8 M. This is the same concentration at which a structural transition was seen by Villanueva and Allen (1983a) from changes in fluorescence, CD, and absorption difference spectra. These investigators interpreted the changes at this low level of denaturant as corresponding to the unfolding of one of two structural domains of the protein, with a concomitant loss of about half of the α-helix content. The abrupt changes in titration seen here for the NMR resonances from histidines-1 and -5 are also consistent with such an unfolding. C(2) proton resonances from histidines-2, -3, and -4 do not sense the transition. The environments of these histidines, therefore, are not significantly altered by the unfolding of the weak domain, either because they are located in the second domain, at some distance from the interface with the first, or because their properties are not strongly influenced by the local protein tertiary structure, or a combination of these two.

In the presence of heparin, the abrupt change in histidine resonance titration occurs at a higher guanidine deuteriochloride concentration (Figure 3). The transition occurs between 0.8 and 1.0 M rather than 0.6–0.8 M denaturant. This finding agrees with the results of Villanueva and Allen (1983a) and lends further support to their proposal of two distinct structural domains within the protein.

Although Villanueva and Allen did not investigate the denaturation behavior of bovine ATIII, there are composition and limited sequence data (Kurachi et al., 1976; Gettins, 1987) as well as NMR data (Gettins, 1987) to suggest that the proteins are very similar structurally as well as functionally. It is thus not unexpected that similar denaturation behavior should be seen for bovine ATIII. This was found to be the case by Fish and co-workers (Fish et al., 1985). Of the six histidine residues present in bovine ATIII, and visible by NMR, three show a smooth titration with increase in Gdn-DCl concentration, while two also titrate downfield up to 0.7 M denaturant and then gradually diminish in intensity as new resonances appear (Figures 4 and 5). The remaining histidine, histidine-2, titrates very slightly upfield, up to 0.7 M Gdn-DCl, and then disappears. The new resonance at 8.48 ppm, which titrates downfield, is probably from histidine-2 in the unfolded structure. In the presence of heparin, there is again a need for a higher Gdn-DCl concentration, between 0.8 and 1.0 M, before the transition occurs. Thus, bovine and human ATIII show analogous behavior in the presence of Gdn-DCl, both in the presence and in the absence of heparin. Furthermore, there seems to be a structural equivalence between histidines-1 and -5 of human ATIII, and histidines-2 and -6 in bovine ATIII. It has been argued previously that this equivalence exists (Gettins, 1987), on the basis of the similarity of the pK_a's of these histidines and of their NMR titration behavior. The present data support this contention.

In contrast to the well-defined partial unfolding of human ATIII caused by low concentrations of Gdn-DCl, the thermal unfolding is characterized by a single denaturation temperature (Figure 6). Heparin stabilizes the protein to thermal denaturation by 9 K under the conditions of the experiment (Figure 7). A similar shift in T_d, the midpoint of the thermal denaturation, has been reported for human ATIII in the presence of heparin (Busby et al., 1981). It would be very instructive to follow the thermal unfolding of ATIII by differential scanning calorimetry, since a comparison of calorimetric and van't Hoff enthalpies of unfolding could differentiate between single- and multiple-step transitions. (Privalov, 1979). A good example of this is provided by Bence-Jones protein, which gives a ratio of 1.9 for these two enthalpies (Zavyalov et al., 1977) in contrast to the value of 1.0 expected for a single-step unfolding.

The failure to regenerate the low-temperature spectrum of human antithrombin III after cooling from a temperature above the denaturation point (Figure 6) reflects the tendency of the unfolded protein to aggregate, as has been reported previously (Busby et al., 1981; Mitra et al., 1982).

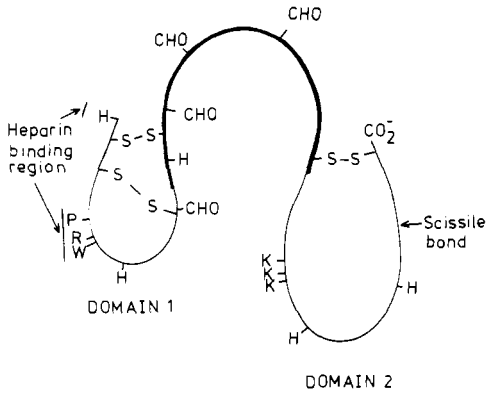

FIGURE 8: Schematic representation of the human ATIII molecule, showing a possible division into two domains and proposed location of the heparin binding site. The histidine residues and sites of glycosylation are shown. The portion of the chain shown by a thick line corresponds to the fragment isolated by Rosenfeld and Danishefsky (1986) and purported to possess heparin and thrombin binding affinity. Lysine residues 290, 294, and 297, proposed by Villanueva (1984) to be the heparin binding site, are indicated. Tryptophan-49, proline-41, and arginine-47 are also shown.

Localization of the Heparin Binding Site. As mentioned previously, there is evidence to support the equivalence of histidine-1 (NMR numbering) in human ATIII to histidine-2 in bovine ATIII and of histidine-5 in human ATIII to histidine-6 in bovine ATIII. In addition, on the basis of a secondary structure prediction for human ATIII (Villanueva, 1984) and the pK_a's of the five histidines (Gettins, 1987), it has been proposed that human ATIII histidine-5 corresponds to the N-terminal histidine and that histidine-1 corresponds to either residue 65 or residue 369. These two histidine resonances in human ATIII, and their equivalents in bovine ATIII, are the only ones to reflect the unfolding of the first domain of ATIII. It is therefore reasonable that both histidines giving rise to these resonances are in this domain. From the location of the three disulfide linkages in human ATIII, between residues 8 and 128, 21 and 96, and 247 and 430, a possible division of the protein into two domains would place residues 1–128 in one domain and residues 239 to the carboxyl terminus in the second domain. This is shown schematically in Figure 8. If this were so, the first N-terminal domain would contain three histidine residues, at positions 1, 65, and 120, while the C-terminal domain would contain the remaining two, at positions 319 and 369. The ^1H NMR resonances labeled 5 and 1 would, on this basis, be assigned to residues 1 and 65, respectively, rather than to residues 1 and 369, respectively, since the latter assignment would place the two Gdn-DCl-sensitive histidines in different domains, whereas it is more likely that they are both in the same domain, the one that is unfolded by low concentrations of Gdn-DCl. However, only the assignment of resonance 5 to the amino-terminal histidine is fairly certain (Gettins, 1987), and so the possible assignment of resonance 1 to histidine-65 will not be considered further.

It is clear that the amino terminus is strongly affected by low levels of guanidine hydrochloride. Since a second histidine residue also reflects the first unfolding transition, which is a major perturbation involving loss of half the α-helix content (Villanueva & Allen 1983a), it is possible that the unfolded region extends much further than a few amino acids and may be domain 1 shown in Figure 8. This is by no means definite, however. The retention of significant tertiary structure, as opposed simply to secondary structure, beyond the first unfolding transition is evidenced by the retention of the high-field-shifted methyl resonances and a number of unexchanged amide proton resonances. If the unfolding were a general structure loosening with loss of helix in all regions of the protein, these resonances might be expected to shift downfield and disappear, respectively.

It has been shown here, and elsewhere (Villanueva & Allen 1983a), that the unfolding domain is stabilized by heparin. It has also been shown previously that the pK_a of the N-terminal histidine is raised by 0.32 pH unit upon binding heparin (Gettins, 1987), whereas the pK_a's of the remaining histidines are little affected. Heparin most probably binds very close to this residue, though without forming a direct salt bridge to the imidazole ring. Given the stabilization to unfolding produced by heparin and the major effect unfolding has on the resonance from the amino-terminal histidine, the most conservative interpretation of these data is to place the heparin binding site, or at least some elements of it, in the N-terminal domain.

The scissile bond in antithrombin is between residues 393 and 394 (Björk et al., 1982) and would thus occur in the intact second domain in the present model. A study on susceptibility of human ATIII to protease cleavage in the presence and absence of heparin concluded that heparin binding results in a conformational change in the N-terminal region which exposes a site in this region to cleavage (Kress & Catanese, 1981). These data are in agreement with the present proposals and with the properties of abnormal and chemically modified ATIII molecules described above. They all suggest the location of the heparin binding site in the N-terminal domain of antithrombin III. In contrast, Villanueva (1984) has proposed the region from residue 289 to residue 301 as a possible heparin binding site, based on the prediction that this region is α-helical and has three lysyl residues positioned to form a positively charged face for interaction with the sulfate groups of a heparin octasaccharide. A third proposal, based on isolation of a fragment of antithrombin III with apparent affinity for heparin, is that the heparin binding site involves residues in the region 104–251 (Rosenfeld & Danishefsky, 1986). However, neither of the last two proposed heparin binding sites is well supported by experimental data. Although chemical modification studies have implicated lysine residues in heparin binding (Rosenberg & Damus, 1973), the particular residues involved are not known, and so lysines-290, -294, and -297, favored by Villanueva (1984), are no more likely to be involved than others. In the case of the fragment isolated by Rosenfeld and Danishefsky (1986), no direct binding studies with heparin were performed, nor was it shown that the conformation of the fragment was the same as in the intact protein. It should also be noted that three of the four glycosylation sites occur in this fragment, yet Rosenfeld and Danishefsky (1984) have shown that the carbohydrate moieties are not required for heparin binding.

In conclusion, therefore, the present data are in accord with all definitive chemical modification studies and sequence data on abnormal antithrombins and support an N-terminal binding site for heparin on antithrombin. In addition, we have presented evidence in support of a two-domain folding pattern for antithrombin III from both human and bovine sources. These proteins appear to be very similar structurally.

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Infrared Studies of Fully Hydrated Saturated Phosphatidylserine Bilayers. Effect of Li^+ and Ca^{2+} †

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ABSTRACT: The thermotropic phase behavior of fully hydrated Na^+ and/or NH_4^+ salts of 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS) was determined by temperature-dependent infrared spectra. The molecular level properties and thermal phase behavior of DMPS–Li^+ complexes were also characterized by infrared spectroscopy. With increasing concentrations of Li^+, the infrared spectra reveal the appearance of a second, more ordered, lipid phase which shows a gel to liquid-crystal transition at significantly higher temperatures (75–95 °C) than the Na^+ or NH_4^+ salts of DMPS (39 °C). Li^+ binds to the phosphate and carboxylate groups of DMPS, resulting in the following changes: (1) water of hydration is lost from both the carboxylate and phosphate groups; (2) there are changes in the conformation of the glycerol backbone but not in the P–O ester bonds of the phosphate group which remain in the gauche–gauche conformation; and (3) the packing of the fatty acyl chains becomes more ordered. In addition, the properties of the DMPS–Ca^{2+} complex were studied by infrared spectroscopy. While the DMPS–Ca^{2+} complex is also characterized by rigidly packed, well-ordered fatty acyl chains, the mode of Ca^{2+} binding to the DMPS head groups differs significantly from that of Li^+ binding. By comparison, with dry DMPS–Ca^{2+} [Casal, H. L., Mantsch, H. H., Paltauf, F., & Hauser, H. (1987) *Biochim. Biophys. Acta* (in press)], the phosphate group undergoes a conformational change, probably to the antiplanar–antiplanar conformation, and loses its water of hydration. In contrast to the DMPS–Li^+ complex, the carboxylate group remains hydrated in the DMPS–Ca^{2+} complex, indicating that Ca^{2+} is chelated by phosphate groups only. Furthermore, in the DMPS–Ca^{2+} complex, one of the ester carbonyl groups is engaged in hydrogen bonding; such a hydrogen bond is not found in DMPS–Na^+, DMPS–NH_4^+, and DMPS–Li^+.

The interaction of metal ions with membrane lipids is of great importance in the control of the structure and function of biological membranes. The interaction of metal ions with anionic phospholipids such as phosphatidylserine (PS)[1] seems to be related to many of the roles which these lipids play in

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[1] Abbreviations: DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine; PS, phosphatidylserine; DSC, differential scanning calorimetry; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid or 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; His, histidine; EDTA, ethylenediaminetetraacetic acid.

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