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Evidence That Arginine-129 and Arginine-145 Are Located within the Heparin Binding Site of Human Antithrombin III

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ABSTRACT: Arginyl residues of human antithrombin III have been implicated to involve in the heparin binding site [Jorgensen, A. M., Borders, C. L., & Fish, W. W. (1985) *Biochem. J. 231*, 59–63]. We have performed chemical modification of antithrombin with (*p*-hydroxyphenyl)glyoxal (HPG) in order to determine the locations of these arginine residues. Antithrombin was modified with 12 mM HPG in the absence and presence of heparin (2-fold by weight to antithrombin). In the absence of heparin, about 3–4 mol of arginines/mol of antithrombin were modified within 60 min, and the modification led to the loss of 95% of the inhibitor's heparin cofactor activity as well as heparin-induced fluorescence enhancement and 50% of its progressive inhibitory activity. In the presence of heparin, the extent of modification was diminished by 30% and modified antithrombin retained approximately 70% of its heparin cofactor activity. Peptide mapping and subsequent sequence analysis revealed that selective HPG modification occurred at Arg^{129} and Arg^{145} and that their modifications were protected upon binding of heparin to antithrombin. We conclude that Arg^{129} and Arg^{145} are situated within the heparin binding site of human antithrombin III.

Antithrombin III (AT-III)[1] is a plasma glycoprotein and is the most important protease inhibitor that regulates the blood coagulation cascade [for reviews, see Rosenberg (1977) and Bjork and Lindahl (1982)]. AT-III inhibits thrombin as well as factors IXa, Xa, and XIa, and its inhibitory activity is greatly enhanced in the presence of heparin, a negatively charged polysaccharide. The precise mechanism of the heparin function remains to be defined. It has however been well established that heparin must bind to AT-III in order to exert its enhancing effect as an anticoagulant (Rosenberg & Damus, 1973; Villanueva & Danishefsky, 1977) and that high-affinity heparin in general displays high anticoagulant activity. Binding of heparin to AT-III induces a conformational change of the inhibitor (Einarsson & Andersson, 1977; Olson et al., 1981) and leads to the exposure of Lys^{236} for chemical modification (Chang, 1989). The consequence of this heparin-induced conformational change is at this stage still debated. It has been proposed either to activate the reactive site of AT-III (Rosenberg & Damus, 1973) or to tighten the heparin–thrombin complex (Olson et al., 1981; Peterson & Blackburn, 1987).

The heparin binding site of AT-III is thought to consist of clusters of basic amino acids. Four lysyl residues, Lys^{107}, Lys^{114}, Lys^{125}, and Lys^{136}, were shown to directly participate in the heparin binding site of AT-III (Peterson et al., 1987; Liu & Chang, 1987a; Chang, 1989). In addition, arginyl residues have also been implicated. A single amino acid replacement at Arg^{47} (Arg to Cys, His or Ser) impairs the heparin binding ability of AT-III congenital variants (Koide et al., 1984; Duchange et al., 1987; Borg et al., 1987). These findings confirm that Arg^{47} is essential for the integrity of the heparin binding site but do not conclusively prove that Arg^{47} is involved in direct heparin binding. Jorgensen et al. (1985) showed that the complete loss of the heparin cofactor activity of AT-III was accompanied by the modification of approxi-

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[1] Abbreviations: AT-III, human antithrombin III; HPG, (*p*-hydroxyphenyl)glyoxal; S-DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanoazobenzene-2′-sulfonic acid; DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanoazobenzene; DABTH, 4-(*N*,*N*-dimethylamino)-4′-thiohydantoylazobenzene; HPLC, high-performance liquid chromatography.

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mately 6–7 of the 22 arginines per polypeptide, but the locations of these modified residues have not been characterized. It also was not clear how many of those 7 modified arginines were involved in heparin binding. In this study, we employed the strategy of protected chemical modification to identify the arginyl residues that participate in the heparin binding site of AT-III.

Experimental Procedures

Materials. AT-III was purified from human plasma by use of heparin–Sepharose chromatography (Koide, 1979). Human α-thrombin (T-6795, >3000 NIH units/mg) and low molecular weight heparin (H-5640, MW 4000–6000) were purchased from Sigma. S-DABITC was synthesized according to the method described (Chang, 1989). (*p*-Hydroxyphenyl)glyoxal (HPG) was obtained from Pierce and 4-(*N*,*N*-dimethylamino)-4′-isothiocyanoazobenzene (DABITC) was purchased from Fluka. Chromozym TH was from Boehringer Mannheim.

Modification of AT-III with HPG (Yamasaki et al., 1980). One milligram of AT-III was allowed to react with 2 mg of HPG in both the absence and presence of heparin (2 mg) in 1 mL of sodium bicarbonate buffer (50 mM, pH 9.0, containing 150 mM NaCl). The reaction was performed at 22 °C. At various periods of time (15, 30, 60, and 120 min), 200 μL of the sample was withdrawn and passed through a disposable G-25 column (PD-10 from Pharmacia, equilibrated with 50 mM ammonium bicarbonate) in order to stop the reaction and remove the excess reagent. Modified samples (collected in 1.2 mL) were used directly for biological assay or were freeze-dried and used for structural characterization. A control sample was processed in parallel without adding HPG and heparin. The number of arginines modified per mole of AT-III was calculated by molar absorption $\Delta\epsilon_{340nm} = 1.83 \times 10^4\ M^{-1}\ cm^{-1}$.

Biological Assay of Modified AT-III. These were performed basically according to the method described by Bjork and Nordling (1979). Chromozym TH (tosyl-Gly-Pro-Arg-pNa) was used as the chromogenic substrate. For heparin cofactor activity, 40 μL of the buffer (50 mM Tris-HCl, pH 8.2, 0.15 M NaCl) containing 600 ng of AT-III and 1 μg of low molecular weight heparin was incubated for 5 min. Thrombin (72 ng in 10 μL of buffer) was added, and the mixture was incubated at room temperature for exactly 2 min. For the progressive inhibitory activity, 600 ng of AT-III was incubated with 72 ng of thrombin in 50 μL of buffer for exactly 15 min. The incubated AT-III/thrombin mixture (25 μL) was then transferred to a cuvette which already contained 975 μL of buffer and 1 μmol of Chromozym TH. Hydrolysis of the substrate was monitored at 405 nm for a period of 2 min. Both activities were calculated by the following formula: $[(A_t - A_{sample})/(A_t - A_{control})] \times 100$. A_t, $A_{control}$, and A_{sample} are activities for thrombin alone, thrombin plus control AT-III, and thrombin plus modified AT-III, respectively. Each sample was analyzed in triplicate, and the data were averaged.

Fluorescence Measurements. The modified AT-III and control sample (30 μg each) was incubated in 2 mL of Tris buffer (50 mM Tris-HCl, pH 8.2, 150 mM NaCl) in the absence or presence of 60 μg of low molecular weight heparin for 10 min before measurement. The intensity of emission at 345 nm was measured at an excitation wavelength of 280 nm on a Perkin-Elmer Model L5-3 fluorescence spectrometer. The fluorescence enhancement was calculated by the formula $(F - F_0)/F_0$, where F is the fluorescence intensity of the sample containing heparin and F_0 represents that of sample in the absence of heparin. Heparin binding capacity of modified AT-III was expressed by taking fluorescence enhancement of control sample as 100%. Results from triplicate analysis of each sample are presented.

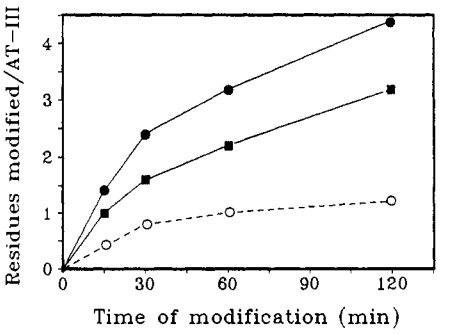

FIGURE 1: Modification of AT-III with (*p*-hydroxyphenyl)glyoxal (HPG). AT-III was modified with 12 mM HPG in the absence and presence of heparin (2-fold by weight to AT-III). Following removal of the excess reagent, the number of arginyl residues modified per mole of AT-III was calculated by a molar absorption coefficient of $\Delta\epsilon_{340nm} = 1.8 \times 10^4\ M^{-1}\ cm^{-1}$. Extent of modification in the absence of heparin (●) and in the presence of heparin (■); the difference as a result of heparin protection (○).

S-DABITC Modification of Modified AT-III (Chang, 1989). Control sample and arginine-modified samples (500 μg each) were dissolved in 0.25 mL of 50 mM sodium bicarbonate (pH 8.3), and 0.25 mL of S-DABITC solution (2 mM in 50 mM sodium bicarbonate) was added. The experiment was carried out in both the absence and presence of heparin (1 mg). Derivatization was performed at 22 °C for 7.5 min followed by desalting on PD-10 column and freeze-drying. S-DABITC-modified AT-IIIs were reduced and carboxymethylated with iodoacetic acid and digested overnight with trypsin (enzyme/substrate weight ratio 1:20) in 250 μL of 50 mM ammonium bicarbonate for HPLC analysis.

Tryptic Peptide Mapping Using the DABITC Precolumn Derivatization Method. Arginine-modified AT-III and control AT-III (250 μg each) were first reduced and carboxymethylated with iodoacetic acid. After desalting on PD-10 column, each sample was then digested with 12.5 μg of trypsin in 50 mM ammonium bicarbonate overnight and freeze-dried. The digested samples were dissolved in 150 μL of water, and 300 μL of DABITC (10 mM in pyridine) was added. The mixture was incubated at 70 °C for 50 min, then 10 μL of PITC was added, and the mixture was incubated for another 15 min. The excess reagent was extracted four times with 1 mL of heptane/ethyl acetate (2:1 v/v). The samples were dried and redissolved in 20% pyridine for peptide mapping on HPLC. For quantitative N-terminal analysis, DABITC-labeled peptides were treated directly with aqueous acid (Chang, 1981; Chang & Tran, 1986) and the released N-terminal DABTH-amino acids were analyzed by the method described (Chang, 1988). For sequence analysis, the DABITC-labeled peptides were treated with trifluoroacetic acid at 54 °C for 5 min, dried, and then subjected to amino acid sequence analysis by an automatic sequencer (Hunkapiller et al., 1983).

Results

Modification of AT-III with HPG. (*p*-Hydroxyphenyl)-glyoxal (HPG) has been reported to be an arginine-specific reagent (Yamasaki et al., 1980). The extent of modification of arginine per mole of protein can be determined at 340 nm (molar extinction coefficient of $1.83 \times 10^4\ M^{-1}\ cm^{-1}$) after removal of the excess reagent. AT-III was modified with 12 mM HPG in the absence and presence of heparin for various periods of time. The extent of modification is shown in Figure 1. In the absence of heparin, approximately 3–4 mol of

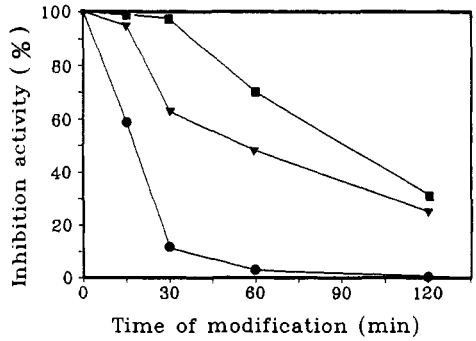

FIGURE 2: Biological activities of HPG-modified AT-III. Heparin cofactor activity (●) and progressive inhibitory activity (▼) of AT-III modified in the absence of heparin; heparin cofactor activity (■) of AT-III modified in the presence of heparin. They were measured according to the methods described under Experimental Procedures. The activity of the control AT-III was taken as 100%.

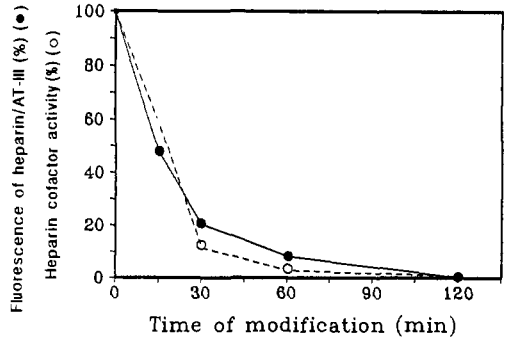

FIGURE 3: Extent of fluorescence enhancement of HPG-modified AT-III induced by heparin binding. Control and modified AT-IIIs (30 μg) were incubated in the absence and presence of heparin (60 μg) in 2 mL of Tris-HCl buffer for 10 min before measurement. The fluorescence enhancement was evaluated as $(F - F_0)/F_0$ (see Experimental Procedures). The fluorescence enhancement of the control AT-III was taken as 100%. The dashed curve represents the decrease of the heparin cofactor activity.

arginines/mol of AT-III were modified after 60 min. The presence of heparin protected about 1 mol of arginine from HPG modification.

Biological Activities of Modified AT-III. The heparin cofactor activity and the progressive inhibitory activity of modified AT-III are presented in Figure 2. After 60-min modification with 12 mM HPG in the absence of heparin, AT-III lost nearly 95% of its heparin cofactor activity, but only 50% of its progressive inhibitory activity was impaired. The decrease of the progressive inhibitory activity upon HPG modification suggested that Arg^{393} at the reactive site of AT-III might also be attacked (Jorgensen et al., 1985). AT-III modified under similar conditions in the presence of heparin retained about 70% of its heparin cofactor activity (Figure 2), clearly indicating that impairment of the heparin binding site by HPG was significantly protected by heparin. The progressive inhibitory activity of AT-III modified in the presence of heparin was not measured, because it was extremely difficult to quantitatively remove the bound heparin from modified AT-III.

Fluorescence Enhancement of Modified AT-III Induced by Heparin. Heparin binding to AT-III induces a conformational change and leads to an increased fluorescence intensity of the inhibitor (Einarsson & Andersson, 1977). For HPG-modified AT-III, the extent of this enhancement in the presence of heparin was sharply reduced. The results are shown in Figure 3. The decrease of the fluorescence enhancement of modified AT-III was found to correlate to its loss of heparin cofactor activity. Compared to the control sample, the 30 min modified

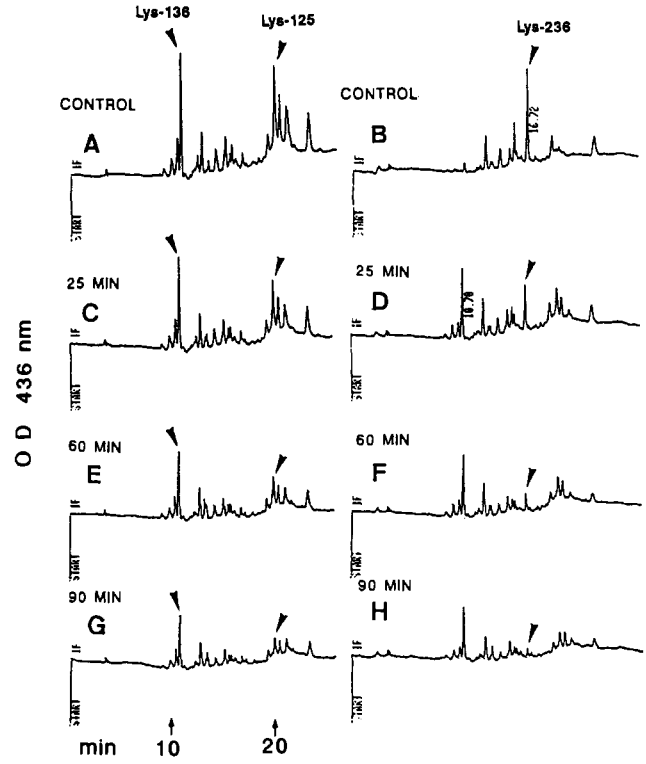

FIGURE 4: Monitoring the heparin binding affinity and the conformation of HPG-modified AT-III by the S-DABITC technique. Control AT-III (A and B); AT-IIIs modified with HPG for 25 min (C and D), 60 min (E and F), and 90 min (G and H) modified with 1 mM of S-DABITC in the absence (A, C, E, and G) and presence (B, D, F, and H) of heparin for 7.5 min. All S-DABITC-modified samples were subsequently reduced–carboxymethylated, followed by tryptic digestion. Peptides containing S-DABITC-labeled lysines were detected at 436 nm. In the absence of heparin, two major color peptides corresponding to labeling at Lys^{136} (residues 133–139) and Lys^{125} (residues 115–129), respectively, were observed (A) (Chang, 1989). In the presence of heparin (B), modification at Lys^{125} and Lys^{136} was protected, but a conformational change of AT-III induced by heparin binding exposed Lys^{236} for S-DABITC modification (residues 236–241). Solvent A was 17.5 mM acetate, pH 5.0. Solvent B was acetonitrile. Linear gradient was 10% to 70% B in 30 min. Column was Vydac C-18 for peptides and proteins. Column temperature was 22 °C.

AT-III displayed only 20% of the fluorescence enhancement upon heparin binding and retained approximately 12% of the heparin cofactor activity. One should however bear in mind that the decreased fluorescence enhancement of modified AT-III could be a consequence of either reduced heparin binding affinity or conformational change of AT-III caused by HPG modification.

Monitoring the Heparin Binding Ability and the Conformational Change of Modified AT-III by the S-DABITC Method (Chang, 1989). Two lysyl residues (Lys^{125} and Lys^{136}) at the heparin binding site of AT-III were selectively labeled by S-DABITC (a lysine-specific, water-soluble color reagent). Upon binding of heparin, Lys^{125} and Lys^{136} were shielded from S-DABITC labeling, and at the same time Lys^{236} was exposed for S-DABITC labeling as a consequence of the conformational change of AT-III. The shielding of Lys^{125} and Lys^{136} has been found to be inversely proportional to the exposure of Lys^{236}, and the reactivities of these three lysyl residues served as useful indicators for both the heparin binding affinity and the conformational change of AT-III (Chang, 1989).

The control AT-III and three HPG-modified AT-IIIs (time-course modification, 25, 60, and 90 min) were analyzed by the S-DABITC method, and the results are shown in Figure 4. The left column of Figure 4 presents the results of the four

Table I: Quantitative N-Terminal Analysis[a] and Sequence Analysis of Tryptic Peptides of AT-III

peptides	N-terminus (pmol) A[b]	B[b]	C[b]	sequences	position
T1	Leu (133)	Leu (67)	Leu (139)	LNCR	126–129
T2	Asp (400)	Asp (240)	Asp (400)	DIPMNPMCIYR	14–24
	Ser (280)	Ser (260)	Ser (220)	SLNPNR	394–399
	Tyr (140)	Tyr (61)	Tyr (36)	YR	260–261
T3	Leu (164)	Leu (79)	Leu (151)	LVSANR	140–145
T4				LFGDK	146–150

[a] DABITC-peptides collected from Figure 4 were dried and directly treated with aqueous acid to release the N-terminal DABTH-amino acids (see Experimental Procedures). [b] (A) Control sample; (B) sample modified with 12 mM HPG; (C) sample modified with 12 mM HPG in the presence of heparin.

samples analyzed in the absence of heparin, and the right column shows the results obtained in the presence of heparin. Several points can be generalized: (1) Modification of Arg obstructed the reactivity of the two lysyl residues (Lys^{125} and Lys^{136}) at the heparin binding site (see the left column of Figure 4). The reactivity of Lys^{125} and Lys^{136} of the 60 min modified AT-III dropped to 18% and 56% of that of the control sample (compare parts A and E of Figure 4). (2) Modification of Arg impaired the ability of heparin to shield Lys^{125} and Lys^{136} of AT-III (compare the left column and the right column of Figure 4). This decreased ability of shielding most likely reflects the reduced binding affinity of heparin. Taking the 25 min modified sample as an example, the extent of shielding of Lys^{125} and Lys^{136} in the presence of heparin was only 38% and 24% (calculated from Figure 4C,D) as compared to greater than 95% of the control sample (calculated from Figure 4A,B). (3) In reference to the control AT-III, the heparin binding capacity of the HPG-modified AT-III evaluated by the S-DABITC method is comparable to that detected by fluorescence enhancement (Figure 3). For instance, the heparin binding affinity of the 60 min modified sample was 11% as judged by the S-DABITC technique (Figure 4E,F) and was 9% as judged by fluorescence enhancement (Figure 3). (4) For each HPG-modified AT-III, the decrease of its heparin binding affinity (evaluated by either the S-DABITC method of fluorescence enhancement) is quantitatively related to the loss of its heparin cofactor activity.

Structural Analysis of HPG-Modified AT-III. The above results strongly suggest that arginyl residues are directly involved in the heparin binding site of AT-III. In order to locate these arginyl residues, three samples (control AT-III, 60-min modification in both the absence and presence of heparin) were reduced–carboxymethylated and digested by trypsin. The tryptic peptides were analyzed by the DABITC precolumn derivatization method (Chang, 1981; Chang & Tran, 1986). Their peptide mappings are shown in Figure 5. Four peptide fractions marked as T1, T2, T3, and T4 in the control AT-III (Figure 5A) were reduced by nearly 50% in the sample modified in the absence of heparin (Figure 5B). Recoveries of these four fractions returned to the level of the control sample when modification was carried out in the presence of heparin (Figure 5C). They were collected and analyzed by quantitative N-terminal analysis (following direct incubation with aqueous acid) (Chang, 1981) and sequence analysis. The structures are summarized in Table I. Fractions T1, T3, and T4 each contained single peptides encompassing sequence positions of 126–129, 140–145, and 146–150 (Figure 6), respectively. These results revealed that Arg^{129} and Arg^{145} were modified by HPG and were protected from modification in the presence of heparin. Attempts to find the peptide containing residues 130–136 were unsuccessful. Fraction T2 contained three peptides, but only Asp-Ile-Pro-Met-Asn-Pro-Met-Cys-Ile-Tyr-Arg (residues 14–24) was diminished after modification and recovered again in the presence of heparin—a result that implied either Arg^{13} or Arg^{24} was at-

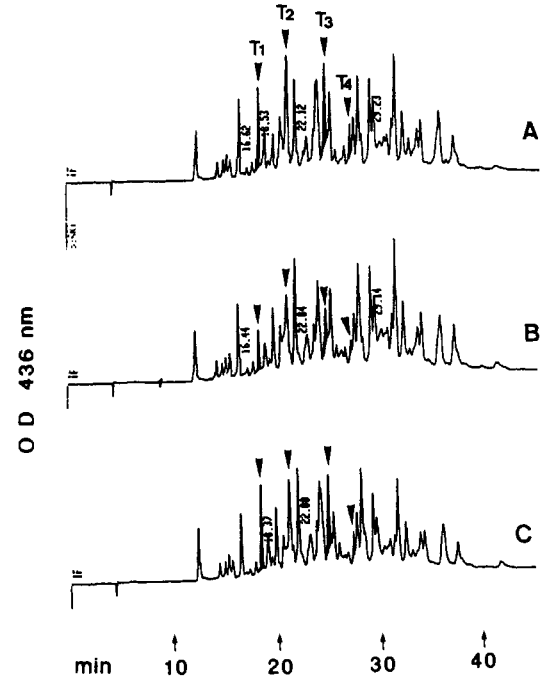

FIGURE 5: Peptide mapping of HPG-modified AT-III. Control AT-III (panel A), AT-III modified with 12 mM HPG (panel B), and AT-III modified with 12 mM HPG in the presence of heparin (panel C) were reduced and carboxymethylated followed by tryptic digestion. Digested samples were analyzed by the DABITC precolumn derivatization method (Chang, 1981). Two micrograms of sample was applied to HPLC for peptide mapping. Solvent A was 17.5 mM sodium acetate, pH 5.0. Solvent B was acetonitrile. Column was Vydac C-18, 5 μm. Column temperature was 22 °C. The gradient was 20% to 70% B in 40 min and 70% to 80% B from 40 to 45 min, was held at 80% B from 45–50 min, and then was returned to 20% B in 2 min. Detector was set at 436 nm. Peptides T1, T2, T3, and T4 were collected and analyzed as described under Experimental Procedures, and the results are given in Table I.

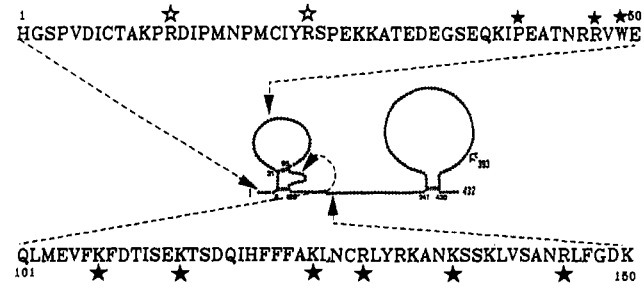

FIGURE 6: Diagram illustrating the two regions of AT-III which have been implicated in the heparin binding site. The reactive site is Arg^{393} (Bjork et al., 1982). Amino acid residues marked with solid stars were shown either to be essential to maintain the integrity of the heparin binding site or to directly participate in the heparin binding site. For details, see Discussion in the text.

tacked by HPG and protected by heparin.

It is relevant to mention that the effort to identify modified arginines was partly complicated by the limited stability of the HPG–arginine adduct. The stability of HPG-modified arginine in model proteins has been documented (Yamasaki et al., 1980). In our experiment, the absorption at 340 nm of HPG-modified AT-III decreased by exactly 50% following reduction–carboxymethylation.

Discussion

Elucidation of the heparin binding site of AT-III is essential to our understanding about the heparin function at the molecular level. In the absence of the three-dimensional structure of the heparin/AT-III complex, the chemical nature of the heparin binding site of AT-III has been largely derived from studies of congenital AT-III variants, chemically modified AT-III, and fragments of AT-III. Two regions of AT-III have been implicated in maintaining the integrity of the heparin binding site (Figure 6). The first region is located at the N-terminal end. Several genetic variants of AT-III which exhibited normal reactive site activity and impaired heparin binding affinity were found to have amino acid substitutions within this region. They were AT-III Toyama and Tours (Arg^{47} to Cys) (Koide et al., 1984; Duchange et al., 1987), AT-III Rouen I (Arg^{47} to His) and Rouen II (Arg^{47} to Ser) (Borg et al., 1987), and AT-III Basel (Pro^{41} to Leu) (Chang & Tran, 1986). Selective chemical modification at Trp^{49} affected only the heparin cofactor activity, but not the progressive inhibitory activity of AT-III (Blackburn et al., 1984). Glu^{34}–Gly^{35}, Glu^{42}–Ala^{43}, and Glu^{50}–Leu^{51} of AT-III were shown to be preferentially attacked by V8 protease, and their cleavages could be partly protected by heparin (Liu & Chang, 1987b). NMR analysis of AT-III denaturation in the presence of heparin also indicated that the heparin binding site is located within this region (Gettins & Wooten, 1987). However, most evidence supporting the involvement of the first region does not unambiguously demonstrate that residues within this domain directly participate in the heparin binding site. For instance, the reduced heparin binding affinity of AT-III Basel is most likely a consequence of the conformational change of the heparin binding site induced by Pro^{41} to Leu replacement (Chang & Tran, 1986).

The second region is located within residues 101–150. Rosenfeld and Danishefsky (1986) first isolated a CNBr peptide (residues 104–251) which possessed heparin binding activity. Smith and Knauer (1987) subsequently isolated a heparin binding peptide encompassing the region consisting of residues 114–156. Computer-generated plots of hydropathy and charge density of AT-III indicated that the region of residues 126–140 was exposed to the exterior surface while the region of residues 123–141 possessed the highest positive charge density within the molecule. If one draws a helical wheel from 107 to 145, all lysines and arginines will be found to line at the same side along the helix (data not shown). The disulfide bond Cys^{8}–Cys^{128} has been reported by our lab to be essential to keep the fully active heparin binding site (Sun & Chang, 1989). Further information was obtained from the chemical modification of AT-III. Four lysyl residues, Lys^{107} (Chang, 1989), Lys^{114} (Liu & Chang, 1987a), Lys^{125} (Peterson et al., 1987; Liu & Chang, 1987a; Chang, 1989), and Lys^{136} (Chang, 1989), within the second region have been shown to directly participate in the heparin binding site. Selective modification of these lysyl residues diminished the heparin binding affinity and heparin cofactor activity of AT-III, and their modifications could be quantitatively shielded upon binding of heparin to AT-III.

We have demonstrated that modification of 3–4 mol of arginine/mol of AT-III led to a nearly quantitative loss of the inhibitor's heparin cofactor activity as well as its heparin binding ability. Subsequent structural analysis revealed that selective modifications occurred at Arg^{129}, Arg^{145}, and Arg^{24} (or Arg^{13}) and that their modifications could be substantially protected by heparin. These data are convincing evidence that Arg^{129}, Arg^{145}, and one arginine at the N-terminal region (Arg^{13} or Arg^{24}) are participating in the heparin binding site of AT-III. These results provide new information and are consistent with our current knowledge about the structure of the heparin binding site of AT-III. Arg^{129} and Arg^{145} are situated within the second region (Figure 6), whereas Arg^{24} (or Arg^{13}) is located within the first region. Thus within the second region, 6 out of the total of 9 Arg/Lys have been identified to be involved directly in the heparin binding site. Our results however do not ascertain whether these arginines interact with the negatively charged group of heparin to form salt bridges or are merely situated within the heparin binding site of AT-III.

Registry No. AT-III, 9000-94-6; HPG, 24645-80-5; L-Arg, 74-79-3; heparin, 9005-49-6.

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Cardiolipin-Depleted Bovine Heart Cytochrome *c* Oxidase: Binding Stoichiometry and Affinity for Cardiolipin Derivatives†

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ABSTRACT: Detergent-solubilized bovine heart cytochrome *c* oxidase requires 2 mol of tightly bound cardiolipin (CL) per mole of monomeric complex for functional activity. Four lines of evidence support this conclusion: (1) Phospholipid depletion shows that two tightly bound CL's must remain associated with cytochrome *c* oxidase in order to maintain full electron transport activity. (2) Removal of the two tightly bound CL's correlates with decreased activity that is restored by reassociation of 2 mol of exogenous CL. (3) CL-depleted cytochrome *c* oxidase has two high-affinity binding sites for 2-[^{14}C]acetylcardiolipin (AcCL), $K_{d,app} < 0.1$ μM, that are not present in enzyme containing endogenous CL. An additional 2–3 lower affinity AcCL binding sites, $K_{d,app} = 4$ μM, are present in the CL-depleted complex, but these sites are also present in enzyme containing endogenous CL. (4) CL, monolysocardiolipin (MLCL), and dilysocardiolipin (DLCL) compete for AcCL binding with approximately the same relative affinities as those measured by the restoration of electron transport activity (MLCL competes much better than DLCL). However, MLCL and DLCL are only 60% and 15% as effective as CL in restoring maximum activity when they are bound to the high-affinity sites. The binding specificity of CL, MLCL, DLCL, and some of their acylated derivatives indicates that the apolar tails are most important for binding, not the polar head group. The presence or absence of hydroxyl groups in CL, MLCL, or DLCL also has little effect upon binding affinities. Binding specificity clearly favors CL since phosphatidylglycerol, phosphatidic acid, and phosphatidylcholine each have very low affinity for the CL binding sites ($K_{d,app} > 20$ μM). We, therefore, conclude that restoration of activity to CL-depleted cytochrome *c* oxidase is highly specific and requires the reassociation of CL, or structurally similar compounds, with two high-affinity binding sites.

Bovine cytochrome *c* oxidase (ferrocytochrome *c*:O_2 oxidoreductase; EC 1.9.3.1) is the terminal enzyme complex of the inner mitochondrial electron transport chain and catalyzes electron transfer from reduced cytochrome *c* to molecular oxygen. The multisubunit enzyme complex spans the inner mitochondrial membrane and is in contact with the membrane phospholipids. Some of these mitochondrial membrane phospholipids copurify with cytochrome *c* oxidase, and the purified complex commonly contains 20–60 mol of PL[1]/mol of enzyme, i.e., bound phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL). PC and PE are easily extracted, or exchanged for detergent (Robinson & Capaldi, 1977; Robinson et al., 1980; Yu et al., 1975; Vik & Capaldi, 1977), but CL is tightly associated and is more difficult to remove.

Several studies have shown that the tightly associated CL is required for maximum electron transport activity of the bovine heart enzyme (Awasthi et al., 1971; Robinson et al., 1980; Fry & Green, 1980). When CL is removed, the resulting CL-depleted enzyme has only 30–50% of the original activity. Recovery of the remaining 50–70% activity is specific for CL and cannot be accomplished by other PL's (Robinson et al., 1980; Robinson, 1982; Dale & Robinson, 1988a). A number of reports support this observation although other PL's have been reported to restore activity to the CL-depleted enzyme (Awasthi et al., 1971; Fry & Green, 1980; Vik et al., 1981). Also, adriamycin, which specifically binds CL, inhibits cytochrome *c* oxidase and other CL-dependent enzymes (Goormaghtigh et al., 1982; Goormaghtigh & Ruysschaert, 1984). Some studies, however, indicate cytochrome *c* oxidase has no

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[1] Abbreviations: DM, dodecyl β-D-maltopyranoside; TX, Triton X-100; MA, molecular activity in μmol of ferrocytochrome *c* oxidized (μmol of cytochrome *c* oxidase)$^{-1}$ s^{-1}; Tris-HCl buffer, tris(hydroxymethyl)-aminomethane base titrated to the appropriate pH with hydrochloric acid; AcCL, 2-acetylcardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-acetyl-*sn*-glycerol; Ac_2MLCL, diacetylated monolysocardiolipin or 1-(3-*sn*-phosphatidyl)-3-(2′-acetyl-3-*sn*-phosphatidyl)-2-acetyl-*sn*-glycerol; Ac_3-DLCL, triacetylated dilysocardiolipin or 1,3-bis(2′-acetyl-3-*sn*-phosphatidyl)-2-acetyl-*sn*-glycerol; CL, cardiolipin; MLCL, monolysocardiolipin; DLCL, dilysocardiolipin; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SucCL, 2-succinylcardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-succinyl-*sn*-glycerol; THP-CL, 2-(tetrahydropyranyl)cardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-(tetrahydropyranyl)-*sn*-glycerol.

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