Interaction of Heparin with Human Angiogenin*

(Received for publication, September 3, 1996, and in revised form, December 9, 1996)

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HT-29 human colon adenocarcinoma cells adhere rapidly to human angiogenin (Ang) via interactions with cell-surface heparan sulfate moieties (Soncin, F., Shapiro, R., and Fett, J. W. (1994) J. Biol. Chem. 269, 8999–9005). Soluble heparin inhibits adhesion, and Ang itself binds tightly to heparin-Sepharose. In the present study, the interaction of Ang with heparin has been further characterized. The basic cluster Arg-31/Arg-32/Arg-33 has been identified as an important component of the heparin binding site. Mutations of these residues, and of Arg-70 as well, decrease both the affinity of Ang for heparin-Sepharose and the capacity of Ang to support cell adhesion. Replacements of four other basic residues do not affect heparin binding. Heparin partially protects Ang from cleavage by trypsin at Lys-60, suggesting that heparin also binds to the region of Ang that contains this residue. The map here determined indicates that the heparin recognition site on Ang lies outside the catalytic center; indeed, heparin has no significant effect on the ribonucleolytic activity of Ang. It also does not influence the angiogenic activity of this protein. Light scattering measurements on Ang-heparin mixtures suggest that 1 heparin chain (mass of 16.5 kDa) can accommodate ~9 Ang molecules. The minimum size required for a heparin fragment to effectively inhibit HT-29 cell adhesion to Ang was determined to be 6 disaccharide units. The implications of these findings for inhibition of Ang-mediated tumor establishment in vivo are discussed.

Cell adhesion and angiogenesis are essential events in the establishment, growth, and dissemination of solid tumors. Molecules that are involved in these processes therefore constitute attractive targets for cancer therapy. Angiogenin (Ang),1 a 14.1-kDa single-chain, basic polypeptide in the pancreatic RNase superfamily (1), is both a potent inducer of angiogenesis (2), and recent findings strongly suggest that it plays a critical role in tumor formation. Thus, non-cytotoxic, anti-human Ang monoclonal antibodies and the Ang-binding protein actin prevent or delay the establishment of HT-29 and other human tumor xenografts in athymic mice (4, 5). This inhibition may reflect interference with the initial attachment of tumor cells to the substratum and/or suppression of subsequent angiogenesis required for nourishment of the growing tumor.

The precise molecular events involved in Ang-induced blood vessel formation and in cell adhesion to Ang remain to be determined. Results to date indicate that angiogenic activity requires both the ribonucleolytic action of Ang (6–8) and binding of the protein to endothelial cells (9–11). Adhesion of tumor cells to Ang is mediated by an as yet unidentified cell-surface heparan sulfate/chondroitin sulfate proteoglycan (3). Treatment of cells with heparinase or with inhibitors of proteoglycan synthesis or secretion decreases adhesion, as does the presence of soluble glycosaminoglycans (GAGs). Moreover, Ang binds tightly to heparin-Sepharose. These observations, apart from their implications for the nature of the adhesion receptor, raise the possibility that heparin itself may regulate or modulate some of the actions of Ang in vivo.

In the present study, physical and functional aspects of the interaction of Ang with heparin have been investigated further. We have examined the heparin recognition site on Ang and the stoichiometry of binding. Purified heparin fragments have been used to define the heparin size requirements for inhibition of tumor cell adhesion to Ang. The effects of heparin on the ribonucleolytic and angiogenic activities of Ang have also been determined.

**EXPERIMENTAL PROCEDURES**

**Materials**

<Gl u-1 Ang and Met-(−1) Ang were obtained from a recombinant expression system in Escherichia coli (12). The two forms differ only at their N termini (<Glu versus Met-Gln) and are indistinguishable with respect to angiogenic and ribonucleolytic activities. The <Glu-1 mutant derivatives R5A, R32A, R66A, and R70A, and the Met-(−1) derivatives K40Q, H13A, R31A, and R33A were from earlier studies (6, 7, 13).> 

**Cell Culture**

HT-29 human adenocarcinoma cells (HTB38 from the American Type Culture Collection) were cultured in 75-cm² culture flasks (Nunc) in Dulbecco’s modified Eagle’s medium (DMEM, Whittaker Bioproducts) containing 5% heat-inactivated fetal bovine serum (HyClone), 50 μg/ml gentamicin, and 200 nM fungizone in a humidified atmosphere of 5% CO₂, 95% air at 37 °C and routinely passaged at a 1:5 ratio.

**Assays**

**Cell Adhesion—Bacteriologic Petri dishes were coated with protein test samples as described (15). Subconfluent cell monolayers were har-
vested with 1 mM EDTA in Dulbecco’s phosphate-buffered saline (DPBS), resuspended in DMEM containing 1 mg/ml bovine serum albumin (BSA fraction V, low endotoxin, Sigma; DMEM-BSA) and centrifuged for 5 min at 400 × g at room temperature. The cell pellet was washed twice with DMEM-BSA and resuspended in the same medium at 20 × 10^6 cells/ml. One ml of the cell suspension was seeded in the coated dishes and incubated in a humidified atmosphere of 5% CO₂, 95% air at 37 °C for the indicated amount of time. The dishes were then washed and fixed, and the cell number was measured as described (15).

**Angiogenic Activity**—The effect of heparin on the angiogenic activity of Ang was assessed with the chicken embryo chorioallantoic membrane (CAM) assay (13, 16). Five-μl aliquots of Ang (5 μg/ml) with or without heparin (10 μg/ml) in DPBS were applied to Thermomax disks and air-dried. The disks were then implanted on the CAM, and angiogenesis was evaluated after 68 ± 2 h. The numbers of positive and negative responses for each sample from two sets of assays were combined, and χ² values were calculated from an outcome contingency table by comparing the test sample with a water control; the associated probabilities, p, were then obtained (11). A value of p < 0.05 identifies a sample as active.

Ribonuclease Activity—The effect of heparin on the ribonuclease activity of Ang was examined with both dinucleotide (CpA) and polynucleotide (tRNA) substrates. In the former case, <Glu-1 Ang (2–4 μM) was incubated with 100 μM substrate in 0.2 M Hepes, pH 7, at 25 °C in the presence or absence of heparin. After 15.5–18 h, the concentrations of Cpa and the products cytidine cyclic 2’ 3’-phosphate and adenosine in the mixtures were determined by C18 HPLC and k₅/Kc values were calculated (6, 17). Assays with tRNA as substrate were performed as described (18), except that the buffer was 0.1 M Hepes and incubations were for 2 h.

Oligonucleotide-directed Mutagenesis

The gene for the Ang triple mutant <Glu-1 R31A/R32A/R33A was prepared by the polymerase chain reaction overlap extension method of Ho et al. (19) as described (11). The template for polymerase chain reaction was the expression plasmid pAng3 (13), and the mutagenic oligonucleotides were 5’-GGATCTGCATGCTTACGGTGGCAGACCCGCGTGCAG-3’ and 5’-GGATCTGCATGCTTACGGTGGCAGACCCGCGTGCAG-3’ (13). The mutant protein eluted 11 min earlier than native Ang during Mono S chromatography and 3 min after Ang during C18 HPLC. Amino acid analysis demonstrated the loss of three arginines, an increase of three alanines, and no other significant changes compared with native Ang.

**Heparin-Sepharose Chromatography**

Protein was loaded onto a 1-ml Hitrap heparin-Sepharose column, which had been equilibrated with DPBS at room temperature. The column was then washed with 3 ml of DPBS, and a 35-ml linear gradient from 0.14 to 1 M NaCl in DPBS was applied. The flow rate was 0.66 ml/min. One-min fractions were collected and their ionic strength measured by conductimetry. Human Ang elutes from the Hitrap column at a flow rate of 15–20 ml/h. Fractions of 4.5 ml were collected and assayed for their uronic acid content by the carbazole reaction (24) and assayed for their effects on HT-29 cell adhesion to Ang. Selected fractions were analyzed by PAGE with Azure A staining (25), which revealed size heterogeneity in all cases. Various pools were lyophilized, dissolved in 0.66 M NaCl, 0.14 M NaCl, and the concentration required for heparin-Sepharose CL4B (3).

**Effect of Heparin on Cleavage of Ang by Trypsin**

Ang (28 μg) was incubated with 0.5 μg of HPLC-purified trypsin (20) at 37 °C in 2 μl of DPBS in the presence or absence of 70 μg of heparin. Reactions were stopped by addition of 1 μl of 1 M Mes, pH 2.2. The digests were dried directly onto amylamine membranes (Sequelon, Millipore) and coupled to the membranes by a carbodiimide reaction (21). After washing with water and methanol, membranes were placed in a Millipore ProSequence and sequencing was performed for five cycles by the 380B/FAPA protocol. PTH-derivatives were quantitated by HPLC (22). The Ang preparation employed in these experiments consisted of a 15:1 mixture of the <Glu-1 and Met(–1) forms. The use of the ε-NH₂ blocked Ang as the primary species facilitated detection of internal sequences that opened up during the early stages of digestion; the presence of a trace quantity of the unblocked protein provided a basis for normalizing yields obtained in the presence and absence of heparin (see legend to Table III).

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**Table I**

| Protein       | [NaCl] | μ    |
|---------------|--------|------|
| <Glu-1 Ang    | 0.64   |
| <Glu-1 R5A    | 0.64   |
| <Glu-1 R32A   | 0.58   |
| <Glu-1 R66A   | 0.65   |
| <Glu-1 R70A   | 0.59   |
| Met(–1) Ang   | 0.68   |
| Met(–1) R31A  | 0.63   |
| Met(–1) R33A  | 0.58   |
| Met(–1) K40Q  | 0.69   |
| Met(–1) H13A  | 0.67   |

**Light Scattering**

Successive aliquots of <Glu-1 Ang (5.5 μl each, containing 2 nmol) were mixed with 2 nmol of heparin in 1 ml of DPBS in a quartz cuvette, and light scattering at 90° was measured at 320 nm with a Perkin-Elmer MPF-3 fluorescence spectrophotometer 1–5 min after each addition. Scattering was constant during this time interval. The observed scattering was corrected for the effect of dilution (in all cases < 10%). Scattering of the individual components (Ang at 30 μM; heparin at 2 μM) was negligible.

**Heparin Fragments**

One gram of heparin was depolymerized by treatment with nitrous acid as described (23). The product mixture was desalted on a 2.6 × 50-cm Sephadex G-10 column in 10% ethanol, concentrated to 5 ml by rotary evaporation, and fractionated by gel filtration on a Sephadex G-50 superfine column (1.6 × 200 cm) in 0.5 M ammonium bicarbonate at a flow rate of 15–20 ml/h. Fractions of 4.5 ml were collected and assayed for their uronic acid content by the carbazole reaction (24) and for their effects on HT-29 cell adhesion to Ang. Selected fractions were analyzed by PAGE with Azure A staining (25), which revealed size heterogeneity in all cases. Various pools were lyophilized, dissolved in 10% ethanol, and rechromatographed on Sephadex G-50 as above. Fractions that eluted in the region of interest were assayed for their uronic acid content, and selected fractions were then lyophilized and reconstituted in water for further analysis.

**Mass Spectrometry**

The molecular masses of heparin fragments were measured by matrix-assisted laser desorption ionization mass spectrometry with Ang as a complexing agent and sinapinic acid as the matrix (26, 27).

**RESULTS**

**Heparin-binding Sites on Angiogenin**

**Effects of Ang Mutations on Affinity for Heparin-Sepharose**—The location of the heparin-binding site on Ang was investigated initially by measuring the affinity for heparin-Sepharose of eight Ang mutants available from previous studies. The derivatives selected contained replacements of basic residues, primarily arginines, from several different regions of the molecule. Basic amino acids are known to be important components of heparin-binding sites in general, and arginine has been shown to have particularly high affinity for sulfate groups on GAGs (28). Some of the mutant proteins had been produced as Met(–1) derivatives, whereas others were in the natural <Glu-1 form (see “Experimental Procedures”). Heparin-Sepharose chromatography of unmutated Met(–1) Ang and <Glu-1 Ang revealed that these two forms elute from heparin-Sepharose at different NaCl concentrations (0.68 M and 0.64 M, respectively; Table I). Thus the behavior of each mutant was compared with that of its corresponding parent. Four of the derivatives examined (R31A, R32A, R33A (Fig. 1A), and R70A) had significantly decreased affinity for heparin-Sepharose: they eluted at NaCl concentrations 0.05–0.10 M lower than for
Ang (Table I). Three other mutants with similarly decreased basicity (R5A, K40Q, and R66A) had virtually unaltered affinity, as did the one His mutant examined, H13A. RNase A, a highly basic Ang homologue, eluted at a substantially lower NaCl concentration, 0.25 M (Fig. 1A).

To assess the overall contribution of the basic cluster Arg-31, -32, and -33, a triple mutant (R31A/R32A/R33A) was prepared by oligonucleotide-directed mutagenesis. It eluted from heparin-Sepharose at a NaCl concentration 0.15 M lower than for the parent (<Glu-1) protein (Fig. 1B). This decrease in affinity is greater than that resulting from any of the three individual mutations, and somewhat less than that expected if their effects are independent and additive.

Effects of Heparin on Cleavage of Angiogenin by Trypsin—Incubation of Ang with 2–3% (w/w) trypsin at 37 °C for 18 h results in cleavage of all 19 theoretically susceptible peptide bonds (1). The capacity of heparin to protect these various sites from trypsin was examined by sequencing the unseparated peptide mixtures produced during the early stages of digestion. Consistent with previous findings (29), the predominant site of cleavage in the absence of heparin was Lys-60: i.e. the major products obtained during the five cycles analyzed were PTH-Asn, -Gly, -Asn, -Pro, and -His, respectively (the sequences of all possible tryptic peptides are shown in Table II). Approximately 5% of the Ang had been hydrolyzed at this site after 2 min (Table III). Three- to 5-fold lower quantities of products indicative of cleavages at Arg-5, Arg-21, Lys-54, Arg-70, Arg-95, Arg-101, and Arg-122 were also found. The most striking effect of heparin at this time was on the reaction at Lys-60, which was suppressed by a factor of about 4, as indicated by the decreases in the yields of Asn, Gly, Asn, Pro, and His in cycles 1–5, respectively. A less pronounced (<2-fold) decrease in cleavage at Arg-101 (yielding Asn-Val-Val-Val-Ala) was also evident. Hydrolysis at the other sites appeared to be virtually unchanged, indicating that heparin did not inhibit the protease itself. The results for 5 min tryptic digests (not shown) were similar. Again, heparin primarily decreased cleavage at Lys-60 (by ~3-fold) and to a lesser extent reduced hydrolysis at Arg-101. In this case, however, cleavage at Arg-31 (producing Arg-Arg) also seemed to be diminished by 2-fold.

Effects of Heparin on the Enzymatic and Angiogenic Activities of Angiogenin

The Ang-heparin interaction was characterized further by examining the effects of heparin on the ribonucleolytic and angiogenic activities of Ang. Heparin was shown previously to inhibit Ang-catalyzed cleavage of tRNA at pH 5.5 with an IC50 value of 700 μg/ml (30). The present study used both tRNA and the dinucleotide CpA as substrates at the more physiological pH of 7. With tRNA, 0.5 mg/ml heparin inhibited by 48%. However, at concentrations of 1.0 and 2.0 mg/ml inhibition increased only to 55% and 63%, respectively, suggesting that heparin acts as a partial inhibitor. The effect on cleavage of CpA was even smaller; both 0.25 and 0.75 mg/ml heparin decreased the kcat/Km value by 15%, from 3.3 M-1 s-1 to 2.8 M-1 s-1.

Heparin did not alter significantly the angiogenic response to Ang on the chicken embryo CAM. In the absence of heparin, 10 ng of Ang produced 51% positive responses (27/53; p = 0.00005). When 50 μg of heparin were included, 48% of the eggs were positive (24/50; p = 0.00014). A water control sample assayed simultaneously yielded 10% positive responses (4/39).
A 15.1 mixture of <Glu-1 and Met(-1) Ang was treated with trypsin for 2 min at 37 °C in the absence (−) and presence (+) of heparin, and the unseparated peptides were sequenced. The yields of PTH-derivatives (in pmol) for the first five cycles are shown. To facilitate comparisons, values listed in "plus" columns have been normalized with respect to those in "minus" columns based on the respective yields of PTH-Met for cycle 1 (44.1 and 34.9 pmol). This PTH-Met derives only from Met(-1) of this preparation of Ang. The values obtained for amino acids corresponding to the sequence NGNPH produced by cleavage at Lys-60 are highlighted. PTH-Asp and PTH-Glu are not shown since peptides were coupled to membranes through their carboxylate moieties.

| PTH-derivative | Cycle 1 | Cycle 2 | Cycle 3 | Cycle 4 | Cycle 5 |
|---------------|---------|---------|---------|---------|---------|
|               | −       | +       | −       | +       | −       | +       |
| Hia           | −a      | 27.0    | −       | 10.1    | −       | 51.0    | 10.0    |
| Asn           | 100.9   | 37.0    | −       | 24.6    | 35.3    | 24.2    | 15.1    | 13.4    |
| Arg           | 41.9    | 43.8    | 22.1    | 22.3    | 20.0    | 18.7    | −       | 22.1    | 18.2    |
| Serb          | −       | 12.4    | 16.4    | 14.3    | −       | 13.5    | 10.7    | 24.9    | 15.5    |
| Thr           | −       | −       | 19.6    | 15.5    | 10.4    | 14.6    | −       | −       | −       |
| Gla           | −       | 13.0    | 18.6    | 13.5    | 10.9    | 12.8    | −       | −       | −       |
| Gly           | −       | 13.0    | 23.5    | −       | −       | −       | −       | −       | −       |
| Ala           | 41.2    | 32.2    | 10.2    | 10.4    | 19.4    | 19.0    | −       | 10.2    | 26.8    | 16.3    |
| Tyr           | 25.4    | 19.3    | −       | 17.6    | 22.7    | 10.4    | 10.8    | 43.1    | 15.1    | 27.2    | 15.6    |
| Pro           | −       | −       | 17.6    | 22.7    | 10.4    | 10.8    | −       | −       | −       | −       |
| Met           | 34.9    | 34.9    | −       | 28.6    | 20.0    | 24.8    | 15.8    | 24.8    | 13.9    | 13.0    | 16.8    |
| Val           | −       | −       | 28.6    | 20.0    | 24.8    | 15.8    | 24.8    | 13.9    | 13.0    | 16.8    |
| Lys           | −       | −       | −       | −       | −       | −       | −       | −       | −       | −       |
| Phe           | 13.0    | −       | −       | −       | 14.5    | 14.5    | 32.1    | 20.9    | 37.4    | 21.7    |
| Ile           | 26.1    | 24.3    | 19.1    | 20.5    | −       | −       | 11.8    | 9.8     | 11.4    | −       |
| Leu           | 10.2    | 12.1    | 11.1    | −       | −       | −       | 11.1    | −       | 23.8    | 16.2    |

—, less than 10 pmol was obtained.

b PTH-Ser values shown also include PTH-d-Ser.

Identification of Inhibitory Heparin Fragments

Intact heparin (50 μg/ml) inhibits adhesion of HT-29 cells to Ang by 60% (3). In contrast, four heparin disaccharides (see “Experimental Procedures”) tested individually at up to 100 μg/ml did not inhibit HT-29 cell adhesion to Ang (data not shown). To determine the minimum size required for a heparin fragment to inhibit effectively, heparin was depolymerized with nitrous acid and the products were separated by gel filtration and assayed for their effect on adhesion. In the chromatographic system used, intact heparin elutes with a Kav value of 0.15, whereas disaccharides elute with a Kav value of 0.85. Heparin fragments that eluted with Kav values between 0.48 (60% inhibition) and 0.67 (no significant inhibition) in all cases showed multiple discrete bands. Rechromatography of various pools of this material yielded several fractions that were composed primarily of a single size species as judged by PAGE (Fig. 4A). Mass spectrometry of these fractions revealed average molecular masses consistent with the presence of 8-, 10-, 12-, 14-, and 16-saccharide products (lanes 2–6, respectively) (Table IV). When tested for their effects on HT-29 cell adhesion to Ang at 50 μg/ml (Fig. 4B), only the dodecamer, tetradecamer, and hexadecamer inhibited appreciably: by 46%, 53%, and 63%, respectively.

DISCUSSION

Heparin binds tightly and specifically to a vast array of disparate proteins, including angiogenesis factors (the fibro-
stained as described above. The heparin fragments. After 60 min, the plates were rinsed, fixed, and obtained by Sephadex G-50 chromatography (containing products with identified fractions of nitrous acid-treated heparin. Three pools of material recently reported by Faham single protein, basic FGF (bFGF). The crystal structure re-
laminin (40), and tenascin (41)). Despite long-standing interest in the heparin-binding sites on antithrombin III (see Refs. 48 and 65), and the crystal structures of FGF complexes with small NH groups. This picture of the heparin binding site on FGF between 8 FGF residues and, primarily, sulfate groups on the GAG. The FGF residues are located on three elements of primary structure, and all but one lie on surface loops. About half of the interactions are ionic and involve Arg or Lys side chains, whereas the others utilize Asn or Gln side chains or main-chain NH groups. This picture of the heparin binding site on FGF agrees substantially with previous proposals based on chemical modification, mutagenesis, and thermodynamic results (43–45), and the crystal structures of FGF complexes with small anions (46, 47).

Analogous structure-function, kinetic, and modeling studies with other heparin-binding proteins suggest that many features of the heparin-bFGF complex are widely shared. Thus, the heparin-binding sites on antithrombin III (see Refs. 48 and 49), thrombin (50, 51), fibronectin (52), and lipoprotein lipase (53) also appear to be assembled from multiple discrete segments and to be rich in arginines and lysines. (Only these two residue types have been closely scrutinized as potential candidates.) The effects of ionic strength on \( K_a \) values for the complexes examined vary widely, indicating that the energetic contributions of salt linkages range, minimally, from ~40% (antithrombin (54)) to >80% (thrombin (55) and mucus proteinase inhibitor (37)).

In the present study, the heparin binding site on Ang was investigated by measuring the effects of mutating various basic residues of Ang on affinity for heparin-Sepharose (Table I, Fig. 1) and by determining which basic residue (i.e. tryptic cleavage sites on the native protein are protected by heparin (Table III). Four single-site mutants (R31A, R32A, R33A, and R70A) bound more weakly than Ang to heparin-Sepharose, and the triple mutant R31A/R32A/R33A had an affinity lower than for any of the individual mutants. In the three-dimensional structure of Ang (56), residues 31–33 lie on \( \alpha \)-helix 2 whereas residue 70 is part of \( \beta \)-strand 3 more than 30 Å away (Fig. 5). The side chains of Arg-31 and -32 extend toward the exterior of the molecule and are fully accessible for interactions. In contrast, the side chain of Arg-33 already engages in multiple hydrogen bonds with the main-chain carbonyl oxygens of Thr-11 and Tyr-14 on \( \alpha \)-helix 1 (56) and is available only for a single additional bond unless the intramolecular linkages are broken upon heparin binding. Such a disruption seems unlikely, however, since removal of these interactions (by Ala substitution) decreases enzymatic activity by 7-fold (13, 56) and yet heparin inhibits only slightly. Arg-70 is also not completely free; its NH1 group is fully accessible for interactions. Indeed, they are components of the enzymatic active site that are predicted to interact directly with phosphate moieties on RNA (7, 8, 56). The failure of mutations of these four accessible basic Ang residues to decrease binding to heparin-Sepharose than does the <Glu-1 form. This part of the protein is not seen in the crystal structure owing to its high flexibility, but is likely to be on the surface.

Four single-site mutations (Arg-5 \( \rightarrow \) Ala, Arg-66 \( \rightarrow \) Ala, His-13 \( \rightarrow \) Ala, and Lys-40 \( \rightarrow \) Glu) do not affect binding to heparin-Sepharose, although all of the residues substituted are accessible. Arg-5 and Arg-66 in native Ang are entirely free of interactions. His-13 and Lys-40 each form a hydrogen bond with another Ang residue but are available for additional ones. Indeed, they are components of the enzymatic active site that are predicted to interact directly with phosphate moieties on RNA (7, 8, 56). The failure of mutations of these four accessible basic Ang residues to decrease binding to heparin-Sepharose, together with the low heparin affinity of the related basic protein RNase A, strongly suggests that binding of Ang to this GAG is specific. Thus, the weakened binding of the Arg-31, -32,

### Table IV

| Lane in Fig. 4A | Measured \( M_r \) | Theoretical \( M_r \)\(^a\) | Number of saccharides |
|----------------|-----------------|-----------------|----------------------|
| 2              | 2192            | 2232.8          | 8                    |
| 3              | 2683            | 2810.3          | 10                   |
| 4              | 3177            | 3387.8          | 12                   |
| 5              | 3610            | 3965.2          | 14                   |
| 6              | 4230            | 4542.7          | 16                   |

\(^a\) Theoretical values correspond to the trisulfated disaccharides (with a terminal residue of 2,5-anhydromannitol-6-sulfate generated by depolymerization with nitrous acid). Differences between measured and theoretical values may reflect lower degrees of sulfation in the fractions analyzed or loss of sulfates upon ionization.
-33, and -70 mutants can be presumed to reflect the loss of specific interactions rather than a change in the ion-exchange properties of the protein.

The effects of these replacements on binding to heparin-Sepharose ($\Delta[NaCl] = 0.04–0.10$) are similar to some of those reported for mutations of putative heparin-binding arginines and lysines in fibronectin (52), lipoprotein lipase (53), and thrombin (57). Much larger changes ($\Delta[NaCl]$ up to 0.75; Ref. 44) have been observed for mutations of bFGF residues known (42) to be part of the contact surface. Thus, the individual interactions may not be as well optimized in Ang and these other proteins as in bFGF, which binds heparin much more tightly. Alternatively, other residues, as yet unidentified, may make much larger contributions to heparin binding.

Consistent with the involvement of Arg-31, -32, -33, and -70 in heparin binding, R31A, R32A, R33A, and R70A are less effective than Ang as substrates for adhesion of HT-29 cells (Fig. 2), a process shown previously to be mediated by a cell-surface heparan sulfate/chondroitin sulfate proteoglycan (3). Mutation of Arg-66 also diminishes cell adhesion, although in this case there is no parallel decrease in binding to heparin-Sepharose. The dissociation of these two effects may reflect chemical differences between the heparin attached to the Sepharose. The dissociation of these two effects may reflect chemical differences between the heparin attached to the Sepharose resin versus the heparan sulfate chains on the proteoglycan. Alternatively, replacement of Arg-66 may affect binding to the chondroitin sulfate or protein portion of the proteoglycan rather than the heparan sulfate chains.

Heparin decreases tryptic cleavage of Ang at Lys-60 by several fold, suggesting that it also binds to the region of Ang that contains this residue. Since a free portion of the heparin chain might sterically hinder the action of trypsin, it is unclear from this finding whether Lys-60 itself interacts with heparin. We note, however, that this residue is on a highly accessible surface loop. Heparin also produces more modest effects on tryptic cleavage at Arg-31 and Arg-101. Failure of heparin to influence digestion at any other sites may reflect the loss of structural integrity of Ang and its capacity to bind heparin once the first few sites have been cleaved.

The present results identify the cluster Arg-31/Arg-32/Arg-33 as a major site of interaction with heparin, and indicate that the GAG chain may extend from this region in the direction of Lys-60, Arg-70, and the N terminus (Fig. 5). The N terminus and the Lys-60 loop are on the same face of Ang as the arginine cluster and, although these various sites are separated by as much as 37 Å, a heparin molecule 8 or more saccharides in length could bridge such a distance. However, Arg-70 is on the opposite face of Ang and cannot contact the same heparin chain as residues 31–33. The weakened binding of R70A Arg to heparin-Sepharose may therefore be due to the loss of interactions between the Arg-70 guanidino group and the loop that contains Lys-60 (see above). It is also possible that Arg-70 may form part of a second heparin-binding site, although simultaneous attachment of two heparin molecules to a single Ang would seem unlikely due to the strong electrostatic repulsion between the two highly charged GAG chains.

The binding density of ~9 Ang/heparin chain determined by measuring light scattering after addition of Ang to heparin translates into 1 Ang molecule/5 or 6 monosaccharide units. Given the heterogeneity of heparin, this rules out the possibility that Ang, like antithrombin III (23), recognizes only a single, highly specific structure. At the same time, it contrasts with the finding that heparin fragments of at least 12 saccharides are required to inhibit HT-29 cell adhesion to Ang (Fig. 4B). This implies that heparin sequences that bind sufficiently tightly to prevent the interaction of Ang with the adhesion receptor may occur relatively infrequently or that they are significantly larger than 5–6 saccharide units. In this regard, it should be noted that Ang is long enough (43 Å) to form contacts over an 8–10-saccharide portion of heparin (i.e., a 10–12-saccharide nitrous acid digestion product of heparin, since the terminal unit is converted to 2, 5-anhydromannitol-6-sulfate).

The partial map of the heparin binding site on Ang here determined, together with the observed functional effects of this GAG, suggests that the interaction of Ang with heparin does not modulate the enzymatic or angiogenic activities of Ang. The active site mutants tested (H13A, K40Q, and R5A) all have unchanged affinity for heparin, and heparin has essentially no influence on Ang-catalyzed cleavage of dinucleotide substrates. It is a somewhat more effective, but still partial, inhibitor of tRNA cleavage, indicating that it may contact some peripheral subsite for RNA binding outside the catalytic center. Heparin has no detectable effect on the capacity of Ang to induce new blood vessels on the CAM, although it interacts with the cluster Arg-31–Arg-33 that is involved in translocation of Ang to the nucleolus of endothelial cells (58), thought to

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3 Enzyme-linked immunosorbent assays demonstrate that all five mutants bind to polystyrene as efficiently as Ang (K. A. Olson, Harvard Medical School, personal communication), largely ruling out the possibility that reduced HT-29 cell adhesion to these proteins is due to decreased coating of the plastic dishes.
be an essential step in the angiogenic mechanism (59). Moreover, it binds near or within the putative cell-binding site of Ang, which is required for angiogenic activity. This site is distinct from the enzymatic active center and includes Asn-109 plus residues in the segment 61–67 (10, 11, 13); proteolytic cleavage of the 60–61 or 67–68 peptide bond, deamidation of Asn-61 or Asn-109, and mutation of Arg-66 to Ala all abolish angiogenic activity without influencing ribonuclease activity appreciably. Although heparin does not contact Arg-66 in this site, it does hinder the action of trypsin at Lys-60, which is either adjacent to or part of this region. Thus the heparin recognition site may overlap the cell-binding site. In this case, the inhibitory effects of heparin and heparin fragments on angiogenic activity of Ang.

Whatever the answer to this question ultimately turns out to be, the inhibitory effects of heparin and heparin fragments on the adhesion of tumor cells to Ang raise the possibility that heparin-related compounds may have therapeutic potential as antagonists of Ang-dependent tumor formation and metastasis. As noted above, Ang has been demonstrated to play a key role in the establishment of some human tumors in an athymic mouse model (4, 5), and part of this role may involve the critical early event of tumor cell attachment. HT-29 cells in culture adhere to Ang remarkably quickly, in fact, much more rapidly than to the common extracellular matrix molecules fibronectin, laminin, and collagen (3). Further studies on the in vivo importance of Ang-mediated tumor cell attachment and on the potential utility of heparin derivatives for inhibiting this process therefore seem warranted at this time.

Acknowledgments—Mass spectrometry data were kindly provided by Dr. P. Juhász at the MIT Mass Spectrometry Facility, which was supported by National Institutes of Health Grant RR 00317 (to Prof. K. Biemann). We thank Dr. K. R. Acharya for providing Fig. 5, Dr. J. F. Riordan for valuable discussions, and Kerrin Green for excellent technical assistance. The support and advice of Dr. B. L. Vallee are gratefully appreciated.

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J. Biol. Chem. 1997, 272:9818-9824.
doi: 10.1074/jbc.272.15.9818

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