A Novel Anti-angiogenic Form of Antithrombin with Retained Proteinase Binding Ability and Heparin Affinity*

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Latent antithrombin, an inactive antithrombin form with low heparin affinity, has previously been shown to efficiently inhibit angiogenesis and tumor growth. We now show that heat treatment similar to that used for preparation of latent antithrombin also transforms antithrombin to another form, which we denote prelatent, with potent anti-angiogenic and anti-tumor activity but with retained proteinase- and heparin-binding properties. The ability of prelatent antithrombin to inhibit angiogenesis is presumably due to a limited conformational change, which may partially resemble that in latent antithrombin. Such a change is evidenced by a different cleavage pattern of prelatent than of native antithrombin by nontarget proteinases. Prelatent antithrombin exerts its anti-angiogenic effect by a similar mechanism as latent antithrombin, *i.e.* by inhibiting focal adhesion formation and focal adhesion kinase activity, thereby leading to decreased proliferation of endothelial cells. The proteinase inhibitory fractions in commercial antithrombin preparations, which have been heat treated during production, also have anti-angiogenic activity, comparable with that of the prelatent antithrombin form.

Angiogenesis, the formation of new capillaries from pre-existing vessels, is important for regulation of many physiological processes, including wound healing, embryogenesis, and female reproductive functions. Deregelation of angiogenesis in diseases such as cancer leads to excessive formation of new blood vessels and tumor progression (1). A number of endogenous angiogenesis inhibitors, specifically inhibiting endothelial cell function and thus also tumor expansion, have recently been identified. Many of these inhibitors are derived by modification of abundant proteins which thereby gain new properties, *e.g.* endostatin, a fragment produced by proteolytic cleavage of collagen XVIII (2) and angiotatin, an analogous fragment of plasminogen (3).

Antithrombin, a plasma proteinase inhibitor of the serpin superfamily, is the major physiological inhibitor of coagulation serine proteinases, primarily thrombin and factor Xa. The anticoagulant polysaccharide, heparin, accelerates antithrombin inhibition of these enzymes up to 4000-fold by binding and activating the inhibitor by means of a specific pentasaccharide sequence (4, 5). Like all serpins, antithrombin traps its target proteinases by exposing a reactive bond, located in a surface loop (Fig. 1) (6), to the enzyme, which initially cleaves this bond as a normal substrate (4, 7). The opening of the reactive-bond loop at the acyl-intermediate stage of this cleavage, however, causes the N-terminal segment of the loop to rapidly insert as a middle strand into the main β-sheet, the A sheet, of the serpin. The proteinase, which is still attached to this segment by an acyl bond, is thereby translocated to the other end of the inhibitor and concurrently inactivated by distortion of the active site structure (8–12).

Full proteolytic cleavage of the reactive-bond loop of antithrombin by target or nontarget proteinases converts the inhibitor to an inactive form with low heparin affinity, in which the cleaved loop is inserted into the A sheet in a similar manner as in the proteinase complexes (Fig. 1) (13, 14). Heat treatment transforms antithrombin to another inactive, low heparin affinity form, denoted latent, in which the intact reactive-bond loop is analogously inserted into the A sheet (Fig. 1) (6, 15). Both cleaved and, more potently, latent antithrombin have recently been found to possess anti-angiogenic properties that are not shown by the native inhibitor (16, 17). These properties presumably are due to the conformational changes caused by loop insertion exposing certain epitopes that interact with appropriate endothelial cell membrane components. Antithrombin-proteinase complexes may have similar anti-angiogenic activity, although this possibility has not been investigated. Latent antithrombin has been shown to exert its anti-angiogenic effect by inducing apoptosis of endothelial cells, caused by disruption of cell-matrix interactions through uncoupling of focal adhesion kinase (17). Due to its anti-angiogenic activity, latent antithrombin efficiently inhibited tumor growth in mouse models (16, 17).

In this work, we show that heat treatment similar to that used for preparation of latent antithrombin converts native antithrombin to a form with potent anti-angiogenic and tumor inhibitory activity but, unlike latent antithrombin, with retained ability to bind proteinases and heparin. A different proteinase cleavage pattern of this form than of native antithrombin indicates that a limited conformational change has been induced by the heat treatment. The anti-angiogenic activity is presumably due to this conformational change, which may be related to that in latent antithrombin. We therefore tentatively denote the new antithrombin form as prelatent. We also show that the fractions with proteinase inhibiting activity*
in commercial antithrombin preparations, which have undergone heat treatment as a viral inactivation step during production, have anti-angiogenic activity similar to the novel prelatent form.

EXPERIMENTAL PROCEDURES

Antithrombin—Human α-antithrombin was purified from plasma by affinity chromatography on heparin-agarose, followed by anion-exchange chromatography (18). Commercial antithrombin preparations were purchased from Baxter (Vienna, Austria) and Pharmacia & Upjohn, Plasma Products (Stockholm, Sweden). Antithrombin concentrations were determined by absorption measurements at 280 nm with the use of an absorption coefficient of 0.65 liters g⁻¹ cm⁻¹ (19). Molar concentrations were calculated from a relative molecular mass of 58,000 (19).

Proteinases—Human α-thrombin, >99% α-form and >90% active by active site titrations (20), was a gift from Dr. John Fenton, New York State Department of Health, Albany, NY. Human factor Xa, predominantly α-form and ~70% active by active site titrations (21) was a gift from Dr. Steven T. Olson, University of Illinois, Chicago, IL.

Heparin—Heparin with high affinity for antithrombin and with an average M₉ of ~8000 and reduced polydispersity, i.e. containing ~26 saccharide units (20), was donated by Dr. Steven T. Olson. The anti-thrombin-binding heparin pentasaccharide (22) was a gift from Dr. M. Petitou, Sanofi Recherche, Toulouse, France.

Preparation of Prelatent Antithrombin—Plasma antithrombin (8–11 mg at a concentration of 3–4 mg/ml) was incubated for 24 h at 60 °C in 10 mM Tris/HCl, 0.5 mM sodium citrate, pH 7.4 (15). After dialysis against 0.02 M sodium phosphate, 0.05 M NaCl, pH 7.4, the sample was applied to a 5-ml HiTrap Heparin-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was eluted at room temperature with a 120-ml gradient to 1.5 M NaCl at a flow rate of 0.5 ml/min. Prelatent antithrombin, eluting at ~1 M NaCl, was concentrated by ultrafiltration and dialyzed against 0.02 M sodium phosphate, 0.1 M NaCl, pH 7.4.

Heparin Affinity Chromatography of Commercial Antithrombin—Commercial antithrombin preparations were dissolved in water and dialyzed against 0.02 M sodium phosphate, 0.05 M NaCl, pH 7.4. An amount of 50 IU of antithrombin, corresponding to 6–8 mg of active inhibitor, was applied to a 5-ml HiTrap Heparin-Sepharose column. The column was eluted and the protein peak appearing at ~1 M NaCl concentrated and dialyzed as described above.

Electrophoresis under Nondenaturing and Denaturing Conditions—Nondenaturing PAGE⁵ and SDS-PAGE were done on 7.5% gels with the Laemmli (23) and Tricine (24) buffer systems, respectively. The gels were stained with Coomassie Brilliant Blue R-250.

Chorionicallantoic Membrane Assay—The chorionicallantoic membrane (CAM) angiogenesis assay essentially followed a previously described procedure (25–27). Briefly, an avascular zone was identified in the chorioallantoic membrane (CAM) by angiography and marked with a black, dark gray, and light gray loop, the residue 325–375 region, and the C-terminal loop of the reactive bond are shown in the native structure. The Aβ-sheet (with the F-loop) is at the front of the molecule. The regions forming the reactive-bond loop, the residue 325–375 region, and the C-sheet in the native protein are indicated in black, dark gray, and light gray, respectively, in all three forms. The side chains of the reactive bond are shown in the native protein and those of the residue 341–346 epitope in all forms. The dashed line in the structure of latent antithrombin indicates missing electron density. Drawn by Dr. Sherry Mowbray, Dept. of Molecular Biology, Swedish University of Agricultural Sciences, based on PDB structures 2ant and 1at (6, 14).

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; CAM, chorionicallantoic membrane; FGF-2, fibroblast growth factor-2; PAE/FGFR-1 cells, porcine aortic endothelial cells overexpressing fibroblast growth factor receptor-1; FAK, focal adhesion kinase; Tricine, N₂-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

CAM, on which were placed filter discs (Whatman, Maidstone, United Kingdom) that had been saturated with 3 mg/ml cortisone acetate (Sigma-Aldrich, St. Louis, MO) and soaked in 30 μl of 0.02 M sodium phosphate, 0.1 M NaCl, pH 7.4, with or without 0.2 μg of fibroblast growth factor-2 (FGF-2; Roche Molecular Biochemicals, Germany) and 0.03, 0.3, 1, or 3 μg of native or prelatent antithrombin. After 3 days of incubation at 38.5 °C, the membrane was cut around the filter and inspected in a light microscope (Eclipse TE 300; Nikon, Tokyo, Japan) at a magnification of 2.5 or 4. Each membrane was assigned a score from 1 (low) to 4 (high), based on the number of vessel branch points (27).

Tumor Growth in Vivo—Animal work was carried out at the animal facility of the Biomedical Center, Uppsala University, and was approved by the local board of animal experimentation and thus performed according to the United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (28). Female, 6-week-old C57BL/6J mice (M & B A/S, Ry, Denmark) were acclimatized and caged in groups of five and were fed a diet of animal chow and drinking water ad libitum. The mice were anesthetized with isoflurane (Forene; Abbott, Solna, Sweden) during all manipulations. T241 fibrosarcoma cells (0.5 x 10⁶ cells in a volume of 50 μl) were injected subcutaneously into the left flank of each mouse.

Animals carrying palpable tumors within 4 days after injection were randomized and received treatment with 1 mg/kg/day of native antithrombin, prelatent antithrombin, or buffer (0.02 M phosphate, 0.1 M NaCl, pH 7.4), given as daily subcutaneous injections in the right flank for 10 days. The tumors were measured once a day in a double-blind procedure, and tumor volumes were calculated by the formula: V/6 x P² x L². Statistical analysis was performed by ANOVA. The mice were sacrificed at the end of the treatment.

Cell Culture—The porcine aortic endothelial cell line overexpressing fibroblast growth factor receptor-1 (PAE/FGFR-1 cells) (29) was cultured in Ham’s F-12 medium (Life Technologies, Taiby, Sweden), supplemented with 10% fetal calf serum (Life Technologies).

Endothelial Cell Proliferation—PAE/FGFR-1 cells were seeded into 24-well dishes (2 x 10⁴ cells/well). After 4 h at 37 °C, the medium was replaced with Ham’s F-12 medium containing 0.1% fetal calf serum, and the cells were incubated for an additional 12 h. At this time, FGF-2 (20 ng/ml), native antithrombin (1 μg/ml), or prelatent antithrombin (1 μg/ml) alone or FGF-2 together with native or prelatent antithrombin at these concentrations were added, and the incubation was continued. The same additions were then made after an additional 2 days. Cell numbers were scored in a Coulter counter (Coulter Electronics, Luton, UK) after a total of 5 days of incubation. All experiments were performed in triplicate.

Focal Adhesion Kinase Activity—PAE/FGFR-1 cells were serum-starved overnight and preincubated for 4 h at 37 °C with native or prelatent antithrombin (3 μg/ml). The cells were then washed and stimulated for 10 min at 37 °C with FGF-2 (100 ng/ml), native antithrombin (3 μg/ml), or prelatent antithrombin (3 μg/ml) alone or with FGF-2 in combination with native or prelatent antithrombin at these concentrations. The cells were lysed in 0.02 M Hepes, 0.15 M NaCl, 1% (w/w) Nonidet P-40, 10% (v/v) glycero, 0.3 mM NaN₃, 0.1% (w/w) aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, pH 7.5, and focal adhesion kinase (FAK) was immunoprecipitated with specific antibodies (Transduction Laboratories, Lexington, KY) accord-
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ing to the protocol from the manufacturer. The precipitates were separated by SDS-PAGE, and the proteins were transferred to a Hybond-C Extra (Amersham Pharmacia Biotech) nitrocellulose membrane. The membrane was treated with anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology, Lake Placid, NY), and immunoreactive proteins were detected by an enhanced chemiluminescence detection system based on a protocol described earlier (30).

Stoichiometry and Affinity of Heparin Binding—Stoichiometries of full-length heparin binding and affinities of full-length heparin or pentasaccharide binding to native and prelatent antithrombin were measured at 25 °C ± 0.2 °C by titrations of the antithrombin forms with the saccharides, as described previously (18, 31, 32). The titrations were monitored by the increase in tryptophan fluorescence that accompanies the interaction (33).

Stoichiometry and Kinetics of Proteinase Inactivation—Stoichiometries of inhibition of α-thrombin by native and prelatent antithrombin were determined by titrating the enzyme with the inhibitor forms and measuring the residual enzyme activity with a chromogenic substrate, as detailed previously (18, 32). Second-order rate constants for inhibition of α-thrombin or factor Xa by the two antithrombin forms alone and in complex with full-length heparin or pentasaccharide were measured at 25 °C ± 0.2 °C under pseudo-first order conditions with catalytic amounts of the saccharides, as in earlier work (18, 31, 32).

Circular Dichroism—Circular dichroism spectra in the far-ultraviolet wavelength region (200–250 nm) of native and prelatent antithrombin were measured at room temperature (22 ± 2 °C) in 0.02 M sodium phosphate, 0.1 mM NaCl, pH 7.4, with a Jasco J-41A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). The measurements were done with a band width of 2 nm in cells with 0.1-cm path lengths and with protein concentrations of 0.2 mg/ml. Mean residue ellipticities were calculated from a mean residue weight of 113 (34).

RESULTS

Isolation of Prelatent Antithrombin—Plasma antithrombin was incubated for 24 h at 60 °C in the presence of citrate at pH 7.4, as in the procedure for isolation of latent antithrombin (15). Affinity chromatography on heparin-agarose with NaCl gradient elution gave two major peaks (Fig. 2), viz. latent antithrombin, eluting at about 0.3 M NaCl (15), and an antithrombin form eluting at about 1.0 M NaCl, i.e. at a similar position as native antithrombin. As detailed below, further characterization of this form showed that it differed from native antithrombin in anti-angiogenic and certain biochemical properties in a manner that prompted us to denote it as prelatent. Prelatent antithrombin had the same mobility as native antithrombin. As detailed below, further characterization of this form showed that it differed from native antithrombin in anti-angiogenic and certain biochemical properties in a manner that prompted us to denote it as prelatent. Prelatent antithrombin had the same mobility as native antithrombin.

Anti-angiogenic Properties of Prelatent Antithrombin—The ability of prelatent antithrombin to inhibit angiogenesis was investigated by a CAM assay. Filter discs were saturated with a constant amount of FGF-2 alone or together with different amounts of native or prelatent antithrombin. The discs were applied to the CAM, and the development of new blood vessels was analyzed after 3 days. Prelatent antithrombin efficiently inhibited FGF-2-induced angiogenesis at an equimolar dose, although not in a 10-fold lower amount (Fig. 4, Table I). In contrast, native antithrombin did not appreciably affect neo-vascularization even at a 10-fold higher level than the lowest effective amount of prelatent antithrombin.

The possibility that prelatent antithrombin might affect tumor growth due to its anti-angiogenic properties was studied in a mouse fibrosarcoma model. C57BL/6j mice were inoculated with T241 fibrosarcoma tumor cells subcutaneously in the left flank. Treatment by daily injections of native antithrombin, prelatent antithrombin, or buffer in the right flank was initiated when the mice had palpable tumors and continued for 10 days. The tumor volume in animals treated with prelatent antithrombin was significantly reduced to 25% of that in animals treated with buffer (Fig. 5). Native antithrombin had an appreciably smaller effect on tumor growth, and the decrease in tumor volume was not significant.

The effect of prelatent antithrombin on endothelial cell proliferation was examined by culturing PAE/FGFR-1 cells together with FGF-2 and in the presence and absence of native or prelatent antithrombin. FGF-2 treatment induced an increase in the number of cells after 5 days of culture to ~150% compared with the control cultures (Fig. 6). The increase in cell number in cultures treated with FGF-2 together with prelatent...
Antithrombin was significantly lower, whereas prelatent antithrombin had no effect in the absence of the growth factor. In contrast, native antithrombin did not affect the FGF-2-induced increase in endothelial cell number.

Latent antithrombin has previously been shown to inhibit angiogenesis by impeding focal adhesion formation and FAK activity (17). A possible similar effect of prelatent antithrombin was investigated by stimulating PAE/FGFR-1 cells with FGF-2 in the presence and absence of native or prelatent antithrombin. The activity state of FAK was analyzed by immunoprecipitation of FAK, followed by immunoblotting with anti-phosphotyrosine antibodies. Incubation of the cells with prelatent antithrombin, but not with native antithrombin, was found to markedly reduce the FGF-2-induced FAK activity (Fig. 7).

Biochemical Properties of Prelatent Antithrombin—Titrations, monitored by the increase in tryptophan fluorescence induced by heparin binding, at high antithrombin concentrations showed that prelatent antithrombin bound full-length heparin (containing ~26 saccharide units) with a stoichiometry of 1:1.0 ± 0.05, identical to the binding stoichiometry of native antithrombin (18, 33). Heparin induced the same about 40% enhancement of tryptophan fluorescence in prelatent as in native antithrombin (18, 33). Heparin induced the same about 40% enhancement of tryptophan fluorescence in prelatent as in native antithrombin (18, 33).

Latent antithrombin is presented in Table I. AT, antithrombin.

### Table I

| Stimulator | Antithrombin | Amount of antithrombin (µg) | Angiogenesis score | No. of embryos |
|------------|--------------|-----------------------------|--------------------|----------------|
| Buffer     | None         | 0                           | 1.2                | 9              |
| FGF-2      | None         | 0                           | 3.0                | 9              |
| FGF-2      | Native       | 3                           | 2.7                | 9              |
| FGF-2      | Prelatent    | 0.03                        | 2.5                | 5              |
| FGF-2      | Prelatent    | 0.3                         | 1.3                | 5              |
| FGF-2      | Prelatent    | 3                           | 1.2                | 9              |

![Image](http://www.jbc.org/)

**Fig. 5.** Inhibition of tumor growth in mice by prelatent antithrombin. Mice with fibrosarcoma tumors in the left flank were treated daily with subcutaneous injections of phosphate buffer, native antithrombin (▲, n = 6), or prelatent antithrombin (■, n = 7) in the right flank. The doses of the proteins were 1 mg/kg/day. Tumor volumes were measured daily. Mean values ± S.E. are shown. Statistical significance of differences: native antithrombin versus buffer, p = 0.05; prelatent antithrombin versus buffer, p < 0.05.

**Fig. 6.** Decrease of FGF-2-induced endothelial cell proliferation by prelatent antithrombin. PAE/FGFR-1 cells were cultured with or without FGF-2 and native or prelatent antithrombin, and the cells were counted after 5 days. Mean values ± S.E. are shown. Statistical significance of differences: FGF-2 versus unstimulated, p = 0.008; prelatent AT versus unstimulated, p = 0.025; FGF-2 + prelatent AT versus FGF-2, p < 0.0001. AT, antithrombin.

**Fig. 7.** Reduction of FAK activity by prelatent antithrombin. PAE/FGFR-1 cells were incubated with or without FGF-2 and native or prelatent antithrombin, lysed, and immunoprecipitated with antibodies against FAK. The precipitates were separated by SDS-PAGE and the bands transferred to a membrane, which was subsequently incubated with anti-phosphotyrosine antibodies. Immunoreactive material was detected by enhanced chemiluminescence. AT, antithrombin.
Prelatent antithrombin inhibited thrombin with a stoichiometry of $1.1 \pm 0.1$, indistinguishable from that of native antithrombin (18). The second-order rate constants at pH 7.4, ionic strength 0.15, for thrombin and factor Xa inhibition by prelatent antithrombin alone and by the complexes of prelatent antithrombin with full-length heparin or pentasaccharide were also identical, within experimental error, to those of native antithrombin (Table III). The pentasaccharide enhancement of the rate of thrombin inhibition by native antithrombin is minimal, less than 2-fold (31, 35), and the inhibition of this enzyme by the antithrombin-pentasaccharide complexes therefore was not investigated.

The far-ultraviolet circular dichroism spectra of native and prelatent antithrombin were experimentally indistinguishable (not shown), in keeping with the extensive functional similarities between the two forms. Despite these similarities, proteolytic digestions gave evidence for a structural difference between the two antithrombin forms. All three enzymes used, chymotrypsin, thermolysin, and subtilisin, digested both native and prelatent antithrombin to species with slightly lower apparent $M_r$ than native antithrombin, i.e. ~52,000 (Fig. 3). N-terminal analyses of these bands indicated that both native and prelatent antithrombin were cleaved by chymotrypsin between Gln$^{299}$ and Lys$^{300}$, by thermolysin between Lys$^{299}$ and Ala$^{300}$, and by subtilisin between Gln$^{299}$ and Lys$^{300}$, as well as between Thr$^{34}$ and Asn$^{45}$. These cleavages are thus all within a 15-amino acid stretch in the N-terminal region of the protein. Most likely, the two forms were also cleaved in the reactive bond loop, i.e. around Arg$^{299}$, as indicated by previous work (15, 36), although the peptide representing the C-terminal end of the protein was not identified. In addition to these identical cleavages in native and prelatent antithrombin, the enzymes specifically digested prelatent antithrombin to fragments with apparent $M_r$ of 42,000–44,000 that were not seen in the corresponding digests of native antithrombin (Fig. 3). N-terminal analyses of these bands showed the same cleavage sites in the N-terminal region as in the $M_r$ ~52,000 bands. The specific cleavages of prelatent antithrombin thus must have occurred in the C-terminal end of the protein, the size of the bands being consistent with in the region between amino acids ~325 and ~375. Proteolytic fragments allowing identification of these specific cleavage sites could not be identified, most likely because such fragments were further cleaved into small peptides.

**An Anti-angiogenic Antithrombin Form in Commercial Preparations**—Two commercial antithrombin preparations, from Pharmacia & Upjohn and Baxter, were subjected to heparin affinity chromatography (Fig. 8). Considerable amounts of stabilizing proteins, eluting in the flow-through fraction, were present in both preparations. The Baxter preparation contained a fraction, comprising ~15% of the total amount of protein bound to the column, that eluted in about the same position as latent antithrombin, whereas no such material was detected in the Pharmacia & Upjohn preparation. Most of the antithrombin in both commercial preparations eluted similarly as native antithrombin prepared in-house. These two fractions with high heparin affinity migrated indistinguishably from the in-house prepared antithrombin in PAGE under nondenaturing conditions well as in SDS-PAGE under reducing conditions (not shown). They were tested in the chorioallantoic membrane assay and, like prelatent antithrombin, were both found to have appreciable anti-angiogenic activity (Table IV).

**TABLE II**

Dissociation equilibrium constants for binding of full-length heparin and pentasaccharide to native and prelatent antithrombin at 25°C, pH 7.4 and ionic strengths 0.15 and 0.3.

| Heparin      | Antithrombin | 0.15 | 0.3 |
|--------------|--------------|------|-----|
| Full-length  | Native       | 28 ± 6 | 320 ± 50 |
|              | Prelatent    | 41 ± 4 | 430 ± 30 |
| Pentasaccharide | Native     | 52 ± 5 | ND*  |
|              | Prelatent    | 57 ± 9 | ND*  |

* ND, not determined.

**TABLE III**

Association rate constants for proteinase inhibition by native and prelatent antithrombin alone and in complex with full-length heparin or pentasaccharide at 25°C, pH 7.4, and ionic strength 0.15.

| Proteinase | Antithrombin | $k_{\text{uncat}}$ | $k_{\text{H}5}$ | $k_{\text{H}5}$ |
|------------|--------------|-------------------|------------------|-----------------|
|            |              | $10^{-3}$ × $k_{\text{uncat}}$ | $10^{-6}$ × $k_{\text{H}5}$ | $10^{-5}$ × $k_{\text{H}5}$ |
|            | Thrombin     | Native            | Prelatent        | Native          | Prelatent       |
|            | Factor Xa    | Native            | Prelatent        | Native          | Prelatent       |
| Thrombin   | Native       | 7.2 ± 0.5         | 18.0 ± 0.3       | ND*             |
|            | Prelatent    | 7.9 ± 0.2         | 18.0 ± 0.7       | ND              |
| Factor Xa  | Native       | 2.2 ± 0.3         | 1.6 ± 0.1        | 7.0 ± 0.2       |
|            | Prelatent    | 2.2 ± 0.1         | 1.6 ± 0.03       | 7.5 ± 0.3       |

* ND, not determined.
are induced in this region in cleaved and latent antithrombin. Previous work has shown that conformational changes, resulting in an epitope comprising residues 341–346, are reflected in an altered sensitivity to proteolysis. As proposed earlier (17), a possible region is the C sheet, which has a different structure in latent and cleaved antithrombin as a result of strand 1 having been dislocated in the latent form (Fig. 1) (6, 14). A similar conformational change could have been induced by heat treatment in latent antithrombin.

The mechanism of the anti-angiogenic effect appears to be similar for latent and prelatent antithrombin in that both forms perturb FGF-2-induced FAK activation (17), consistent with similar structural changes of the two molecules being responsible. Activation of FAK is known to protect against programmed cell death (38). The decreased number of endothelial cells in cultures treated with FGF-2 together with prelatent antithrombin is therefore likely to be due to apoptosis induced by uncoupling of FAK. FAK resides in focal contacts and its activity is induced by integrin-mediated ligation of cells to the extracellular matrix, as well as by growth factors (38, 39). The exact mechanism by which latent and prelatent antithrombin perturb FAK activation remains to be determined. However, we have shown previously that FGF-2-induced focal adhesions dissolved when cells were treated with latent antithrombin (17). There is no structural feature such as an RGD sequence in antithrombin that would allow a prediction of how the protein interferes with focal adhesion formation. Moreover, as prelatent but not latent antithrombin binds proteases and heparin with similar ability as native antithrombin, these two properties cannot be essential for the anti-angiogenic effect.

Prelatent antithrombin was shown to efficiently retard fibrosarcoma growth in mice to an extent comparable with that of latent antithrombin (17). This anti-tumoral effect most likely is due to the inhibition of endothelial cell function. In agreement, the extent of inhibition of FGF-2-induced angiogenesis in the CAM assay by a dose of prelatent antithrombin equimolar to that of FGF-2 was more efficient than that we have observed earlier for the tumor growth inhibitor, endostatin (27). Moreover, previous work has shown that treatment with latent antithrombin leads to a reduction in vessel length and diameter and to a reduced vascularization of fibrosarcomas (17). The moderate effect of native antithrombin on tumor growth most likely is due to conversion to the prelatent, or possibly latent, form than of FGF-2 was more efficient than that we have observed earlier for the tumor growth inhibitor, endostatin (27). Moreover, previous work has shown that treatment with latent antithrombin leads to a reduction in vessel length and diameter and to a reduced vascularization of fibrosarcomas (17). The moderate effect of native antithrombin on tumor growth most likely is due to conversion to the prelatent, or possibly latent, form that of FGF-2 was more efficient than that we have observed earlier for the tumor growth inhibitor, endostatin (27). Moreover, previous work has shown that treatment with latent antithrombin leads to a reduction in vessel length and diameter and to a reduced vascularization of fibrosarcomas (17). The moderate effect of native antithrombin on tumor growth most likely is due to conversion to the prelatent, or possibly latent, form. Instead these properties presumably are due to the heat treatment having induced an altered conformation in prelatent antithrombin that is akin to those of the cleaved and latent forms. Because of the high structural similarity between native and prelatent antithrombin, the possibility remains that not all antithrombin molecules have undergone this conformational change and that the anti-angiogenic ability is only shown by a fraction of the protein in the prelatent preparation. However, in this case, this active component must have an unprecedentedly high anti-angiogenic ability.

The main common structural characteristic of cleaved and latent antithrombin is that the reactive-bond loop is no longer accessible on the surface of the protein but is inserted as a middle strand into the main sheet (Fig. 1) (6, 14). The anti-angiogenic properties of the two forms are thus most likely due to conformational changes resulting from this loop insertion. However, the greater effect of latent than of cleaved antithrombin (17) indicates that the anti-angiogenic ability is not due to structural alterations directly in the vicinity of the inserted loop but instead involves changes in another region where the two forms differ in structure. The potent anti-angiogenic activity of prelatent antithrombin, which binds proteinases and therefore must have an exposed reactive-bond loop, supports this conclusion and indicates that analogous conformational changes can be induced without loop insertion. The digestion pattern of prelatent antithrombin by nontarget proteinases is consistent with such changes having been induced in the region between residues 325 and 375. In the crystal structures of native and latent antithrombin, this region forms strands 6 and 5 of $\beta$-sheet A and the large segment connecting the two strands, containing helix I (Fig. 1) (6). The structures reveal certain differences between latent and native antithrombin in this region, although these differences apparently do not lead to the same proteolytic sensitivity in latent antithrombin as that observed here for prelatent antithrombin (15). Moreover, previous work has shown that conformational changes, resulting in the exposure of an epitope comprising residues 341–346, are induced in this region in cleaved and latent antithrombin (Fig. 1) (37). However, the structural changes that are responsible for the anti-angiogenic activity may also have occurred in another region of prelatent antithrombin, without being reflected in an altered sensitivity to proteolysis. As proposed earlier (17), a possible region is the C sheet, which has a different structure in latent and cleaved antithrombin as a result of strand 1 having been dislocated in the latent form (Fig. 1) (6, 14). A similar conformational change could have been induced by heat treatment in prelatent antithrombin.

Analysis of the crystal structure of prelatent antithrombin may aid in delineating those conformational features of both latent and prelatent antithrombin that are responsible for the anti-angiogenic activity of the two forms.
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