Complex-dependent Inhibition of Factor VIIa by Antithrombin III and Heparin*

(Received for publication, September 24, 1992)

Jeffrey H. Lawson‡, Saulius Butenas, Natalie Ribarik, and Kenneth G. Mann§
From the Department of Biochemistry, University of Vermont, Burlington, Vermont 05405

The regulation of the factor VIIa-tissue factor complex is essential for control of the hemostatic response. However, the role of the inhibitor antithrombin III in the regulation of factor VIIa has remained in question. The inhibition of factor VIIa activity by antithrombin III and heparin in the presence and absence of tissue factor was evaluated using the fluorescent substrate m-LGR-nds. Our data show that the activity of recombinant human factor VIIa is inhibited by antithrombin III in the presence of heparin at a rate of $1.7 \times 10^5$ mol$^{-1}$ s$^{-1}$. In the presence of tissue factor, the rate constant for this reaction increases to $5.6 \times 10^5$ mol$^{-1}$ s$^{-1}$. A 1:1 stoichiometric complex between factor VIIa and antithrombin III, with an apparent molecular weight of 110,000, was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A heterogeneous mixture of factor VIIa products with molecular weights between 50,000 and 80,000, most likely representing proteolytically degraded factor VIIa-antithrombin III complexes, was also observed.

The factor VIIa-tissue factor complex appears to play a major role in the initiation of the blood coagulation process (1–3). The expression of this complex requires the activation of the zymogen factor VII to the enzyme factor VIIa, the expression of tissue factor on a cell surface, and the preparation of the appropriate membrane environment. A fully formed complex efficiently activates the plasma zymogens factor X and factor IX to their respective enzyme forms, ultimately leading to the generation of α-thrombin.

Antithrombin III is the major inhibitor of coagulation processes. However, although the inhibition of factor VIIa by antithrombin III has been reported, the role of antithrombin III has remained controversial (4–6). A study by Jesty (4) reported that neither factor VII nor factor VIIa in the presence or absence of tissue factor was inhibited by antithrombin III. However, Broze and Majerus (5) and Kondo and Kisiel (6) reported that factor VIIa was inhibited slowly by anti-

thrombin III in the presence of heparin. These authors concluded that this reaction proceeded so slowly that it was probably of little physiological significance (5). These authors also observed that tissue factor exhibited no protective effect in preventing antithrombin III inhibition of factor VIIa (5). Of the known plasma inhibitors of blood coagulation enzymes, tissue factor pathway inhibitor (TFPI) has been the most well characterized inhibitor of the factor VIIa-tissue factor complex (7, 8). TFPI is a protease inhibitor that consists of three tandem Kunitz-type domains and may require the binding of factor Xa to TFPI before the inhibitor can interact with the factor VIIa-tissue factor complex (9, 10). In this setting, inhibition of the factor VIIa-tissue factor complex occurs only after factor Xa has been generated in the reaction, although the work of Callander et al. (11) has challenged the notion that factor Xa binding to TFPI is required prior to the binding of TFPI to the factor VIIa-tissue factor catalytic complex. These latter investigators reported that TFPI binds to and inhibits the factor VIIa-tissue factor complex directly with a dissociation constant of 11.9 nM in the absence of factor Xa, and a dissociation constant of 4.5 nM in the presence of factor Xa. These studies concluded that factor Xa is not an obligate requirement for the inhibition of the factor VIIa-tissue factor complex by TFPI but that factor Xa only enhances the overall binding affinity of TFPI for the catalytic complex.

The evaluation of the activity of factor VIIa and its inhibition has been hampered by the lack of sensitive and direct reporters of factor VIIa activity. This lack of a sensitive and direct method for quantitating factor VIIa activity has limited quantitative studies of the antithrombin III inhibition of factor VIIa. We have recently characterized and reported new synthetic substrates for factor VIIa enzymatic activity (12, 13). Our data demonstrated that the active site of factor VIIa is incompletely formed in the absence of Ca$^{2+}$ and tissue factor. The inhibition of factor VIIa by antithrombin III and heparin was directly evaluated using this fluorescent substrate technology.

**EXPERIMENTAL PROCEDURES**

**Materials**—The fluorogenic substrate 6-(methylene)sulfonfyl-n-Leu-Gly-Arg)-amino-1-naphthenediyethylsulfonamide (m-LGR-nds) was synthesized as previously described (12). 1-Palmitoyl-2-oleoyl phosphatidylserine (PS) and 1-palmitoyl-2-oleoyl phosphotidilcholine (PC) were purchased from Sigma. Phospholipid vesicles (PCPS) composed of 75% PC and 25% PS were prepared as described (14).

**Proteins**—Antithrombin III was purified from human plasma as previously described (15). Heparin sodium injection (USP) from beef lung was purchased from Organon, Inc. Recombinant human coagulation factor VIIa was purchased from Novo Pharmaceutics. Recombinant human tissue factor was provided as a gift from Dr. Shu-Len Liu, Hyland Division, Baxter Healthcare Corp.

The concentration of antithrombin III was calculated using a molecular weight of 58,000 and an extinction coefficient ($E_{280}^m$) of 0.65 (16). The concentration of factor VIIa was calculated using a molecular weight of 50,000 and an extinction coefficient ($E_{280}^m$) of 1.39 (17). The concentration of tissue factor was determined by amino acid analysis.

* The abbreviations used are: TFPI, tissue factor pathway inhibitor; m-LGR-nds, 6-(methylene)sulfonfyl-n-Leu-Gly-Arg)-amino-1-naphthenediyethylsulfonamide; PS, 1-palmitoyl-2-oleoyl phosphatidylserine; PC, 1-palmitoyl-2-oleoyl phosphotidilcholine; HBS, Hepes-buffered saline; PAGE, polyacrylamide gel electrophoresis; FVIIa, factor VIIa; TF, tissue factor.
Factor VIIa Inhibition by Antithrombin III in the presence of tissue factor. Panel A illustrates the inhibition of fVIIa enzymatic activity by antithrombin III and heparin in the presence of TF as a function of time. Panel B plots \( \ln[A/(A - J)] \) versus time, where \( A \) represents the concentration of free inhibitor at a particular time in the reaction, and \( J \) represents the concentration of initial inhibitor (14). The slope equals \( k_i \). 

| Inhibitor | Enzyme | Second-order rate constant 
|-----------|--------|--------------------------|
| ATIII | Factor VIIa | 1.7 \times 10^6 |
| ATIII-heparin | Factor VIIa | 4.5 \times 10^6 |
| ATIII-heparin | Factor VIIa-tissue factor | 5.6 \times 10^3 |

* No reaction observed.

The inhibition of Factor VIIa by antithrombin III in the presence of tissue factor was evaluated, and the rates of factor VIIa inhibition for each concentration of antithrombin III were monitored over time following the addition of antithrombin III and heparin to the reaction mixture. Experiments evaluating the inhibition of factor VIIa (800 nM) by antithrombin III (1.6 \mu M) and heparin in the absence of tissue factor were done in a manner similar to that described above. Data were reduced and rate constants estimated by the method of Downing et al. (19).

**Table I** presents the calculated second-order rate constants for the inhibition of factor VIIa by antithrombin III (ATIII) and heparin.

![Graph A](image1.png)

**Graph A** shows the inhibition of factor VIIa by antithrombin III. Panel A illustrates the inhibition of fVIIa enzymatic activity by antithrombin III and heparin in the presence of TF as a function of time. Panel B plots \( \ln[A/(A - J)] \) versus time, where \( A \) represents the concentration of free inhibitor at a particular time in the reaction, and \( J \) represents the concentration of initial inhibitor. The slope equals \( k_i \).
stant(s) for factor VIIa inhibition by antithrombin III in the presence and absence of tissue factor and heparin. These data illustrate that factor VIIa is inhibited by antithrombin III-heparin. Furthermore, under the experimental conditions used, the addition of tissue factor enhanced the rate of factor VIIa inhibition 33-fold over that observed in the presence of heparin alone. These data provide quantitative evidence that the formation of the factor VIIa-tissue factor complex increases the susceptibility of factor VIIa for inhibition by antithrombin III. The enhanced rate of factor VIIa inhibition, in the factor VIIa-tissue factor complex, is unique when compared with other procoagulant enzyme complexes. In other procoagulant complexes the proteases are protected from antithrombin III inhibition when complexed to their respective cofactors (22). The evaluation presented here can only be considered an initial venture into establishing the appropriate kinetic parameters to adequately describe the factor VIIa-tissue factor reaction with antithrombin III-heparin. The interactions of other coagulation enzyme complexes with heparin-antithrombin III are complex functions with concentration-dependent interaction for all species. The rate constants reported here are appropriate only for the specific conditions described.

The nature of the factor VIIa-antithrombin III inhibitor complex was evaluated by subjecting various reaction mixtures to SDS-PAGE/immunoblot analysis in the presence and absence of tissue factor and heparin. Fig. 2 demonstrates that a number of detergent stable species with mass greater than factor VIIa are observed when factor VIIa is treated with antithrombin III and heparin in the presence of tissue factor (lanes 6 and 8). The largest species (a) has an apparent molecular weight of 110,000, a value consistent with the predicted molecular weight of a 1:1 stoichiometric complex between factor VIIa and antithrombin III. A heterogeneous mixture of factor VIIa-related products with molecular weights between 50,000 and 80,000 are also observed (b). These products most likely represent proteolytically degraded factor VIIa-antithrombin III complexes and free factor VIIa not covalently linked to antithrombin III (c). From kinetic studies of substrate hydrolysis (Fig. 1A), under native conditions, all of the factor VIIa is functionally inactive under this set of conditions represented by the mixture in lanes 6 and 8. Thus, the inhibition process probably involves the formation of an inhibited non-covalent factor VIIa-antithrombin III complex followed by the subsequent formation of a covalent complex between the enzyme and inhibitor.

**DISCUSSION**

In this report we have reevaluated the inhibition of factor VIIa by antithrombin III and heparin in the presence and absence of tissue factor. We have previously demonstrated that factor VIIa undergoes an active site transition upon binding of both calcium and tissue factor, which enhances the fundamental catalytic properties of the enzyme (12). Data presented in this report illustrate that the interaction of the protease factor VIIa with the cofactor also enhances the susceptibility of factor VIIa for inhibition by antithrombin III at least 33-fold.

The highest rate constant observed in this report for the inhibition of factor VIIa-tissue factor by antithrombin III-heparin, $5.6 \times 10^5$ M$^{-1}$ s$^{-1}$, is small relative to that reported for the inhibition of $\alpha$-thrombin by antithrombin III-heparin ($1.5 - 4 \times 10^7$ M$^{-1}$ s$^{-1}$) (23-25). The value observed for the factor VIIa-tissue factor reaction is in the range of that observed for factor Xa (0.6-1.5 $\times 10^4$ M$^{-1}$ s$^{-1}$) (26, 27) and that reported for factor XIIa (4 $\times 10^4$ M$^{-1}$ s$^{-1}$) (28). The rates of inhibition of $\alpha$-thrombin and factor Xa by antithrombin III are significantly depressed by formation of complexes with their respective cofactors, thrombomodulin and factor Va (29, 30). In contrast, the rate of inhibition of factor VIIa by antithrombin III is positively influenced by complexation with tissue factor and further enhanced by the addition of heparin. Thus, in contrast to other coagulation serine proteases, circulating factor VIIa is relatively resistant to antithrombin III-heparin inhibition, whereas the complex of factor VIIa-tissue factor is 33-fold more susceptible to antithrombin III inhibition.

The biological significance of the antithrombin III-heparin inhibition of factor VIIa-tissue factor is not known. In the last several years, a number of investigators have concentrated on the influence of the tissue factor pathway inhibitor (TFPI) on the regulation of factor VIIa-tissue factor activity (3, 8, 11). However, the data reported here, when evaluated in respect to the relative concentrations of TFPI and antithrombin III in plasma, suggest that the role of antithrombin III as a factor VIIa-tissue factor inhibitor requires further exploration. The concentration of antithrombin III in plasma, $3 \mu M$ (22) is more than 1000 times that reported for the concentration for TFPI in normal adults (2.4 nM) (31). To our knowledge, no investigator studying the TFPI inhibition process has yet published data identifying the rate constant for inhibition of the factor VIIa-tissue factor complex by this inhibitor. Broze and colleagues (8) reported values for factor Xa inhibition by TFPI which would indicate that the second-order rate constant for this reaction is $9 \times 10^5$ M$^{-1}$ s$^{-1}$. If one assumes a similar rate constant for TFPI inhibition of factor VIIa, using the blood concentrations of TFPI and antithrombin III, one would conclude that the relative velocity of inhibition of antithrombin III-heparin toward factor VIIa-tissue factor would exceed that for TFPI by a factor of 7.4. Even if one assumes that the TFPI inhibition of the factor VIIa-factor Xa-tissue factor complex may occur at a diffusionally limited rate (approximately $10^4$ M$^{-1}$ s$^{-1}$), it would still be concluded that the relative efficiency of antithrombin III-heparin inhibition of factor VIIa-tissue factor would be approximately equivalent to the efficiency of TFPI pathway in

---

**Fig. 2. Immunoblot analysis of factor VIIa inhibition by antithrombin III and heparin in the presence and absence of tissue factor.** SDS-PAGE analysis of factor VIIa inhibition by antithrombin III and heparin in the presence and absence of TF. Lanes: 1, fVIIa; 2, antithrombin III; 3, fVIIa + antithrombin III/heparin (5-s incubation); 4, fVIIa + antithrombin/heparin (30-min incubation); 5, fVIIa/TF/PCPS + antithrombin III/heparin (5-s incubation); 6, fVIIa/TF/PCPS + antithrombin III/heparin (30-min incubation); 7, fVIIa/TF (solved in 0.8% octyl glucoside) + antithrombin III/heparin (5-s incubation); 8, fVIIa/TF (solved in 0.8% octyl glucoside) + antithrombin III/heparin (30-min incubation).
regulating this important reaction of blood clotting. For the
diffusional reaction, the relative efficiency of anti-
thrombin III-heparin to TFPI for inhibition would be approx-
imately 0.7.

REFERENCES

1. Nemerson, Y. (1988) Blood 71, 1-5
2. Edgington, T. S., Mackman, N., Brand, K., and Ruf, W. (1991) Thromb.
Hemostasis 66, 67-79
3. Rapaport, S. I. (1993) Thromb. Hemostasis 66, 6-15
4. Jessy, J. (1976) Arch. Biochem. Biophys. 185, 165-173
5. Broze, G. J., and Majerus, P. W. (1980) J. Biol. Chem. 255, 1242-1247
6. Kondo, S., and Kiesel, W. (1987) Thromb. Res. 46, 325-330
7. Rapaport, S. I. (1989) Blood 73, 359-365
8. Broze, G. J., Girard, T. J., and Novotny, W. F. (1990) Biochemistry 29,
7539-7546
9. Girard, T. J., Warren, L. A., Novotny, W. F., Likert, K. M., Brown, S. G.,
Miletich, J. P., and Broze, G. J. (1989) Nature 338, 518-520
10. Broze, G. J., Warren, L. A., Novotny, W. F., Hinich, C. A., Girard, T. J.,
and Miletich, J. P. (1988) Blood 71, 335-343
11. Callander, N. S., Rao, L. V. M., Nordfang, O., Sandset, P. M., Warn-
cramer, B., and Rapaport, S. I. (1992) J. Biol. Chem. 267, 876-882
12. Lawson, J. H., Butenas, S., and Mann, K. G. (1992) J. Biol. Chem. 267,
4834-4843
13. Butenas, S., Orfeo, T., Lawson, J. H., and Mann, K. G. (1992) Biochemistry
31, 5399-5411
14. Higgins, D. L., and Mann, K. G. (1983) J. Biol. Chem. 258, 6503-6508
15. Damus, P. S., and Rosenberg, R. D. (1976) Methods Enzymol. 45, 662-669
16. Nordenman, B. Nystrom, C., and Bjork, I. Eur. J. Biochem. 78, 289
17. Rajaji, S. P., Rapaport, S. I., and Brown, S. F. (1981) J. Biol. Chem. 256,
253-259
18. Lawson, J. H., and Mann, K. G. (1991) J. Biol. Chem. 266, 11317-11327
19. Downing, M. K., Bloom, J. W., and Mann, K. G. (1978) Biochemistry 17,
2649-2653
20. Laemmli, U. K. (1970) Nature 277, 680-685
21. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.
A. 76, 4350-4354
22. Rosenherg, R. D., and Rosenberg, J. G. (1989) J. Clin. Invest. 74, 1-12
23. Jordan, J. F., Beeler, D., and Rosenberg, R. D. (1979) J. Biol. Chem. 254,
2902
24. Jordan, R. F., Oota, G. M., Gardner, W. T., and Rosenberg, R. D. (1980)
J. Biol. Chem. 255, 10061
25. Griffith, M. J. (1982) J. Biol. Chem. 257, 7360
26. Scott, C. F., and Colman, R. W. (1989) Blood 73, 1373
27. Olson, S. T., and Shore, J. D. (1989) Thromb. Haemostasis 62, 361
28. Piatker, B. A., Schapira, M., and Colman, R. W. (1985) Blood 66, 198
29. Miletich, J. P., Majerus, D. W., Majerus, P. W. (1978) J. Clin. Invest. 62,
824-831
30. Eason, C. T. (1989) J. Biol. Chem. 264, 4743-4746
31. Novotny, W. F., Brown, S. G., Girard, T. J., Miletich, J. P., and Broze, G.
J. (1989) Blood 74, 2914 (abstr.)