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Effect of Heparin on Thrombin Inactivation by Antithrombin-III

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The inactivation of thrombin by heat and by its physiological inhibitor, antithrombin-III, shows quite different dependence on heparin concentration. Heparin at 250 μg/ml protects thrombin against heat inactivation, and thrombin behaves heterogeneously in this reaction. In the absence of heparin, the thermodynamic activation parameters change with temperature ($\Delta H^* = 733$ kJ/mol and 210 kJ/mol at 50 and 58°C respectively). When heparin is present, heat inactivation of the protected thrombin species proceeds with $\Delta H^* =$ 88 kJ/mol and is independent of temperature in the same range. On the other hand, heparin at 0.125–2.5 μg/ml accelerates the thrombin–antithrombin-III reaction. Thrombin does not show heterogeneity in this reaction and the time courses at any heparin concentration and any temperature between 0 and 37°C appear to follow first-order kinetics. Activation enthalpy is independent of heparin concentration or temperature, $\Delta H^* =$ 82–101 kJ/mol, varying slightly with antithrombin-III concentration and thrombin specific activity. Heparin seems to exert its effect by increasing activation entropy. On the basis of these data we suggest a mechanism of action of heparin in the thrombin–antithrombin-III reaction which accounts for all the important features of the latter and seems to unify the different hypotheses that have been advanced.

Although the effect of heparin on blood coagulation has been known for more than half a century, its exact mechanism of action has still not been elucidated. It is well documented, however, that the maximal activity of this anticoagulant requires a plasma factor (Brinkhous *et al.*, 1939; Gerendás *et al.*, 1949), designated now as antithrombin-III (heparin cofactor). Antithrombin-III is a glycoprotein of mol.wt. 65000 (Abildgaard, 1968; Miller-Andersson *et al.*, 1974). Its function in plasma is rather wide-ranging. Antithrombin-III inactivates all the serine proteinases involved in blood coagulation (Damus *et al.*, 1973). As to its mechanism of action, only thrombin inactivation is well documented. It has been demonstrated that the inhibitor forms an irreversible, 1:1 stoicheiometric complex with the enzyme. In the complex, thrombin loses its clotting as well as its esterolytic activity (Abildgaard, 1969). Antithrombin III is also termed in the literature 'progressive antithrombin', since the inhibition of thrombin activity is not instantaneous. The kinetics of enzyme inactivation are, however, changed in the presence of heparin. Heparin accelerates the rate of complex-formation between thrombin and antithrombin-III.

As to the mechanism of action of heparin in this reaction, two hypotheses have been proposed: (i) heparin binds to antithrombin-III and activates it (Rosenberg & Damus, 1973), (ii) heparin binds to thrombin, inducing a conformational change of the enzyme, which renders thrombin more susceptible to its inhibitor (Machovich, 1975). A third mechanism has also been discussed, namely that the enzyme and its inhibitor may be bridged by heparin (Machovich *et al.*, 1975*a*; Gitel, 1975; Danishefsky *et al.*, 1977). This possibility, however, has been disproved by Hatton & Regoeczi (1977). The contradictory data and hypotheses are further complicated by other findings. There is disagreement about the binding of heparin to thrombin. One thrombin molecule may bind two or more molecules of heparin (Machovich *et al.*, 1975*b*) or even three or four heparin molecules (Li *et al.*, 1974) with a dissociation constant in the range 100–10 nM (Li *et al.*, 1974) or even 10–1 nM (Smith, 1977). Antithrombin-III is also able to bind two or three heparin molecules with different dissociation constants, in the range 1–0.1 μM (Einarsson & Andersson, 1977). In addition, heparin seems to be the most effective at an equimolar concentration to thrombin (Smith, 1977). On the other hand, it has been demonstrated that heparin in a concentration lower by more than one order of magnitude than that of thrombin or antithrombin-III accelerates enzyme inactivation by its inhibitor (Björk & Nordeman, 1976; Machovich *et al.*, 1977). Further complications may arise from the fact that heparin is rather heterogeneous (McDuffie *et al.*, 1975; Lam *et al.*, 1976; Höök *et al.*, 1976).

On the basis of these data, it is not yet possible to make a final decision about the mechanism of action of heparin in the thrombin–antithrombin-III reaction.

Therefore, in the present paper, we have examined the effect of heparin on thrombin inactivation in the presence or absence of antithrombin-III from a thermodynamic viewpoint and we suggest a hypothesis which may resolve the contradictions outlined above.

Experimental

Materials

Sulphopropyl-Sephadex C-50 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Fibrinogen (human, grade L) was the product of Kabi AB, Stockholm, Sweden. Heparin (bovine lung, with a specific activity of 125 units/mg) was obtained from G. Richter Pharmaceuticals, Budapest, Hungary: 1 unit is defined as 1/130mg of the International Standard Heparin Preparation (Jaques *et al.*, 1973). This commercial heparin was further purified by the method of Rodén *et al.* (1972) and dissolved in 0.1M-NaCl. Other chemicals were purchased from Reanal Fine Chemicals, Budapest, Hungary.

Methods

Crude bovine thrombin (Parke-Davis, Detroit, MI, U.S.A.) was further purified by chromatography on sulphopropyl-Sephadex C-50 by the method of Lundblad *et al.* (1975). The specific activity of α-thrombin obtained was 1800–2000NIH units/mg of protein. Immediately before experimentation thrombin was gel-filtered on a column (1.1cm × 20cm) of Sephadex G-25 equilibrated with 0.05M-sodium phosphate buffer, pH7.4.

Antithrombin-III, purified from human plasma, was the product of the American Red Cross Fractionation Center, Bethesda, MD, U.S.A. The final product of the inhibitor protein, over 95% purity, was freeze-dried and before experimentation dissolved in 0.1M-NaCl, at pH7.0.

Thrombin activity was assayed in an 0.3ml final volume: 0.2ml of fibrinogen solution (500μg of protein) was prewarmed at 37°C and 0.1ml of thrombin solution (in 0.0375M-sodium phosphate buffer, pH7.4, containing 0.05M-NaCl) was added. Clotting time was measured at 37°C in glass tubes with the Hyland Clotek System (Hyland Laboratories, CA, U.S.A.). Thrombin activity, expressed in NIH units, was calculated from a standard calibration curve established with U.S. Standard Thrombin (human, lot H-1).

Antithrombin-III activity was measured in silicone-treated tubes containing 0.2ml of 0.1M-sodium phosphate buffer, pH7.4, 0.2ml of heparin or 0.1M-NaCl and 0.2ml of thrombin dissolved in 0.05M-sodium phosphate buffer, pH7.4. After prewarming at the appropriate temperature, 0.2ml of antithrombin-III solution was added and the reaction mixture was incubated at 0–37°C for 0–60min. After various times, 0.1ml portions were taken and assayed for thrombin activity as described above.

Thermal-denaturation experiments were carried out under conditions similar to those described for antithrombin assay, i.e. 0.2ml of 0.05M-sodium phosphate buffer, pH7.4, 0.2ml of 0.1M-NaCl, 0.2ml of heparin or 0.1M-NaCl was prewarmed at the appropriate temperature and thereafter 0.2ml of thrombin dissolved in 0.05M-sodium phosphate buffer, pH7.4, was added and incubated at 50–58°C. As a control for heparin effect, experiments were also carried out at 37°C. After various times, 0.1ml samples of reaction mixture were taken and clotting time was determined as detailed above.

Protein was determined by the method of Lowry *et al.* (1951) with human serum albumin as a standard.

Concentrations were calculated from mol.wts. of 39000, 65000 and 11000 for α-thrombin (Mann *et al.*, 1973), antithrombin-III (Miller-Andersson *et al.*, 1974) and heparin (Hilborn & Anastassiadis, 1971).

Results

Heat inactivation of thrombin

Heat-inactivation kinetics, which had proved to be a valuable tool to study thrombin–heparin interaction (Machovich *et al.*, 1975*b*), were used in the present investigation. Thrombin was incubated at 50–58°C in 0.025M-sodium phosphate buffer, pH7.4, either alone or together with 250μg of heparin/ml. In the absence of heparin, thrombin denaturation follows first-order kinetics at any temperature (Fig. 1) and the rate constants cover a range of almost two orders of magnitude. Addition of heparin resulted in a dissociation of each curve into two phases (Fig. 2), in accordance with the earlier results (Machovich *et al.*, 1975*b*; Machovich & Arányi, 1977). A detailed analysis of the dependence on heparin concentration suggested some heterogeneity of thrombin with respect to heparin sensitivity during heat inactivation. Moreover, the data could be reconciled with a model suggesting rapid equilibrium between heparin and the heparin-sensitive form of thrombin and their 2:1 complex. First-order rate constants of the second phases were used for determination of activation parameters of thermal denaturation of the thrombin–heparin complex. Parameters of the first phases could be determined with much less accuracy and the kinetic order could not be reliably assigned them. They were omitted from further analysis.

Arrhenius plots for heat inactivation are different in character in the presence and absence of heparin (Fig. 3). When free thrombin is inactivated, activation

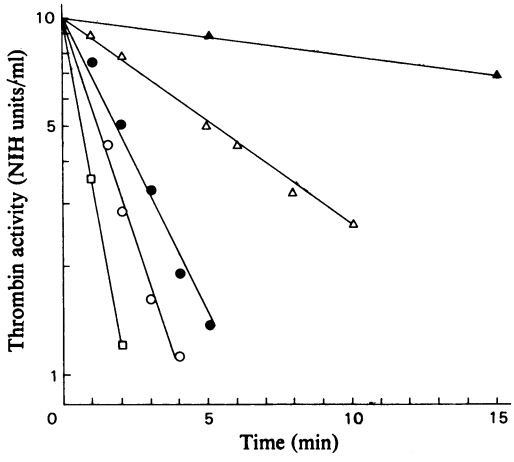

Fig. 1. *Time course of heat inactivation of thrombin at various temperatures*
Thrombin (7.5 μg of protein/ml) in 0.025 M-sodium phosphate buffer, pH 7.4, containing 0.05 M-NaCl was incubated for 0–15 min at 58°C (□), 56°C (○), 54°C (●), 52°C (△) and 50°C (▲). Remaining thrombin activity was determined as described in the Experimental section. Results are presented on a semi-logarithmic scale.

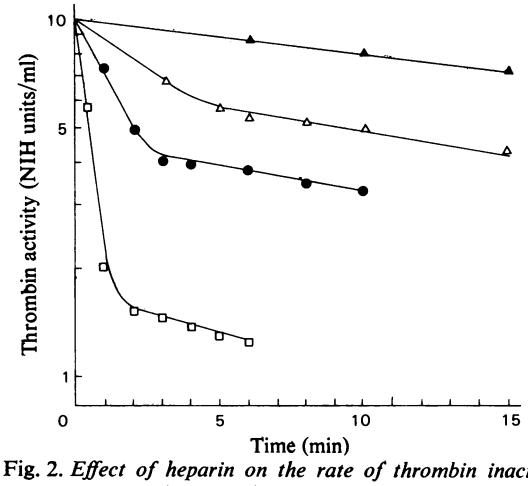

Fig. 2. *Effect of heparin on the rate of thrombin inactivation at various temperatures*
Thrombin in the presence of heparin (250 μg/ml) was incubated as detailed in Fig. 1. □, 58°C; ●, 54°C; △, 52°C; ▲, 50°C.

enthalpy depends strongly on temperature ($\Delta H^* =$ 733 kJ/mol at 50°C and 210 kJ/mol at 58°C), as usual with proteins (Tanford, 1970) because of heat-capacity changes accompanying the exposure of buried side chains to solvent. In contrast, an Arrhenius plot for inactivation in the presence of heparin is linear over the same temperature range, with a much smaller value for ΔH^*, 88 kJ/mol.

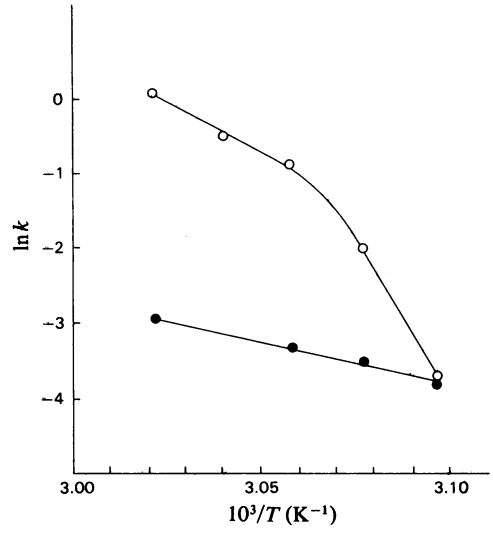

Fig. 3. *Arrhenius plot of heat inactivation of thrombin*
First-order rate constants were determined from Fig. 1, in the absence of heparin (○) and from the second phases of the curves of Fig. 2, in the presence of 250 μg of heparin/ml (●).

These data suggest that thrombin conformation in the activated state may be very close to the native conformation in the presence of heparin, i.e. interaction with heparin stabilizes the overall structure. On the other hand, the large ΔH^* value at 50°C and its negative temperature coefficient in the absence of heparin indicate that an extensive conformational change occurs between the native and the denatured state, whereas the activated state assumes a fairly compact structure.

Inactivation of thrombin by antithrombin-III and heparin

Thrombin inactivation by antithrombin-III was easily measurable between 0 and 37°C. Under our experimental conditions when antithrombin-III was in a 5-fold or greater excess over thrombin, the disappearance of clotting activity followed pseudo-first-order kinetics, the apparent rate constant being proportional to antithrombin-III concentration (Figs. 4 and 5). The actual values were slightly dependent on thrombin concentration and specific activity, and also showed batch-to-batch variation. This dependence was not examined systematically, but we always used the same thrombin concentration, and, if

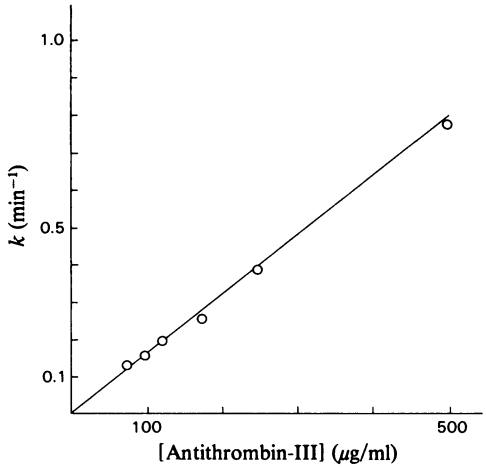

Fig. 4. *Dependence of apparent first-order rate constant of the thrombin–antithrombin-III reaction on antithrombin-III concentration*

Thrombin (7.5 μg of protein/ml) in 0.0375 M-sodium phosphate buffer, pH 7.4, containing 0.05 M-NaCl was incubated at 25°C in the presence of various concentration of antithrombin-III. Clotting time (remaining thrombin activity) was determined at different times for estimation of the rate constants.

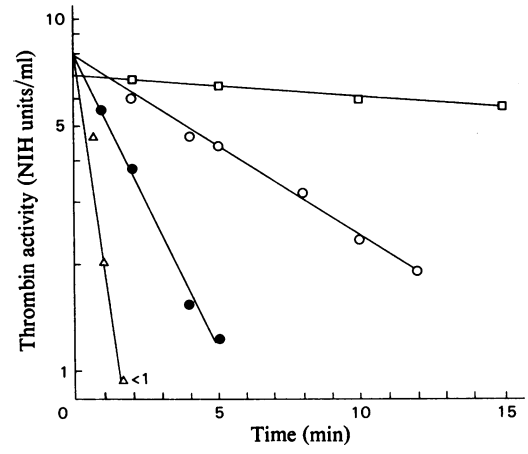

Fig. 5. *Time course of thrombin inactivation by antithrombin-III at various temperatures*

Thrombin (7.5 μg of protein/ml) and antithrombin-III (120 μg of protein/ml) in 0.0375 M-sodium phosphate buffer, pH 7.4, containing 0.05 M-NaCl was incubated for 0-60 min at 37°C (△), 25°C (●), 15°C (○) and 0°C (□). Remaining thrombin activity was determined as described in the Experimental section.

numerical comparison was to be made, the same thrombin stock solution was used for each assay.

In the presence of heparin at very low concentration (0.125 μg/ml), the rate of inactivation increased at each temperature; the time course still followed first-order kinetics within the measurable range (Fig. 6). Under our experimental conditions heparin at a concentration of 5 nM influenced the rate of inactivation of thrombin (results not shown). Hence heparin at a molar concentration one-tenth that of thrombin is effective in promoting the inactivation of thrombin by antithrombin-III. This result agrees with the report of Björk & Nordenman (1976) and with our own findings (Machovich *et al.*, 1977).

Arrhenius plots of the thrombin–antithrombin-III reaction are linear in both the presence and the absence of 0.125 μg of heparin/ml over a range of almost 40K (Fig. 7*a*). Furthermore, the plots are parallel to each other, with $\Delta H^* = 101$ kJ/mol. This value is relatively low, compared with heat-inactivation data for free thrombin (Fig. 3).

In another series of experiments, the effect of alteration of heparin concentration was studied. Again linear and parallel Arrhenius plots were obtained (Fig. 7*b*); their intercept increased with heparin concentration. Activation enthalpy was 82 ± 8 kJ/mol in this case. Absolute values of activation entropy as well as free energy depend on the

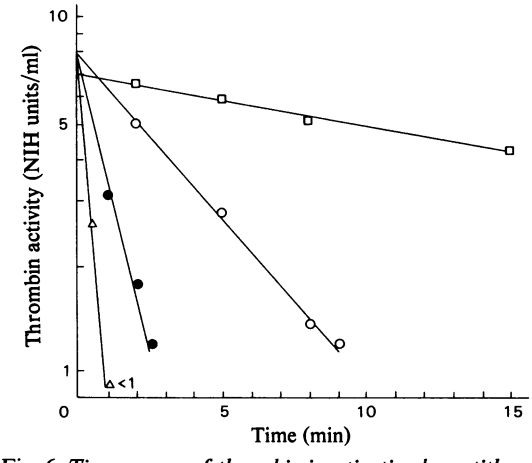

Fig. 6. *Time course of thrombin inactivation by antithrombin-III in the presence of heparin at various temperatures*

Thrombin and antithrombin-III were incubated in the presence of heparin (0.125 μg/ml) as detailed in Fig. 5. (△), 37°C; (●), 25°C; (○), 15°C; (□), 0°C.

choice of the constant A, which relates the rate constant to the activation parameters (Tanford, 1970).

$$k = A\, e^{-\Delta H^*/RT}\, e^{\Delta S^*/R}$$

They are therefore somewhat arbitrary. The value RT/Nk (where N is Avogadro's number, and k is

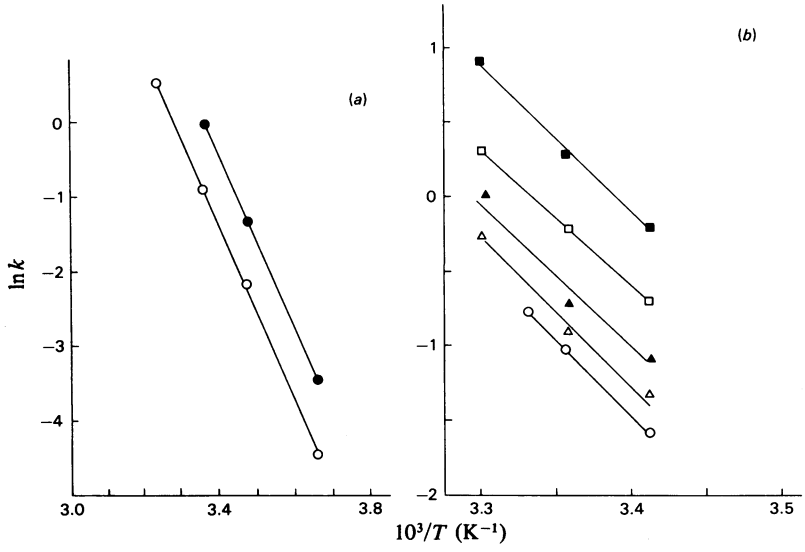

Fig. 7. *Arrhenius plot of the thrombin–antithrombin-III reaction*
Apparent first-order rate constants were determined from the curves of Fig. 5 and Fig. 6 (*a*) and from another series of experiments (*b*). Heparin added (μg/ml): none (○), 0.125 (●), 0.25 (△), 0.5 (▲), 1.2 (□), 2.5 (■).

Planck's constant) is frequently substituted for A. In this case, we have $\Delta G^* = 84\,kJ/mol$, $\Delta S^* = 56\,J \cdot mol \cdot K^{-1}$ at 25°C for the thrombin–antithrombin-III reaction; $\Delta G^* = 101\,kJ/mol$, $\Delta S^* = 2.15\,kJ \cdot mol \cdot K^{-1}$ at 50°C for heat inactivation of thrombin in the absence of heparin; $\Delta G^* = 102\,kJ/mol$, $\Delta S^* = -4\,J \cdot mol \cdot K^{-1}$ in the presence of 250 μg of heparin/ml. However, variation of ΔS^* with heparin concentration can be reliably derived from the data. Heparin (2.5 μg/ml) decreases activation entropy of the thrombin–antithrombin-III reaction by $11\,J \cdot mol \cdot K^{-1}$ and results in a 3.6-fold acceleration of the reaction independent of temperature.

We conclude that in the thrombin–antithrombin-III reaction heparin exerts its effect mainly by increasing activation entropy, but with little, if any, effect on activation enthalpy, in contrast with its effect on thrombin in heat-inactivation conditions.

Discussion

Thrombin does not exist in blood circulation, but when it is formed from its precursor, prothrombin, it may bind immediately to heparin. Antithrombin-III, on the other hand, circulates in blood mainly in the free form in substantial quantity with a half-life of 2.8 days (Marciniak, 1974; Collen *et al.*, 1977). When thrombin and antithrombin-III collide, an inactive enzyme–inhibitor complex is formed. In the presence of heparin, the activation entropy of this complex-formation increases, whereas activation enthalpy does not change. As ΔS^* reflects the difference between the randomness of the activated and native states, its increase by the action of heparin can be interpreted in terms of heparin rendering the initial state more ordered in the thrombin–antithrombin-III reaction.

Although heparin may induce a conformational change either in thrombin (Machovich, 1975; Hatton & Regoeczi, 1977; Smith, 1977; Machovich *et al.*, 1978) or in antithrombin-III (Rosenberg & Damus, 1973; Einarsson & Andersson, 1977), according to our thermodynamic data its function may be an 'ordering effect', i.e. several thrombin and antithrombin-III molecules can bind to heparin, thus giving an ordered structure. It should be emphasized, however, that the thermodynamic parameters also allow a model in which thrombin molecules alone are placed on the heparin template, facilitating the interaction between enzyme and its inhibitor. The enzyme and its inhibitor, held in juxtaposition by their common effector, react and the newly formed thrombin–antithrombin-III complex is released from the heparin, allowing it to act catalytically. Nevertheless, the molecular parameters permit one heparin molecule to bind several thrombin and antithrombin-III molecules in a structure similar to a polyribosome and thus release is not essential to explain how heparin can influence thrombin inactivation by antithrombin-III at a molar ratio greater than or equal to 1. The first thrombin molecule on the heparin template may initiate the binding of an antithrombin-

III molecule, since antithrombin-III shows a higher affinity to the thrombin–heparin complex than to thrombin or heparin alone (Hatton & Regoeczi, 1977; Smith, 1977).

This suggestion is compatible with all the essential findings concerning heparin action and the three hypotheses described in the literature. This proposal can explain: (i) the tight binding of thrombin to heparin and the induced conformational change (Pálos, 1949; Markwardt & Walsmann, 1959; Li *et al.*, 1974; Machovich *et al.*, 1975*a*,*b*; Machovich, 1975; Arányi *et al.*, 1977; Hatton & Regoeczi, 1977; Smith, 1977; Machovich & Arányi, 1977; Machovich *et al.*, 1978); (ii) the strong interaction between antithrombin-III and heparin (Rosenberg & Damus, 1973; Miller-Andersson *et al.*, 1974; Einarsson & Andersson, 1977); (iii) the bridge formation through heparin between enzyme and its inhibitor (Danishefsky *et al.*, 1977).

Our present suggestion also explains a number of observations: (i) one molecule of heparin is able to promote the interaction of several thrombin molecules with antithrombin-III (Björk & Nordenman, 1976; Machovich *et al.*, 1977), since heparin has repeated sequences on its helical structure (Yuan & Stivala, 1975); (ii) only heparin molecules of over 6000 mol.wt. are effective in anticoagulation, since a shorter heparin chain cannot accommodate both thrombin and antithrombin-III. Heparin, which consists of 8–15 disaccharide units (Kiss, 1976), is twisted into a regular but loose helical coil with four disaccharide units in each loop. The total length of the four disaccharides is about 4.2 nm; however, in solution an ordered and more compact conformation has been found (Yuan & Stivala, 1975). Since antithrombin-III is an ellipsoid with semi-axes of 1.9, 3.7 and 5.2 nm (Furugren *et al.*, 1977), one molecule of heparin may bind several of antithrombin-III, or a greater number of thrombin molecules (the molecular weight of thrombin being less than that of antithrombin-III) or both thrombin and antithrombin-III.

Heparin, at a higher concentration than that of thrombin may form a complex with the enzyme, in which two or more heparin molecules are bound to one molecule thrombin. Native thrombin structure is highly stabilized in this complex. For some unknown reason, only a fraction of thrombin is able to form this latter complex with heparin. Both activation enthalpy and entropy for thermal denaturation are dramatically changed in this complex. The reversibility of this interaction suggests that the other, physiologically significant complex, which forms at low heparin concentrations, is also reversible, but by no means proves the release of inactive enzyme-inhibitor complex and recycling of heparin. It is noteworthy that at least two and probably three or more heparin-binding sites should be present on a thrombin molecule, and further, one heparin molecule may also bind several thrombin molecules. Thus, depending on absolute and relative concentrations, a wide variety of thrombin–heparin complexes can exist and react in solution or in plasma.

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