# ON THE KINETICS OF THE THROMBIN-CONTROLLED POLYMERIZATION OF FIBRIN

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Received 5 April 1982

## 1. Introduction

In the currently accepted model of the thrombin (EC 3.4.21.5)-induced fibrinogen/fibrin conversion [1–3] the initial step consists of the splitting-off of 2 fibrinopeptides A from the Aα-chains of fibrinogen, followed by the linear polymerization of the fibrin monomers to yield protofibrils. The subsequent release of fibrinopeptide B from these protofibrils is assumed to lead to the formation of fibers through lateral aggregation. In a final step, fibers are linked to form the three-dimensional clot.

In the following we shall restrict ourselves to the earliest stages of the polymerization, effected by the release of only fibrinopeptide A.

In a remarkable gel-filtration study Smith [4] observed that the thrombin-induced polymerization of fibrin at pH 7.4 and ionic strengths of 0.36 and 0.21 M can be instantly stopped by the addition of the thrombin-inhibitor hirudin [5]. To explain the inhibitory effect it was proposed that under these experimental conditions only peptide A is released and that the polymerization proceeds via the rapid self-associations of monofunctional, monomeric and polymeric intermediates [4] (see fig.1).

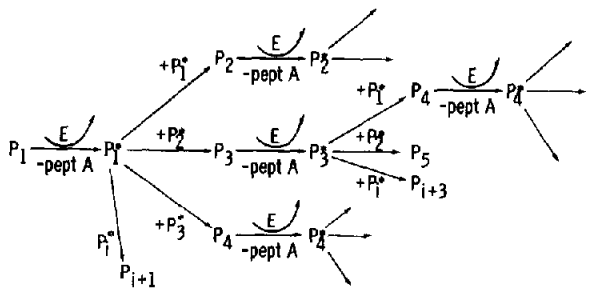


Fig.1. Polymerization scheme for monofunctional fibrins according to [4]. $P_j$ and $P_j^*$ stand for the inactive and active $j$-fold aggregates, respectively, whereas E stands for the enzyme.

Here, we confirm the observation of the inhibitory effect of hirudin by light scattering measurements [4]. Moreover, we developed a steady-state kinetic model of the polymerization, which allows us to relate the progress of the weight-average $M_r$, as obtained from light scattering, to the degree of release of fibrinopeptide A.

## 2. Experimental methods and results

Human fibrinogen was purified as in [6] with an additional DEAE-purification step to remove high $M_r$ material. Purity was checked by SDS–PAGE. Thrombin was lot no. 0056296 from Merck of 50 NIH-units/mg. Hirudin, grade IV, was from Sigma (St Louis MO).

Thrombin activity, $E_a$, was estimated at 405 nm and 37°C from the rate of splitting of H-D-Phenylalanyl–L-pipecolyl–L-arginine–*p*-nitroanalide dihydrochloride (S-2238 from Kabi).

Light scattering was measured at 633 nm with an instrument developed in this laboratory by Vreeman and Van Markwijk. After mixing enzyme and substrate the intensity of the scattered light was measured almost instantaneously at 9 angles at different reaction times and extrapolated to zero scattering angle to obtain the reciprocal apparent weight-av. $M_r$ (fig.2). The data were then recalculated to obtain the plot of the apparent weight-av. $M_r$ ($\overline{M}_r$-app.) as a function of time. It can be shown (A. V., in preparation) that under the present experimental conditions these app. $M_r$-values closely approximate the true weight-average.

Some typical $M_r$ growth curves at 0.5 M NaCl, 0.05 M Tris (pH 7.4) and 20°C are collected in fig.3. Under these experimental conditions the progress of the weight-av. $M_r$ is approximately sigmoidal. Moreover the experiment at $E_a = 35.5 \times 10^{-3}$. min$^{-1}$

*Published by Elsevier Biomedical Press*
00145793/82/0000–0000/$02.75 

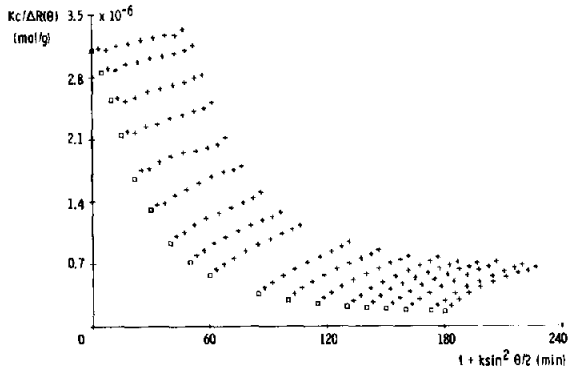


Fig.2. Time- and angle-dependency of the quantity $Kc/\Delta R(\theta)$, where $K$ is Rayleigh's constant, $c$ fibrinogen concentration and $\Delta R(\theta)$ the excess scattering at angle $\theta$. (□) Extrapolated reciprocal apparent weight-av. $M_I$ at zero angle (cf. [11]). Experimental conditions: 0.5 M NaCl, 0.05 M Tris (pH 7.4) and 20°C. Fibrinogen was 0.556 mg/ml; thrombin act. $15.4 \times 10^{-3}$. $min^{-1}$.

demonstrates that hirudin indeed is able to completely inhibit polymerization, as originally observed in [4]. Similar inhibitions have been observed at the lower ionic strengths of 0.35 and 0.15 M.

Parallel to the light-scattering measurements the release of fibrinopeptides A and B was followed by HPLC. To this end, at different reaction times 200 μl samples were drawn from the reaction mixture in the light-scattering cuvet and heated at 90°C for 2 min to deactivate thrombin and to precipitate fibrinogen and fibrin [7]. After centrifugation, 70 μl of he supernatant was injected on a 3.9 mm × 30 cm μm

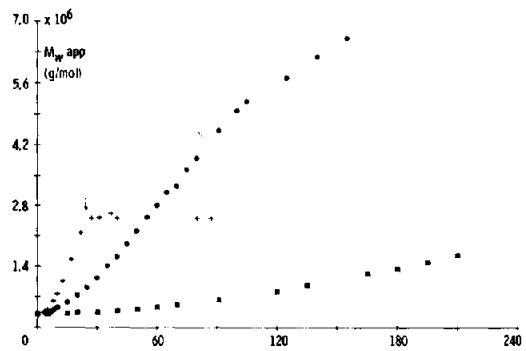


Fig.3. Some typical progress curves for the thrombin-induced polymerization of fibrin. Experimental conditions: 0.5 M NaCl, 0.05 M Tris (pH 7.4). Fibrinogen: (■) 0.803 mg/ml; (●) 0.660 mg/ml; (+) 0.473 mg/ml. Thrombin act.: (■) $3.80 \times 10^{-3}$. $min^{-1}$; (●) $18.7 \times 10^{-3}$. $min^{-1}$; (+) $35.5 \times 10^{-3}$. $min^{-1}$; (↓) indicates the moment of hirudin addition.

Bondapak $C_{18}$ column (Waters Assoc.). Separation of the fibrinopeptides was satisfactory with an eluent consisting of 80% 0.01 M ammonium phosphate (pH 2.90) and 20% acetonitrile [8] at a flow rate of 2 ml/min.

## 3. Discussion

The finding that hirudin is able to instantly inhibit the polymerization of fibrin implies that under the prevailing experimental conditions the kinetics of the process is completely enzyme-controlled. In [4] it was suggested that at the relatively high ionic strengths as used here, the rate of proteolysis is the rate-determining step in the enzymatic fibrinogen/fibrin conversion. As a consequence, fibrin monomers with just one A-peptide split off, rapidly dimerize with other monofunctional monomers to form a blocked dimer. Dimers in turn can be activated by the splitting off of a further A-peptide to yield the activated, monofunctional dimer. These rapidly form inert trimers and tetramers through associations with other activated monofunctional monomers and dimers, and so on (see fig.1). This reaction scheme does not contradict those proposed in [3,9].

The rate equations for the above reaction scheme read:

$$d[P_1]/dt = -\nu'[P_1] \quad (1)$$

$$d[P_j]/dt = -\nu'[P_j] + (k_s/2)\sum_{i=1}^{j-1}[P^*_{j-i}]\,[P^*_i]$$

$$j = 2,3,4,..... \quad (2)$$

and

$$d[P^*_j]/dt = \nu'[P_j] - k_s[P^*_j]\sum_{i=1}^{\infty}[P^*_i]$$

$$j = 1,2,3,..... \quad (3)$$

where:

$[P_j]$ = the number concentrations of the inert species of the degree of polymerization $j$;
$[P^*_j]$ = that of the corresponding monofunctional species;
$k_s$ = a universal association constant.

The factor 1/2 in the second term on the right side of eq. (2) is introduced to avoid double counting of aggregated species [10]. Further, equal substrate properties are accepted for monomeric and polymeric $P_j$-particles. In agreement with this assumption $\nu'$ in eq. (1)–(3) is defined by:

$$\nu' = V_{max}/\left(K_m + \tilde{\sum}_{j=1} [P_j]\right) \quad (4)$$

with $K_m$ the Michaelis-Menten constant.

The hirudin experiment demonstrates that the polymerization instantly stops after the addition of the inhibitor, and that therefore the concentrations of activated species, $[P_j^*]$, must be vanishingly low. Introducing the steady-state condition $d[P_j^*]/dt = 0$ into eq. (3) then immediately leads to the following set of equations for the rate of change of inert particles:

$$d\,\tilde{\sum}_{j=1} [P_j]/dt = (\nu'/2)\,\tilde{\sum}_{j=1} [P_j] \quad (5)$$

and

$$d[P_j]/dt = -\nu'[P_j] + (\nu'/2)\sum_{i=1}^{j-1} [P_{j-i}]\,[P_i]/\tilde{\sum}_{j=1} [P_j]$$

$$j = 2,3,4,..... \quad (6)$$

Equation (6) can be integrated to yield:

$$[P_j]/\left(\tilde{\sum}_{j=1} [P_j]\right)^2 = \left(1 - \tilde{\sum}_{j=1} [P_j]/[P_1^0]\right)^{j-1}/[P_1^0] \quad (7)$$

in which $[P_1^0]$ is the number concentration of fibrinogen at the start of the reaction and $\tilde{\sum}_{j=1} [P_j]$ can be calculated from the integrated eq. (5).

The weight-av. $M_r$ is defined by (11):

$$\bar{M}_r = M_1\,\tilde{\sum}_{j=1} j^2 [P_j]/\tilde{\sum}_{j=1} j[P_j] \quad (8)$$

where: $M_1 = M_r$ of the monomer

Inserting eq. (7) into eq. (8) and carrying through the summation finally gives:

$$\bar{M}_r/M_1 = (1 + \alpha)/(1 - \alpha) \quad (9)$$

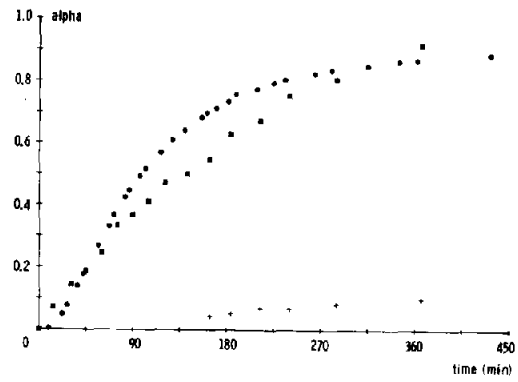


Fig.4. Comparing the progress of the weight-average $M_r$ of polymerizing fibrin to the rate of fibrinopeptide-A release. Experimental conditions: 0.5 M NaCl, 0.05 M Tris (pH 7.4) and 20°C. Fibrinogen: 0.337 mg/ml; thrombin act. $2.17 \times 10^{-3}$. $min^{-1}$. (•) $(\bar{M}_r - M_1)/(\bar{M}_r + M_1)$ as measured by light scattering; (■) and (+), release of fibrinopeptide A and B, respectively.

in which $\alpha$ is the degree of fibrinopeptide A release, defined as:

$$\alpha = \tilde{\sum}_{j=1} 2(j-1)\,[P_j]/2[P_1^0] \quad (10)$$

Eq. (9) allows the independent measurements of the growth of the weight-av. $M_r$ and the release of fibrinopeptide A to be compared. As fig.4 shows, there is indeed a fair correspondance of the plots of $(\bar{M}_r - M_1)/(\bar{M}_r + M_1)$ (eq. (9)) and the degree of peptide A release as measured by HPLC. This observation gives support to the polymerization scheme originally proposed in [4], this the more so since it is seen that the release of fibrinopeptide B strongly falls behind that of peptide A.

Deviations are currently investigated in terms of fibrinogen/fibrin complex formation, non-ideality effects and the influence of the small release of peptide B on the functionality of the aggregating species.

**Acknowledgements**

We are indebted to the Netherlands Foundation for Chemical Research (SON) for financial support, to K. J. Slangen for his assistance with the HPLC experiments and to F. Haverkate and W. Nieuwenhuizen from the Gaubius Institute at Leyden for stimulating discussions.

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