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Temperature Dependence of Fibrin Polymerization: A Light Scattering Study†

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ABSTRACT: The aggregation of fibrin occurring in a fibrinogen solution upon addition of the enzyme thrombin has been studied prior to the sol–gel transition at different temperatures by means of dynamic light scattering and simultaneous measurement of the released fibrinopeptide A (FPA). The evolution of the polymer distribution with time was found to be independent of the temperature. The analysis of the experiments yields the explanation: with increasing temperature the rate of FPA release increases because it involves an activation energy, whereas the aggregation rate of the fibrin monomers decreases because it is exothermic. The light scattering experiments show that the state of aggregation is a chemical equilibrium that can be shifted by the addition of the tetrapeptide Gly-Pro-Arg-Pro. From dynamic light scattering data it is possible to derive the probability of bond formation between fibrin molecules and from it the aggregation enthalpy. For 30 °C a value of −19 kcal/mol was obtained.

The experimental work described and interpreted here was undertaken with the purpose to gain insight into the temperature dependence of the polymerization process going on in a fibrinogen solution after the addition of the enzyme thrombin. The attention was focussed on the following aspects: enzyme kinetics, evolution of the polymer distribution, polymerization enthalpy and the question of chemical equilibrium, and possible temperature dependence of the monomer conformation. As in previous work (Wiltzius et al., 1982b), the evolution of the polymer distribution in the pregelation phase is investigated by means of static and dynamic light scattering and simultaneous determination of the number of the enzymatically activated binding sites through measurements of the number of the released fibrinopeptides A (FPA).

The dependence of the enzymatic step upon temperature can be interpreted in terms of Michaelis–Menten kinetics. The temperature dependence of the spontaneous aggregation of the monomers is interpreted under the assumption of a chemical equilibrium. Although the reaction proceeds, no evidence was found for nonequilibrium states, at least in the early stages of the pregelation phase and for moderate thrombin concentrations. The chemical equilibrium can be shifted by variation of temperature, concentration, or pH (Scheraga, 1983) or by addition of the competitive peptides Gly-Pro-Arg or Gly-Pro-Arg-Pro (Laudano & Doolittle, 1978). A quantitative understanding of this equilibrium requires knowledge of the polymerization enthalpy. The derivation of this quantity from calorimetric measurements is insofar problematic as the corrections that have to be made for chemical processes occurring together with the aggregation introduce a considerable uncertainity (Rozenfeld et al., 1976; Sturtevant et al., 1955). It will be shown that a combination of the light scattering experiments with measurements of the FPA release permits a specific determination of the polymerization enthalpy devoid of chemical interference with the aggregation process.

The investigation of monomer solutions by means of dynamic light scattering indicates a change of the conformation of the monomers with temperature. Since such a change might have an influence on the accessibility of the binding sites, it is likely to be a further temperature-dependent factor influencing the aggregation.

Experimental Procedures

Sample Preparation. A sample consisting of a solution of 2 mL of human fibrinogen (Imco, Stockholm, 2 mg/mL in 0.05 Tris–0.1 M NaCl buffer, pH 7.4) was rapidly thawed to the temperature desired for the experiment and transferred into a cylindrical quartz cell. For each experiment, 250 μL of a thrombin solution with a concentration of 0.01 NIH unit/mL was freshly prepared from a stock solution with a concentration of 1 NIH unit/mL (bovine thrombin, Roche, Basel, Switzerland) and then added to the fibrinogen solution under gentle shaking of the cell.

Great care was taken to assure that the thrombin concentration was the same in each experiment: control runs have shown that the evolution of the scattering intensity with time is perfectly reproducible. Prior to the light scattering experiments, large contaminant particles were sedimented by centrifugation at 10000*g* during 120 s at the desired tem-

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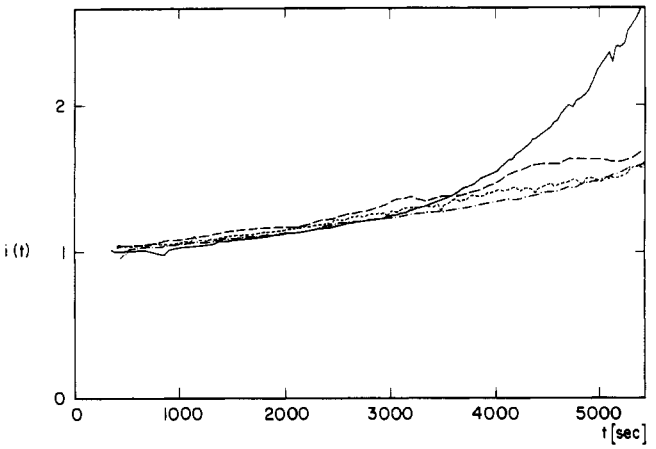

FIGURE 1: Time dependence of the intensity $i(t)$ of the scattered light (see eq 2), after addition of thrombin at time $t = 0$: (---) 10, (—) 20, (--) 30, and (-·-) 37 °C. Scattering angle $\theta = 90°$.

perature. The measurements started about 5 min after the addition of thrombin. The temperature of the scattering cell was controlled to within ±0.1 °C. The low thrombin concentration permitted the observation of the evolution of polymerization in the pregelation phase during a time span up to about 240 min.

In order to take a data point, intensity and autocorrelation function of the scattered light were measured during 30 s at the scattering angle $\theta = 90°$. At each chosen time point during the polymerization, a 50-μL sample was collected from the scattering cell. The thrombin action in this sample was stopped by adding 50 μL of hirudin (concentration 1 ATU/mL, Pentapharm AG, Basel, Switzerland), and the sample was frozen at −20 °C for later FPA analysis. In separate light scattering runs it was verified that the stopping of the aggregation process is complete and sufficiently fast to justify the assumption that the state of aggregation as observed by light scattering at any time point is determined by the release of FPA up to this time point. The FPA analysis was performed by a specific radioimmunoassay in the same way as described in previous papers (Hofmann & Straub, 1977; Wiltzius et al., 1982b).

Light Scattering Apparatus. The light scattering apparatus and the data analysis are the same as in our previous work (Wiltzius et al., 1982a).

RESULTS

Intensity of the Scattered Light. The following designations are used: $n_s^T(t)$ = number concentration of the s-mer at time t for a run conducted at temperature T; $n_0 = n_1(0)$ = initial fibrinogen concentration; M_s = molecular weight of the s-mer; q = absolute value of the scattering vector; $S_s(q)$ = form factor of the s-mer. The intensity scattered by a polydisperse solution of polymers at the scattering angle θ can be written as (Berne & Pecora, 1976)

$$I(t,q) = k\sum_{s=1}^{\infty} M_s^2 n_s^T(t) S_s(q) \tag{1}$$

where k is a proportionality constant.

For our purpose, it is not necessary to know the absolute intensity $I(t,q)$. The desired information is contained in the ratio

$$i(t) = \frac{I(t,q)}{I(0,q)} = \frac{\sum_{s=1}^{\infty} M_s^2 n_s^T(t) S_s(q)}{M_1^2 n_1^T(t) S_1(q)} \tag{2}$$

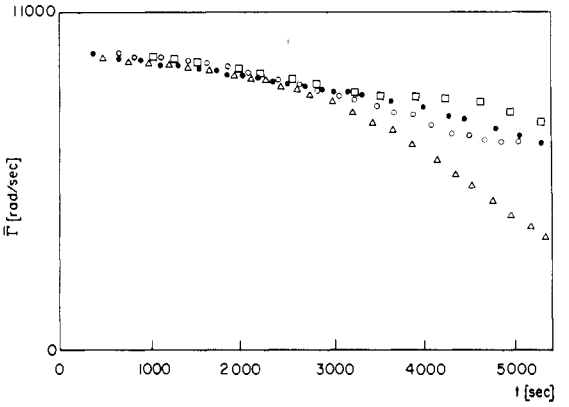

FIGURE 2: Time dependence of the line width $\bar{\Gamma}(t)$ of the scattered light (scattering angle $\theta = 90°$). The $\bar{\Gamma}$ values were corrected for the explicit temperature dependence of the translational diffusion constant (see eq 5): (●) 10, (△) 20, (○) 30, and (□) 37 °C.

The parameter q can be omitted since the scattering angle and the wavelength were kept constant. Figure 1 shows the scattered intensity vs. time, normalized according to eq 2 for four different temperatures.

Surprisingly, the observed increase of the normalized intensity with time does not depend upon the temperature for times shorter than a certain limit, which (for the thrombin concentration used) is about 3200 s, whereas for times above this limit the curves for different temperatures take a different course. Note that the temperature dependence of the scattering intensity, especially at longer times, is not monotonic.

Line Width of the Scattering Light. From the autocorrelation function of the scattered intensity at $\theta = 90°$, a mean line width $\bar{\Gamma}$ can be extracted. The definition of this measureable quantity for a polymer solution is (Berne & Pecora, 1976)

$$\bar{\Gamma}(t,q) = \frac{\sum_{s=1}^{\infty} M_s^2 n_s^T(t) S_s(q) \Gamma_s}{\sum_{s=1}^{\infty} M_s^2 n_s^T(t) S_s(q)} \tag{3}$$

where Γ_s is the line width of the light scattered by the s-mers alone. Γ_s is related to the translation diffusion constant D_s of the s-mers by the relationship:

$$\Gamma_s = D_s q^2 \tag{4}$$

The dependence of D_s upon size of the s-mer and upon the viscosity η of the solvent is given by the Stokes–Einstein relation:

$$D_s = \frac{kT}{6\pi\eta(T) r_s} \tag{5}$$

where r_s is the apparent hydrodynamic radius of an s-mer. Figure 2 represents a plot of the measured line width $\bar{\Gamma}(t)$ vs. t for four temperatures. For direct comparison, the data have been corrected for the explicit temperature dependence of D_s [factor $T/\eta(T)$]. The function $\bar{\Gamma}(t)$ does not depend upon the temperature for times shorter than the above-mentioned limit of about 3200 s. For times above this limit, the curves for different temperatures deviate from each other. There is a clear analogy to the behavior of the scattered intensity.

Temperature Dependence of the Aggregation Pattern. One might suspect at first that the dependence of the curves $i(t)$ and $\bar{\Gamma}(t)$ upon the parameter temperature (Figures 1 and 2) might be a manifestation of a change of the geometry of the aggregation pattern with temperature. In earlier work, we

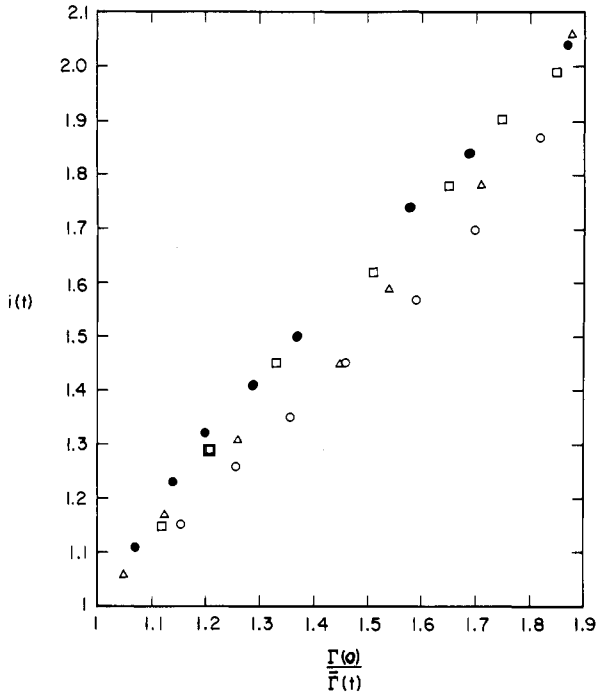

FIGURE 3: Intensity vs. inverse mean line width measured at a scattering angle $\theta = 90°$. The data are taken from Figures 1 and 2. (●) 10, (△) 20, (○) 30, (□) 37 °C.

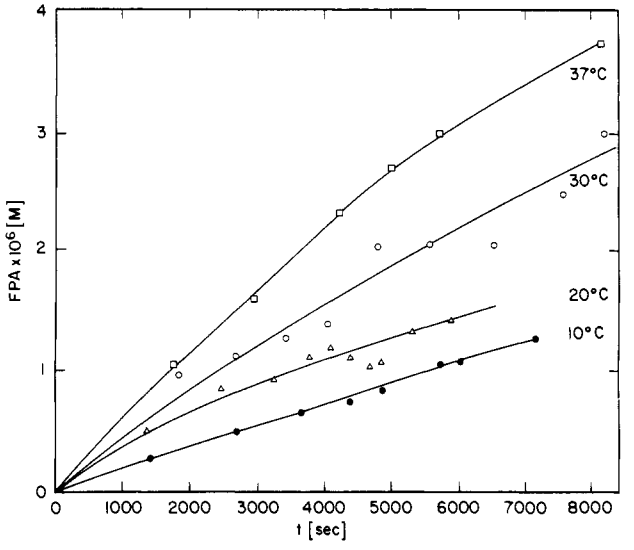

FIGURE 4: Concentration of released FPA vs. time. In order to guide the eye, an exponential function was traced through the data points. (●) 10, (△) 20, (○) 30, and (□) 37 °C.

have shown that a plot of $i(t)$ vs. $\Gamma(0)/\bar{\Gamma}(t)$ is sensitive to such a change (Wiltzius et al., 1982a). For example, it makes a difference in this plot whether the monomers aggregate end to end or in staggered overlap. The experimental data plotted in Figure 3 are taken from runs conducted at different temperatures (10, 20, 30, and 37 °C). Within experimental error the points are on the same curve, thus suggesting that the temperature has little or no effect upon the aggregation pattern.

Release of Fibrinopeptide A. Figure 4 represents the result of the measurement by RIA (Hofmann & Straub, 1977) of $[FPA]/\Phi_0$ vs. time t for the four different temperatures given above ([FPA] is the concentration of the released FPA's, and Φ_0 is the initial fibrinogen concentration). The release rate $v = d([FPA]/\Phi_0)/dt$ increases with temperature, indicating that the enzymatic step has an activation energy. Note that

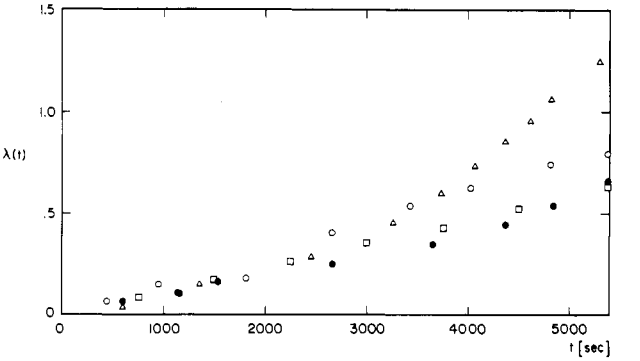

FIGURE 5: Number of occupied binding sites per monomer, $\lambda(t)$, calculated from the mean line width $\bar{\Gamma}(t)$ measured at $\theta = 90°$, using eq 6: (●) 10, (△) 20, (○) 30, and (□) 37 °C.

these curves do not contain any indication that the enzyme kinetics might enter a new regime after 3200 s. Thus, it cannot be the enzymatic step that causes the deviations between the curves for different temperatures in Figures 1 and 2.

Number of Binding Sites Involved in a Bond per Monomer. According to earlier work the polymers scatter like rodlike aggregates (Casassa, 1955; Blombäck & Laurent, 1958; Wiltzius et al., 1982a). In this case the number of used binding sites per monomer unit, λ, can be determined from measurements of the line width $\bar{\Gamma}$ (Wiltzius et al., 1982b):

$$\lambda \simeq 2[1 - \bar{\Gamma}(t)/\Gamma(0)] \tag{6}$$

Using the experimental data from Figure 2, we obtain the function $\lambda(t)$ depicted in Figure 5 for the four temperatures 10, 20, 30, and 37 °C. As expected on the basis of Figures 1 and 2, the curves $\lambda(t)$ for different temperatures do not significantly deviate from each other for times below 3200 s, whereas beyond this time the deviations become quite striking.

Interpretation of the Data. (A) Enzymatic Step. We interpret the time dependence of the FPA release by thrombin (Figure 4) in terms of Michaelis–Menten kinetics: (a) thrombin reversibly binds to fibrinogen to form a thrombin–fibrinogen complex; (b) this complex can decompose irreversibly into fibrin plus FPA plus thrombin. We consider *the early stage* of the reaction and neglect the number of fibrin molecules with both FPA's split off. The FPA concentration can then be written as

$$[FPA(t)] \simeq \frac{K_p[Th_0]\Phi_0}{K_M + \Phi_0} t \tag{7}$$

$[Th_0]$ is the total thrombin concentration and Φ_0 is the initial concentration of fibrinogen monomers, K_M and K_p are the Michaelis–Menten constants. The initial slope of $[FPA(t)]$, i.e., the quantity $K_p[Th_0]\Phi_0/(K_M + \Phi_0)$, can be extracted from the experimental data represented in Figure 4. This at least enables us to check whether the values of K_p and K_M given by Martinelli & Scheraga (1980) are compatible with our measurements. For a temperature of 25 °C, these authors give $K_p = 7.3 \times 10^{-10}$ M L (NIH unit)$^{-1}$ s^{-1} and $K_M = 9.2 \times 10^{-6}$ M. For the thrombin concentration used in our experiments, $[Th_0] = 1$ NIH unit/L, one obtains $K_p[Th_0]\Phi_0/(K_M + \Phi_0) = 5.8 \times 10^{-10}$ M/s. This is to be compared with the initial slope of the curve $[FPA(t)]$ for 25 °C interpolated from Figure 4, which is equal to 3.4×10^{-10} M/s.

(B) Aggregation as a Thermodynamic Equilibrium. There is considerable experimental evidence that the aggregation corresponds to thermodynamic equilibrium, at least in the pregelation stage. For example, it has been observed that fibrin polymers dissociate *reversibly* with increasing temperature (Laskowski et al., 1952; Wiltzius, 1981). Even more striking

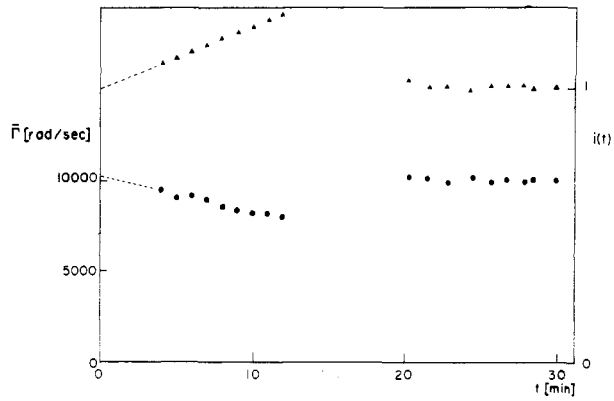

FIGURE 6: $i(t)$ (▲) and $\bar{\Gamma}(t)$ (●) vs. time. At 12 min after the addition of thrombin the enzyme activity was quenched with 50 μL of hirudin (1 ATU/mL), and the tetrapeptide Gly-Pro-Arg-Pro was added to reach a concentration of 10^{-3} M. The intensity and the line width Γ were monitored for other 10 min. The intensity was corrected for the effect of dilution due to addition of hirudin and Gly-Pro-Arg-Pro.

evidence is provided by the following experiment. By means of light scattering we have observed that fibrin polymers dissociate almost completely, if the peptide Gly-Pro-Arg-Pro is added to the polymer solution in a molar concentration large compared to the initial fibrinogen concentration (Figure 6). These peptides compete with the binding sites that are unmasked by the FPA release. The dissociation of the polymers proceeds in a time that is short compared to the time required to take a data point, i.e., short compared to 1 min. This experiment shows that a bond between two monomer units, once formed, does not last forever and that the aggregation equilibrium can be (reversibly) shifted in a short time. In a forthcoming paper we shall show that the aggregation equilibrium in a fibrin polymer solution can also be shifted by the addition of fibrinogen. In this experiment it is the complementary binding site on the fibrinogen molecule (it does not need activation) that competes with the same binding site on the fibrin molecule.

Experimentally, it is possible to gain some insight into this equilibrium by correlating the number of used binding sites per monomer unit, λ, as taken from dynamic light scattering data (see eq 6), with the number of activated binding sites, as determined simultaneously by measurements of the released FPA. In Figure 7 the number of binding sites involved in a bond per monomer unit, λ, is plotted vs. $[FPA]/\Phi_0$ for four runs, each taken at a different temperature. The number λ is derived from the experimental data plotted in Figure 5, with eq 6, and the number $[FPA]/\Phi_0$ from Figure 4. From the observation that the aggregation process immediately stops when the action of thrombin is inhibited by the addition of hirudin, we may conclude that each data point corresponds to a stable (or at least a metastable) state of the polymer solution. Thus, the curves in Figure 7 reflect the fact that this state depends upon the temperature. Let us now assume that each data point corresponds to an equilibrium state. Coniglio et al. (1982) have shown that the elementary expression for the probability of bond formation (eq 8) can be successfully

$$p_b = \frac{e^{-G_b/(kT)}}{e^{-G_b/(kT)} + e^{-G_u/(kT)}} \tag{8}$$

applied to polymer solutions. G_b and G_u designate the Gibbs free energy of the bound and unbound state, respectively. With $G = H - TS$ one has

$$\ln (1/p_b - 1) = \frac{H_b - H_u}{kT} - \frac{S_b - S_u}{k} \tag{9}$$

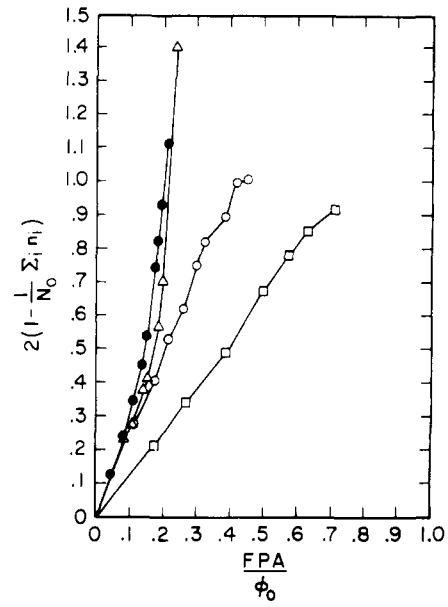

FIGURE 7: Number of occupied binding sites per monomer unit, $\lambda = 2\,[1 - \bar{\Gamma}(t)/\Gamma(0)]$ (from Figure 5) vs. $[FPA]/\Phi_0$ (from Figure 4): (●) 10, (△) 20, (○) 30, and (□) 37 °C.

In a polymer solution the probability p_b of bond formation can be written as

$$p_b = \lambda/f \tag{10}$$

Here λ is the number of binding sites involved in a bond per monomer initially in solution, and f is the functionality of the fibrin monomer. Thus, p_b is experimentally accessible through the measurement of λ (see eq 5), provided f is known. The mean functionality f of the monomers in solution is not constant but increases with the released FPA. In earlier work, we have shown that one can introduce an "effective functionality", which is equal to 2 in the early stage of the aggregation and finally increases to 4 when the gel point is approached (Wiltzius et al., 1982b). For a given value of $[FPA]/\Phi_0$, the dependence of the state of aggregation (as characterized by λ) upon the temperature T can be taken from the experimental data represented in Figure 7 by intersecting the curves with the vertical lines $[FPA]/\Phi_0$ = constant. Using the assumption $f = 2$ (early stage), one then obtains p_b from eq 10. According to eq 9, a plot of $\ln (1/p_b - 1)$ vs. $1/T$ should be a straight line, the slope of which should yield the binding enthalpy $\Delta H = H_u - H_b$ and be independent of the value of the parameter $[FPA]/\Phi_0$. Figure 8 represents such plots for the values 0.05, 0.1, 0.15, 0.2, and 0.24 of the parameter $[FPA]/\Phi_0$. Evidently, the data do not show the predicted straight lines. However, there is one important feature that cannot be overlooked: The initial slope of the curves does not seem to depend significantly upon the parameter $[FPA]/\Phi_0$, indicating indeed that it might correspond to the binding enthalpy ΔH.

Before trying to extract ΔH from this plot, one has to make sure that the result is insensitive with respect to a change of the assumption from $f = 2$ to $f = 3$ or even to $f = 4$. Evaluating the data for the latter f values, one finds that the curves in the plot $\ln (1/p_b - 1)$ vs $1/T$ are shifted along the ordinate axis with a negligible change of the slope. Thus, the assumption $f = 2$ underlying Figure 9 is not relevant in the determination of ΔH. The change of the curves with increasing value of the parameter $[FPA]/\Phi_0$ indicates that to a first approximation the initial slope can be taken from the straight line connecting the 37 °C point with the 30 °C point. The

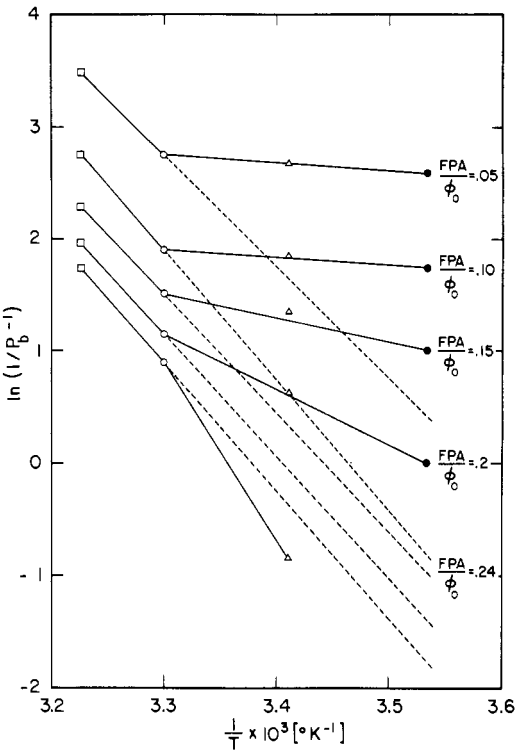

FIGURE 8: ln $(1/p_b - 1)$ vs. $1/T$. p_b was calculated from the data from Figure 7 and eq 10 for $f = 2$. The continuous lines connect the data points, and the broken lines represent what one expects on the basis of eq 9.

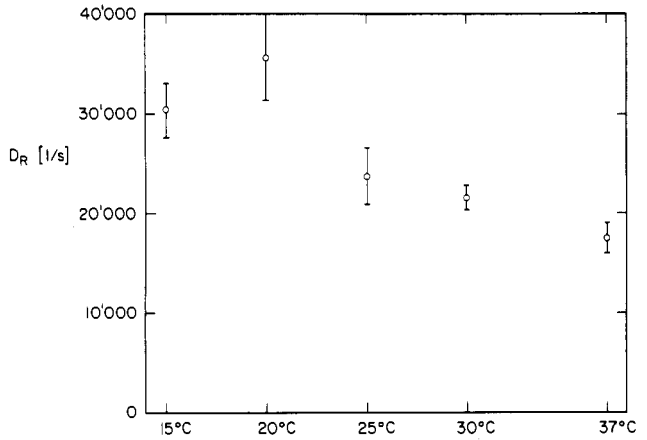

FIGURE 9: Rotational diffusion constant D_R of fibrinogen monomer vs. temperature. The data points are the mean values from the measurements, and the error bars represent the standard deviation.

thus determined slopes are the same to within about 10% for the different values of the parameter $[FPA]/\Phi_0$. The corresponding value of the binding enthalpy ΔH is about −19 kcal/mol. The agreement with the calorimetric data of Rozenfeld et al. (1976) (−24 kcal/mol) is surprisingly good. This indicates that our analysis of the light scattering data is on the right track, at least for temperatures above 30 °C. However, the interpretation of the deviations of the curves in Figure 8 from a straight line for temperatures below 30 °C is still a matter of speculation. For example, they might arise from a temperature dependence of the aggregation enthalpy ΔH or from a temperature dependence of the effective functionality caused by a conformational change of the monomers. The following paragraph contains some evidence for the second possibility.

(*C*) *Temperature Dependence of the Rotational Diffusion Constant of the Fibrinogen Monomer.* If the above-mentioned temperature dependence of the conformation of the monomer is sufficiently large, it should manifest itself in a temperature dependence of the hydrodynamic parameters as they can be determined by means of quasielastic light scattering. The rotational diffusion constant D_R is a more sensitive indicator of a conformational change than the translational diffusion constant D_T. We measured D_R for a fibrinogen monomer solution in the temperature range between 20 and 37 °C. The line width Γ of the depolarized light scattering is given by (Berne & Pecora, 1976)

$$\Gamma = 6D_R + D_T q^2 \qquad (11)$$

The chosen scattering angle ($\theta = 5°$) was sufficiently small to permit neglection of the contribution of the translational diffusion, $D_T q^2$, to the line width. Figure 9 shows the temperature dependence of D_R. In order to eliminate the temperature dependence of the viscosity of the solvent, the D_R values are reduced to the viscosity for 20 °C. The value measured at 20 °C, $D_R = 35700 \pm 4500$ s^{-1}, is in good agreement with flow-birefringence data (Edsall et al., 1947). Since is has been shown that the release of fibrinopeptides does not measurably change the hydrodynamic parameters (Wiltzius & Hofmann, 1980), one can conclude that the data in Figure 9 are also valid for fibrin.

One might speculate that the increase of D_R with decreasing temperature is caused by a folding of the protruding part of the α chains. Since the N-terminal end of these chains might interact with the binding sites on the D domains, it is conceivable that these could be masked by this folding. Thus, the effective functionality (which in the first place is a function of $[FPA]/\Phi_0$) might also depend upon the temperature.

Discussion

Temperature Independence of the Early Stage of the Aggregation. It is surprising that there exists a time range ($t <$ 3200 s for the thrombin concentration used in our experiments) where the raw data characterizing the evolution of the aggregation do not exhibit a significant temperature dependence (Figures 1 and 2), thus indicating that the number of used binding sites per monomer unit, the function $\lambda(t)$, and with it the evolution of the polymer distribution do not depend upon the parameter temperature (Figure 5). At least qualitatively this temperature independence might be explained as follows.

In the early stage the aggregation process involves only two steps with a significant temperature dependence, namely, the enzymatic step (FPA release) and the spontaneous aggregation. (The FPB release is negligible.) Apparently, these two temperature dependencies "compensate" in such a way that the dependence of the function $\lambda(t)$ upon the parameter temperature is too small to be observed. On the one hand, the rate of FPA release increases with increasing temperature because it is thermally activated (see, e.g., Figure 4); on the other hand, the probability of bond formation (at a fixed value of $[FPA]/\Phi_0$) decreases with increasing temperature (Figure 7) because the aggregation enthalpy is negative. For the nonmonotonic temperature dependence at the data stage ($t >$ 3200 s), we cannot offer an explanation as yet.

Enthalpy of the Spontaneous Aggregation. The determination of the enthalpy of aggregation, ΔH, from calorimetric data is problematic since the steps one has to make to induce the aggregation may already as such involve exothermic or endothermic processes. Therefore, our determination of ΔH from light scattering data might be of interest. However, the underlaying assumptions have to be remembered; namely, (a) the value of the functionality $f = 2$, and (b) the rod-like aggregates.

Table I: Measured and Discussed ΔH Values

authors	experiments	pH	temp (°C)	ΔH (kcal/mol)
Sturtevant et al., 1955	calorimetry	6.08	25	−19
Sturtevant et al., 1955	calorimetry	6.88	25	−44.5
Rozenfeld et al., 1976	calorimetry	7.2	25	−24.1
present work	light scattering	7.4	30–37	−19

authors	theory	pH	temp (°C)	ΔH (kcal/mol)
Sturtevant et al., 1955	hydrogen-bond formation is assumed	7.4	25	−55

The first assumption turns out to be unimportant since the ΔH value resulting from our analysis is almost the same for $f = 2$ and $f = 4$. The second assumption is a realistic basis for the interpretation of light scattering data as has been demonstrated by several authors (Casassa, 1955; Blombäck & Laurent, 1958; Wiltzius et al., 1982a). The ΔH values measured and discussed by various authors are summarized in Table I.

The temperature dependence of the enthalpy of aggregation cannot be taken from our data since we cannot exclude that the deviations of the curves in Figure 8 from a straight line might be due (at least partially) to a temperature dependence of the effective functionality.

Registry No. Gly-Pro-Arg-Pro, 67869-62-9; thrombin, 9002-04-4.

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