Biophysical Journal Volume 75 October 1998 1973–1979

Cl^- Regulates the Structure of the Fibrin Clot

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ABSTRACT The differences between coarse and fine fibrin clots first reported by Ferry have been interpreted in terms of nonspecific ionic strength effects for nearly 50 years and have fostered the notion that fibrin polymerization is largely controlled by electrostatic forces. Here we report spectroscopic and electron microscopy studies carried out in the presence of different salts that demonstrate that this long-held interpretation needs to be modified. In fact, the differences are due entirely to the specific binding of Cl^- to fibrin fibers and not to generic ionic strength or electrostatic effects. Binding of Cl^- opposes the lateral aggregation of protofibrils and results in thinner fibers that are also more curved than those grown in the presence of inert anions such as F^-. The effect of Cl^- is pH dependent and increases at pH $>$ 8.0, whereas fibers grown in the presence of F^- remain thick over the entire pH range from 6.5 to 9.0. From the pH dependence of the Cl^- effect it is suggested that the anion exerts its role by increasing the pK_a of a basic group ionizing around pH 9.2. The important role of Cl^- in structuring the fibrin clot also clarifies the role played by the release of fibrinopeptide B, which leads to slightly thicker fibers in the presence of Cl^- but actually reduces the size of the fibers in the presence of F^-. This effect becomes more evident at high, close to physiological concentrations of fibrinogen. We conclude that Cl^- is a basic physiological modulator of fibrin polymerization and acts to prevent the growth of thicker, stiffer, and straighter fibers by increasing the pK_a of a basic group. This discovery opens new possibilities for the design of molecules that can specifically modify the clot structure by targeting the structural domains responsible for Cl^- binding to fibrin.

INTRODUCTION

Fibrinogen circulates in the plasma as a dimer of three chains, $(A\alpha B\beta\gamma)_2$, covalently linked by disulfide bonds (Doolittle, 1984; Binnie and Lord, 1993). After a vascular lesion, fibrinogen is converted into fibrin monomers by the fast form of thrombin, which cleaves the $A\alpha$ chain and releases fibrinopeptide A (Di Cera et al., 1997). The monomers aggregate to form two stranded protofibrils promoting a second highly specific cleavage by the fast form of thrombin at the $B\beta$ chain leading to the release of fibrinopeptide B. These polymers are produced by specific interactions between sites exposed by removal of the fibrinopeptides in the central region of the molecule with complementary sites at the ends of the molecule (Shainoff and Dardik, 1983; Doolittle, 1984; Budzynski, 1986; Medved et al., 1993). The protofibrils then aggregate to form thicker fibers (Hantgan and Hermans, 1979; Hantgan et al., 1980; Higgins et al., 1983; Lewis et al., 1985). This leads to formation of a gel, or fibrin clot, that anchors platelets to the site of injury and initiates processes that stop the bleeding and promote wound repair and healing.

Fibrin polymerization has been studied intensively over the past 50 years because of the availability of large quantities of fibrinogen for biochemical and biophysical characterization. Ferry was the first to demonstrate that fibrin clots formed at different ionic strengths are dramatically different, with fibers becoming apparently thinner at high pH and salt concentration (Ferry and Morrison, 1947). This seminal observation has been reproduced by numerous other investigators (Shulman et al., 1953; Latallo et al., 1962; Nair et al., 1986; Kaminski et al., 1991; Blomback et al., 1992; Weisel and Nagaswami, 1992). Many other factors, including Ca^{2+} and other plasma proteins, exert a significant influence on clot structure (Carr et al., 1985, 1986; Carr, 1988; Mihalyi, 1988; Weisel and Nagaswami, 1992; Blomback et al., 1994). Surprisingly, however, these previous studies have not assessed the role of possible specific ion binding interactions in fibrin polymerization. In Ferry's original experiments (Ferry and Morrison, 1947), and in practically all subsequent studies (Shulman et al., 1953; Latallo et al., 1962; Nair et al., 1986; Kaminski et al., 1991; Blomback et al., 1992; Weisel and Nagaswami, 1992), the ionic strength was changed with NaCl or other salts containing Cl^-.

When the effect of different salts on clot structure was first examined (Vindigni and Di Cera, 1996), it was found that the concentration of Cl^- present in solution is the most important variable that controls the size of fibrin fibers. Clots grown at different ionic strength in the presence of NaF, instead of NaCl, were found to retain the same structure in the ionic strength range from 100 to 500 mM. This recent observation may have a profound impact on our mechanistic understanding of fibrin polymerization and the factors that regulate it. If specific binding interactions of Cl^- determine the size and shape of fibrin fibers, the nonspecific electrostatic components invoked in previous analyses become of marginal importance, and identification of a specific structural domain responsible for Cl^- binding may

Received for publication 30 March 1998 and in final form 12 June 1998.
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0006-3495/98/10/1973/07 $2.00

provide new targets for drugs selectively designed to interfere with fibrin polymerization.

The potential importance of the Cl^- effect in both physiological and pharmacological terms, as well as the possibility of unraveling aspects of the basic molecular mechanism governing fibrin polymerization, have prompted a more thorough characterization using a combination of spectroscopic and electron microscopy measurements. This yields a direct demonstration of the large and significant effect of Cl^- on the fibrin clot and clarifies the role of protons and fibrinopeptide B release in fibrin polymerization.

MATERIALS AND METHODS

Human fibrinogen was obtained from Enzyme Research (South Bend, IN) and further purified using a Pharmacia PD-10 desalting column and a gelatin-lysine affinity column (Bio-Rad, Hercules, CA). Ancrod snake venom from Sigma Chemical Co. (St. Louis, MO) was dissolved in the desired buffer and used at a concentration of 0.15 IU/ml equivalent in terms of clotting activity to 4 nM thrombin. The activity of ancrod pertaining to the exclusive release of fibrinopeptide A was checked directly by high-pressure liquid chromatography analysis of the release of fibrinopeptides (Vindigni and Di Cera, 1996). Under all conditions, thrombin was found to release both fibrinopeptides A and B, whereas ancrod released only fibrinopeptide A. Measurements of turbidity over the pH range 6.5–9.0 were carried out at 25°C under conditions of 10 mM 2-[*N*-Cyclohexylamino]ethanesulfonic acid (CHES), 5 mM Tris, 5 mM bisTris, 0.1% polyethylene glycol (PEG), 1 mM EDTA by monitoring the increase in absorbance of the sample as a function of time at 350 nm. The triple buffer ensured buffering over the entire pH range with minimal perturbations in the ionic strength of the solution (Ellis and Morrison, 1982). The concentration of various anions, used as Na^+ salts, was changed under all solution conditions at an ionic strength of 200 mM kept constant with NaF.

The wavelength dependence of the turbidity of a fibrin gel was used to calculate the average mass-length ratio of fibrin fibers (Carr and Hermans, 1978; Hantgan and Hermans, 1979). Spectra were recorded in the wavelength range from 350 to 700 nm. The turbidity τ defined as the absorbance times ln10 is a measure of the intensity decrease of transmitted light due to scattering. For rod-like particles with a diameter that is small compared with the wavelength λ, τ should vary as λ^{-3} and should go to zero as $\lambda \rightarrow \infty$ (Casassa, 1955). The mass-length ratio is derived directly from the slope of the plot (Carr and Hermans, 1978; Casassa, 1955). This behavior was observed at low fibrinogen concentrations and in the presence of Cl^- (see Results). When the diameter grows relative to λ, the plot is no longer linear, but the quantity $c\tau^{-1}\lambda^{-3}$, where c is the fibrinogen concentration, changes linearly with λ^{-2}, and the intercept of the plot gives the mass-length ratio of the fibers (Carr and Hermans, 1978). This behavior was observed at high fibrinogen concentrations and in the presence of F^-.

Clots were prepared for scanning electron microscope experiments by fixation, dehydration, critical point drying, and sputter coating with gold as described previously (Langer et al., 1988; Weisel, 1992). The clots were formed by addition of thrombin to 3 μM fibrinogen to give a final concentration of 1 nM under the different conditions described in the Results. Clotting was allowed to proceed for 1.5–2 h at room temperature. Specimens were observed and photographed using a Philips XL20 scanning electron microscope.

RESULTS

Fig. 1 reports the asymptotic values of turbidity of clots grown as a function of the concentration of different anions, with the ionic strength kept constant with NaF. No change is observed in the presence of acetate or cacodylate, thereby proving that F^- behaves as a good inert anion because its properties are identical to those of bulky anions. The inert behavior of F^- is to be expected from consideration of its hydration properties (Collins, 1995, 1997), and the data in Fig. 1 support conclusions drawn in previous studies using this anion (Ayala and Di Cera, 1994). Results similar to those seen for cacodylate and acetate are obtained with organic phosphates such as 2,3-diphosphoglycerate and inositol hexaphosphate (data not shown). On the other hand, an almost complete inhibition of lateral aggregation of fibrin is found in the presence of Cl^- or Br^- around the concentration of 200 mM. An even larger effect is seen in the presence of I^- or ClO_4^-, which inhibit lateral aggregation around 100 mM. The observed salt-specific effect is also observed under physiological conditions of pH and temperature. These measurements prove unequivocally that the concentration of the physiological anion Cl^- plays a key role in determining the thickness of fibrin fibers.

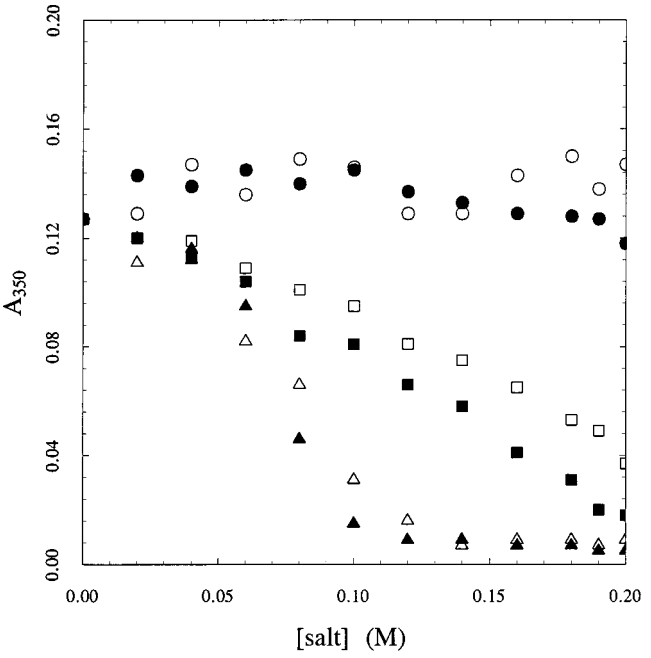

FIGURE 1 Turbidity of fibrin clots developed in the presence of different salts, at I = 200 mM kept constant with NaF. Experimental conditions are as follows: 4 nM thrombin, 0.25 μM fibrinogen, 5 mM Tris, 0.1% PEG, 1 mM EDTA, pH 8.0 at 25°C. Salts are as follows: ○, sodium cacodylate; ●, CH_3COONa; □, NaBr; ■, NaCl; △, NaI; ▲, $NaClO_4$.

This conclusion is directly supported by scanning electron microscopy of clots made in the presence of Cl^- or F^-. Direct visualization of the clots is important to verify the assumptions relating turbidity to fiber diameter and to gain more detailed structural information on clot structure. These experiments were done at fibrinogen concentrations (generally, 3 μM) higher than those of the turbidity measurements just described to obtain clots that were stable enough to withstand the preparation for microscopy and to mimic more closely physiological concentrations (~7 μM). We found that, at these concentrations, fibrinogen is not soluble

in NaF buffers, so all experiments were done with 0.05 M NaCl in the buffers. For example, clots were made at 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, and 0.50 M NaCl and at the same NaF concentrations all containing 0.05 M NaCl. Thus, comparisons could be made of clots either with the same concentration of different anions or with the different ions but the same total ionic strength. Clots were made at pH 6.5, 7.5, 8.0, and 9.5. Interestingly, clots made in the presence of NaF were uniformly more stable mechanically than those made in NaCl, which had a greater tendency to collapse during preparation for microscopy. These properties of fibrinogen in NaF buffers may also be relevant to the preparation of crystals for x-ray diffraction analysis.

Some examples of electron micrographs from these experiments are shown in Fig. 2. As indicated by the turbidity measurements, the fibers formed in NaF are thicker than those formed in NaCl. The mean diameter of fiber bundles is 148 ± 81 nm for clots made in NaF as compared with 60 ± 22 nm for clots formed in NaCl. The pores in the NaF clots are considerably larger than those in NaCl clots. Estimates of fiber densities, calculated from counts of number of fibers in random directions across the images, are 47 fibers bundles per 10^6 nm^3 for NaF clots versus 116 fiber bundles per 10^6 nm^3 for NaCl clots. Fibers in NaF clots are generally more straight or gently curved, whereas those in NaCl clots often display sharp bends or turns. The differences in fiber structure gives clots formed in NaCl more irregularity in the overall appearance of their pore structure.

It has long been known that fibrin clots formed at high pH have low turbidity. This notion was fostered by pioneering studies carried out in the presence of Cl^- concentrations of 100–500 mM (Ferry and Morrison, 1947). In the presence of 200 mM Cl^-, an increase in pH brings about a decrease in turbidity (Fig. 3). However, in the presence of 200 mM F^-, the turbidity remains significant at high pH and actually exceeds that of fibers grown at low pH in the presence of Cl^- or F^-. This result is reinforced by electron microscopy images shown in Fig. 4. At pH 9.5, the differences between the appearance of clots formed in NaF and NaCl are even more striking than at lower pH. The clots formed in NaF are made up of very thick fiber bundles, and fibers associate laterally to form very complex aggregates. Clots formed in NaCl at pH 9.5 are made up of very thin fibers, with a much larger number of branch points, so that the pore size is very small. The pore sizes are usually relatively uniform in these

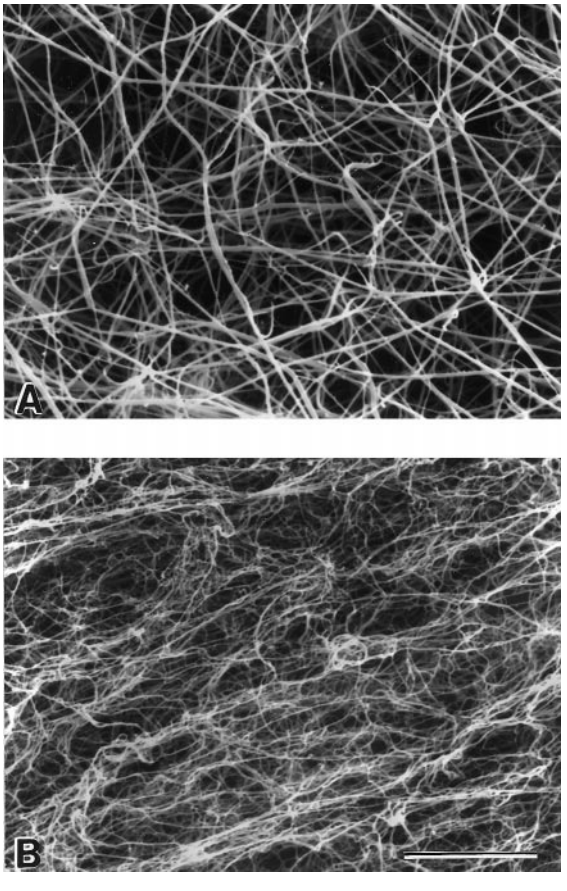

FIGURE 2 Scanning electron micrographs of fibrin clots grown in the presence of NaCl or NaF. Experimental conditions are as follows: 4 nM thrombin, 3 μM fibrinogen, 50 mM Tris, pH 7.5 at 22°C, plus the following salts: (*A*) 150 mM NaF, 50 mM NaCl; (*B*) 200 mM NaCl. Bar, 5 μm.

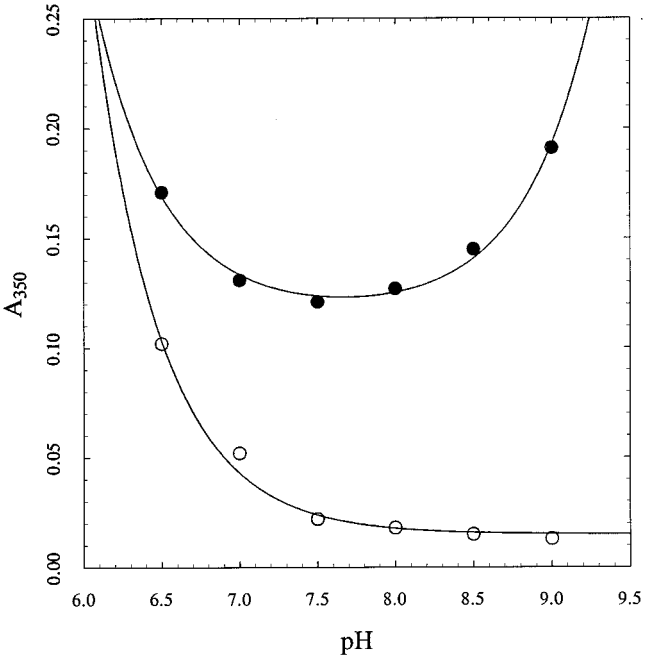

FIGURE 3 Turbidity of fibrin clots developed in the presence of 200 mM NaCl (○) or NaF (●) as a function of pH. Experimental conditions are as follows: 4 nM thrombin, 0.25 μM fibrinogen, 10 mM CHES, 5 mM Tris, 5 mM bisTris, 0.1% PEG, 1 mM EDTA at 25°C. The pH dependence of the turbidity was analyzed according to the empirical expression $\tau = \tau_0[1 + ([H^+]/K_1) + (K_2/[H^+])]$, where τ_0 is the pH-independent value of τ and K_1 and K_2 are the dissociation constants of two ionizable groups. The equation assumes that the turbidity is directly proportional to the formation of lateral aggregates that is promoted by protonation of one group in the acidic pH range and deprotonation a second group in the basic pH range. The best-fit parameter values for the data are s follows: for NaF, $\tau_0 = 0.116 \pm 0.002$, $pK_1 = 6.16 \pm 0.04$, and $pK_2 = 9.18 \pm 0.03$; for NaCl, $\tau_0 = 0.015 \pm 0.002$, $pK_1 = 7.27 \pm 0.09$, and $pK_2 > 10$.

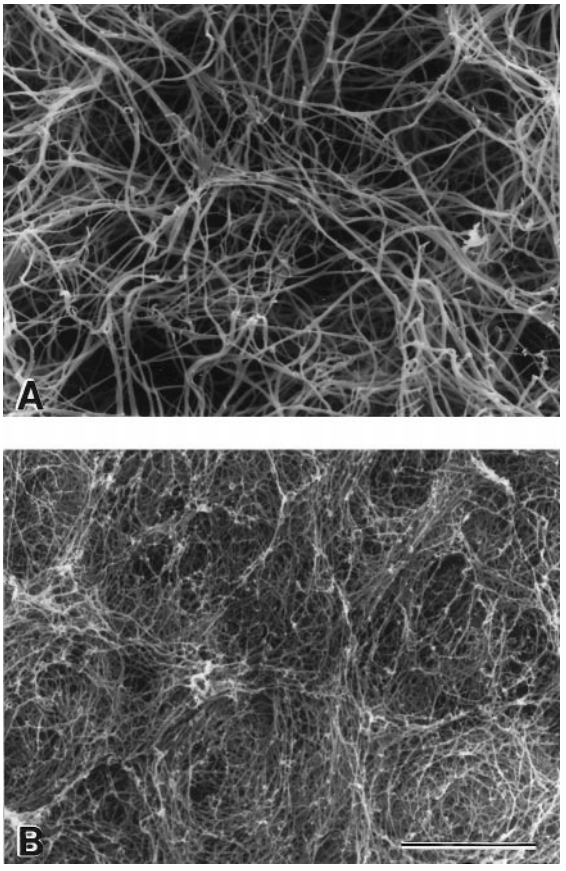

FIGURE 4 Scanning electron micrographs of fibrin clots grown in the presence of NaCl or NaF. Experimental conditions are as follows: 4 nM thrombin, 3 μM fibrinogen, 10 mM CHES, 5 mM Tris, 5 mM bisTris, pH 9.5 at 22°C, plus the following salts: (*A*) 150 mM NaF, 50 mM NaCl; (*B*) 200 mM NaCl. Bar, 5 μm.

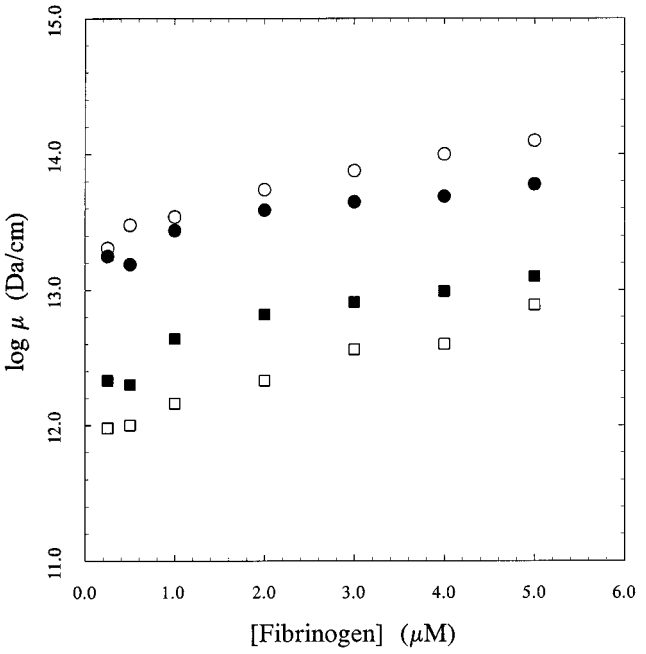

FIGURE 5 Mass-length ratio μ of fibrin fibers as a function of the fibrinogen concentration. Data were derived from the wavelength dependence of the turbidity of fibrin clots according to the theory of light scattering of rod-like particles (Casassa, 1955). Experimental conditions are as follows: 4 nM thrombin (●, ■) or ancrod (○, □), 5 mM Tris, 0.1% PEG, 1 mM EDTA, pH 8.0 at 25°C, in the presence of 200 mM NaCl (□, ■) or NaF (○, ●).

clots. The mean diameter of fiber bundles at pH 9.5 is 164 ± 75 nm for clots made in NaF as compared with 55 ± 12 nm for clots formed in NaCl. Estimates of fiber densities are 40 fiber bundles per 106 nm^3 for NaF clots and 182 fiber bundles per 106 nm^3 for NaCl clots.

The pH dependence of the turbidity in the presence of F^- is reminiscent of the pH dependence of K_m for an enzyme-substrate interaction in which two ionizable groups control optimal binding, with one group being protonated and the other being deprotonated. By analogy, an empirical expression can be derived for the analysis of the data in Fig. 3 that expresses the pH dependence of the turbidity in terms of two ionizable groups. One such group has $pK_a = 6.2$ and must be protonated for optimal aggregation, whereas the other group has $pK_a = 9.2$ and must be deprotonated. Cl^- increases the pK_a of both groups, but its effect is predominant on the second group, which increases its pK_a well beyond 10 in the presence of the anion, thereby opposing deprotonation and lateral aggregation of fibrin fibers.

Elucidation of the role of Cl^- also helps us understand the function of the release of fibrinopeptide B in the polymerization of fibrin fibers. Fig. 5 shows the mass-length ratio of fibrin fibers grown from digestion of fibrinogen with thrombin or ancrod. The mass-length ratio of fibers produced without the release of fibrinopeptide B is lower than that of fibers grown when both fibrinopeptides A and B are released. This result suggests a connection between fibrinopeptide B cleavage and lateral aggregation, although other results indicate that the sites exposed by fibrinopeptide B release mainly reinforce linear polymerization (Blomback et al., 1978; Hantgan and Hermans, 1979; Laudano and Doolittle, 1980; Hantgan et al., 1983; Shainoff and Dardik, 1983; Veklich et al., 1993; Weisel et al., 1993; Gorkun et al., 1994). However, when the same experiments are carried out in the absence of Cl^- and in the presence of F^-, the effect of fibrinopeptide B release is reversed. Fibers grown in ancrod are thicker than those grown in the presence of thrombin. This means that fibrinopeptide B release plays no direct role in lateral aggregation. The effect becomes even more appreciable when the fibrinogen concentration increases and reaches its physiological range, thereby demonstrating the importance of this mechanism for fibrin polymerization in vivo.

DISCUSSION

Individual ions may be systematically classified as chaotropes or kosmotropes by the sign of the Jones-Dole viscosity B coefficient (Jones and Dole, 1929) that correlates with charge density and the strength of interaction with water molecules (Collins, 1997). The effect of different

anions on fibrin polymerization is consistent with a binding interaction the strength of which follows the properties of the anion in the Jones-Dole scale. Kosmotrope anions, such as F^-, cacodylate, acetate, and organic phosphates, bind water molecules tightly, taking up a greater size of their anhydrous volume. This property makes them little reactive with proteins. On the other hand, chaotrope ions, such as Cl^- and ClO_4^-, bind water molecules weakly, allowing an easy removal of them from the complex with a limited steric impairment. Among the anions of physiological relevance, Cl^- is the only chaotrope and tends to interact with basic groups of proteins with significant affinity. The effects reported in this study on fibrin polymerization reflect this tendency of Cl^- to interact with protein moieties.

Fibrin monomers polymerize via complementary binding sites to form two-stranded protofibrils. The structure of some of the complementary polymerization sites of fibrin has been determined recently (Spraggon et al., 1997; Yee et al., 1997). Protofibrils can be formed after the release of either fibrinopeptide A or B (Hantgan and Hermans, 1979; Shainoff and Dardik, 1983; Weisel, 1986), whereas the sequential release of A and then B fibrinopeptides significantly enhances lateral aggregation by allowing intermolecular interactions of the carboxyl-terminal α chains (Veklich et al., 1993; Gorkun et al., 1994). Cl^- exerts its physiological role by impairing lateral aggregation of protofibrils and maintains a smaller size of the fibers. The influence of pH on these effects may be related to the ionization and calorimetric changes that occur on polymerization (Mihalyi, 1954a,b; Sturtevant et al., 1955). Lateral aggregation of fibrin fibers is promoted by two ionizable groups. One group with $pK_a = 6.2$ must be protonated and the other with $pK_a = 9.2$ must be deprotonated for optimal aggregation. We can only speculate on the nature of these groups from their pK_a values. The pK_a of 6.2 is most likely due to a histidine residue. A comparison of the amino-terminal sequence of the β chain of fibrinogen from different species show His16 as a highly conserved residue (Laudano and Doolittle, 1980; Doolittle, 1983). A critical role for this residue in end-to-end polymerization was proposed by Shimizu et al. (1983, 1986) from affinity chromatography experiments on fibrinogen fragments. The exposure of the Gly15-His16-Arg17 sequence after the release of fibrinopeptide B is essential for the formation of central polymerization sites that interact with sites at the end of the molecule (Shainoff and Dardik, 1983; Medved et al., 1993). Laudano and Doolittle (1980) reported the presence of specific sites on the fibrinogen molecule for the Gly-His-Arg-Pro peptide and emphasized the importance of His in the sequence. Quite likely, His16 of the β chain of fibrinogen plays a key role in lateral aggregation and must be protonated for optimal coupling with a negatively charged moiety on the complementary site. In the presence of Cl^-, the pK_a of this histidine is increased and aggregation is similarly enhanced at low pH in NaCl or NaF. The other Cl^--linked ionizable group with $pK_a = 9.2$ may be a lysine, tyrosine, arginine, or the amino terminus of any of the chains. The lateral aggregation of fibrin increases with the group in the deprotonated state, whereas Cl^- tends to increase the pK_a, promote protonation, and oppose lateral aggregation.

Although identification of the ionizable groups remains elusive, specific domains of the fibrin monomer can be targeted with anionic ligands to mimic the physiological effect of Cl^-. This information can be exploited to search for ligands that specifically inhibit fibrin polymerization by binding to the domain responsible for Cl^- binding. The feasibility of this approach is demonstrated already by the drastic effect on fibrin polymerization observed in the presence of I^- or ClO_4^-, two strongly chaotropic anions that interact with fibrin more strongly than the physiological anion Cl^-. Aggregation is severely reduced in the presence of these anions. Phosphate and carbonate, other physiologically important anions, are kosmotropic and have little effect on fibrin polymerization.

Our results on the role of Cl^- also provide new insights into the role of fibrinopeptide B release in fibrin polymerization, which has so far been controversial. Fibrinopeptide B cleavage exposes an aggregation site independent of that uncovered by fibrinopeptide A release (Shainoff and Dardik, 1979). Blomback et al. (1978) proposed that the release of fibrinopeptide A controls the end-to-end polymerization, whereas cleavage of fibrinopeptide B promotes lateral aggregation of protofibrils and formation of fibrin fibers. Dyr et al. (1989) suggested an enhancement for polymerization sites unmasked by fibrinopeptide A release due to release of fibrinopeptide B. However, Laudano and Doolittle (1980) found no inhibition of aggregation of fibrin by tetrapeptide analogs to the amino-terminal sequence of the β chain exposed after the release of fibrinopeptide B. Furthermore, structural studies showed that release of either fibrinopeptide triggers similar modes of aggregation (Weisel, 1986; Mosesson et al., 1987). These controversial findings can be partially reconciled by recent findings on the role of the carboxyl-terminal α chains in lateral aggregation (Veklich et al., 1993; Gorkun et al., 1994), but it is also necessary to consider the differences in experimental conditions used and the previously unrecognized role of Cl^- in fibrin polymerization.

The role of fibrinopeptide B in determining the clot structure depends on the particular anion present in solution and the Cl^- concentration. In the presence of Cl^-, the release of fibrinopeptide B tends to increase the mass-length ratio of fibrin fibers, especially at high fibrinogen concentrations. In the absence of Cl^-, or in the presence of F^-, the effect of fibrinopeptide B cleavage is actually reversed, such that fibrin fibers grow thicker when only fibrinopeptide A is released, demonstrating that there is no direct relationship between fibrinopeptide B cleavage and lateral aggregation. In view of the effect of Cl^- and fibrinogen concentration, as well as Ca^{2+} and other factors, on the size of the fibrin fibers (see Fig. 5), it is not difficult to envision large discrepancies in experimental data collected on clot structures under conditions in which the NaCl or fibrinogen

concentrations are not strictly comparable. In previous studies, the fibrinogen concentration varied significantly, being very low for light-scattering studies and very high for electron microscopy analysis, as did the Cl^- concentration, which ranged from 50 to 500 mM. Small changes in NaCl concentration may yield opposite effects on the release of fibrinopeptide B, whereas experiments carried out at high fibrinogen concentrations will tend to exacerbate the effect of Cl^-.

In the absence of Cl^-, thick fibers are formed, but Cl^- inhibits lateral aggregation. Under physiological conditions in the presence of Cl^-, fiber thickness can be regulated by fibrinopeptide B cleavage, which leads to release of the αC domains to promote lateral aggregation (Gorkun et al., 1994). Thus, Cl^- allows the production of fibers of appropriate thickness for different conditions to guarantee the optimal mechanical properties of the fibrin scaffold.

This work was supported in part by National Institutes of Health research grants HL30954 (J.W. Weisel) and HL49413 and HL58141 (E. Di Cera). E. Di Cera is an Established Investigator of the American Heart Association and Genentech.

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