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Acceleration of Surface-Dependent Autocatalytic Activation of Blood Coagulation Factor XII by Divalent Metal Ions†

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ABSTRACT: The effect of divalent metal ions on the rate of dextran sulfate dependent autocatalytic activation of human blood coagulation factor XII was studied at pH 7.4 and 25 °C. Zn^{2+} and Cu^{2+}, but not Co^{2+}, increased the rate of factor XII activation induced by dextran sulfate with optimum effects at approximately 5 and 1 μM, respectively, while Ca^{2+} acceleration required much higher concentrations (millimolar). Further investigation of the effect of Zn^{2+} on factor XII activation demonstrated a complete dependence on the presence of dextran sulfate, lack of inhibition by soybean trypsin inhibitor, the appearance of α-XIIa as the primary reaction product, and reaction kinetics characteristic of an autocatalytic process. These results were consistent with Zn^{2+} affecting only the rate of surface-mediated factor XII autoactivation. The initial turnover velocity of dextran sulfate induced factor XII autoactivation increased linearly with factor XII concentration in the absence of Zn^{2+} up to 0.9 μM factor XII but showed saturation behavior over this same concentration range in the presence of 5 μM Zn^{2+}, indicating that Zn^{2+} increased the reaction rate primarily by lowering the apparent K_m. Comparison of the kinetics of autoactivation at $\mu = 0.15$ and 0.24 revealed that the enhancement in the apparent k_{cat}/K_m brought about by Zn^{2+} increased from 19-fold to 520-fold, respectively, due to a differential dependence of the Zn^{2+}-stimulated and unstimulated reactions on ionic strength. Evidence that enhanced binding of factors XII and α-XIIa to dextran sulfate contributed to the Zn^{2+} rate enhancement was provided by the observation that factors XII and α-XIIa were eluted at higher ionic strengths from a dextran sulfate–agarose column in the presence of 5 μM Zn^{2+} than in its absence. α-Factor XIIa bound more tightly to dextran sulfate–agarose than factor XII in the presence and absence of Zn^{2+}, while β-factor XIIa, which lacks the surface binding domain, did not bind to the column under the same conditions. The rate of dextran sulfate dependent contact activation in normal human plasma, but not in factor XII, prekallikrein, or high molecular weight kininogen deficient plasmas, was stimulated by Zn^{2+}, suggesting a possible role for metal ions in promoting surface-dependent contact activation reactions under physiological conditions as well as in model systems.

Surface-dependent activation of factor XII is thought to be the initial event in the intrinsic blood coagulation cascade (Griffin & Cochrane, 1979). Factor XIIa activates factor XI and prekallikrein in reactions stimulated by surfaces and the protein cofactor HMW-kininogen[1] (Griffin & Cochrane, 1976a). Evidence has been presented that surfaces may participate in factor XII activation by inducing conformational changes in factor XII that enable it to act directly on its protein substrates (Kurachi et al., 1980; Heimark et al., 1980; Ratnoff & Saito, 1979a,b) and by enhancing the susceptibility of factor XII to proteolytic activation (Griffin, 1978; Rosing et al., 1985), as well as by serving as a site for assembly of the proteins involved in these reactions. Surface- and HMW-kininogen-dependent proteolytic activation of factor XII by

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[1] Abbreviations: HMW-kininogen, high molecular weight kininogen; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CK, chloromethyl ketone; PEG, poly(ethylene glycol); DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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kallikrein, and of prekallikrein by factor XIIa (reciprocal activation), is thought to amplify the rate of factor XII activation during contact activation (Cochrane et al., 1973; Griffin & Cochrane, 1976a; Meier et al., 1977). In addition to kallikrein, rabbit factor XIIa (Wiggins & Cochrane, 1979) and human (Silverberg et al., 1980) factor XIIa activate factor XII in surface-dependent reactions. Several surfaces that induce contact activation of plasma also promote autocatalytic activation of factor XII, including quartz, kaolin, ellagic acid, sulfatides, and dextran sulfate (Silverberg et al., 1980; Espana & Ratnoff, 1983; Tans et al., 1983). Kinetic studies of factor XII proteolytic activation induced by sulfatides have shown that the reaction can be quantitatively described by autocatalytic activation of factor XII by factor XIIa rather than by contaminating kallikrein (Tans et al., 1983). The kinetics of factor XII activation by kallikrein and factor XIIa have been compared in model systems as alternative routes of factor XIIa formation. With dextran sulfate or sulfatides as the activating surfaces, second-order rate constants for factor XII autoactivation are at least 2 orders of magnitude slower than those for factor XII activation by kallikrein (Tankersley & Finlayson, 1984; Rosing et al., 1985). Although the effect of HMW-kininogen on the rates of these reactions has not been clearly established, it has been suggested that kallikrein is the major activator of factor XII in normal plasma (Tankersley & Finlayson, 1984). Factor XII autoactivation, however, has been proposed to account for factor XII activation in prekallikrein-deficient plasma (Miller et al., 1980).

Several recent studies suggest that metal ions may influence contact activation reactions involving factor XII and HMW-kininogen, which could alter the relative rates of the surface-dependent reactions in which they participate. Our previous observation that insoluble complexes formed between ellagic acid and metal ions such as Zn^{2+} and Cu^{2+} were responsible for the activity of ellagic acid as an activating surface suggested a possible role for metal ions in surface interactions of contact activation (Bock et al., 1981). The specific binding of HMW-kininogen to stimulated platelets, which mediates factor XI binding (Greengard et al., 1986), requires zinc ions and is potentiated by Ca^{2+} at the concentrations of these ions found in normal plasma (Greengard & Griffin, 1984). The use of zinc chelate chromatography in factor XII purification (Weerasinghe et al., 1981, 1985; Pixley & Colman, 1986) indicates that this protein binds zinc, which could be related to the development and expression of its activity. Shimada et al., (1984) recently reported that zinc accelerated the sulfatide-dependent rate of factor XII activation by kallikrein but inhibited activation of prekallikrein by factor XIIa.

The present studies were undertaken to determine whether divalent metal ions, particularly zinc, played a role in surface-induced factor XII dependent contact activation reactions. The dextran sulfate induced autocatalytic activation of factor XII in solution was chosen as a model reaction for this investigation. We demonstrate that zinc enhances the rate of factor XII autoactivation up to 520-fold and that this effect is correlated with zinc promoting the binding of factors XII and α-XIIa to the dextran sulfate surface. The physiological relevance of these findings is suggested from the enhancement by zinc of the rate of factor XII dependent contact activation reactions in plasma which may involve factor XII autoactivation as a component.

Materials and Methods

Dextran sulfate (sodium salt, $M_r \sim 500\,000$) was purchased from Pharmacia, and dextran sulfate–agarose was from Pierce Chemical Company, Rockford, IL. Soybean trypsin inhibitor (SBTI)–agarose was prepared as described previously (Bock et al., 1985). Chelex 100 was from Bio-Rad Laboratories, Richmond, CA, the chromogenic substrate S-2302 was from AB Kabi Diagnostica, Stockholm, Sweden, and the irreversible serine protease inhibitor D-Phe-Phe-Arg-CK (FFRCK) was obtained from Calbiochem-Behring Corp., La Jolla, CA. Normal and deficient human plasmas were obtained from George King Biomedical, Overland Park, KS. Reagent-grade zinc chloride and copper chloride were from Sigma, while zinc sulfate and cobalt chloride were obtained from Baker. Ultrapure calcium chloride was obtained from Merck.

Purification and Characterization of Factor XII. Factor XII was purified from normal human plasma by published procedures (Griffin & Cochrane, 1976b) with some modifications: the initial plasma dialysis step was replaced by a partial purification step using poly(ethylene glycol) precipitation (Bock & Shore, 1983), and the second DEAE-Sephadex column was replaced with DEAE-Sepharose CL6B. Polybrene was omitted from the buffers in the final SP-Sephadex chromatography, which served to eliminate it from the final product. The purified protein was dialyzed against storage buffer consisting of 5 mM sodium acetate, pH 5.3, containing 0.15 M NaCl. For long-term storage, 1.0 mM EDTA, 10 mM benzamidine, and 0.02% NaN_3 were included and the solution was frozen at −70 °C. SDS gel electrophoresis on 10% gels in the Laemmli buffer system gave a single band (M_r ~80 000) under reducing conditions. Although specific clotting activities of the purified protein ranged between 43 and 110 units/mg, no differences were noted among preparations with respect to the kinetics of dextran sulfate induced autoactivation.

The extinction coefficient of factor XII was determined from measurements of the absorbance and refractive index of a solution of approximately 4 mg/mL in 6 mM sodium acetate buffer, pH 6.0, containing 0.5 mM EDTA and 0.15 M NaCl. Refractive index measurements were performed by using a C. N. Wood differential refractometer at 546 nm and 25 °C. Aliquots of the factor XII solution were diluted in 0.02 M Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl, for measurements of the absorbance at 280 nm. Assuming 0.189 ± 0.005 mL/g at 546 nm for the refractive index increment of representative proteins (Sober, 1970), an $E^{1\%}_{280}$ of 14.1 ± 0.6 was determined for factor XII. This was indistinguishable from the value reported for bovine factor XII by a related technique (Fujikawa et al., 1977) and somewhat lower than the value of 17 used by Tankersley and Finlayson (1984) for human factor XII. A molecular weight of 80 000 was used for calculations of the molar factor XII concentration.

Preparation of α-Factor XIIa. QAE-Sephadex chromatography has been previously shown to separate α- and β-XIIa generated by kallikrein proteolysis of factor XII (Silverberg et al., 1980). Essentially the same procedure was used to separate α-XIIa from β-XIIa and dextran sulfate after complete autoactivation under conditions where α-XIIa was the primary reaction product. One-tenth volume of 0.2 M Tris-HCl and 2 mM EDTA, pH 8.0, buffer was added to a 0.2 mg/mL solution of factor XII in storage buffer containing 1 mM EDTA to raise the pH, and autoactivation was initiated at room temperature by addition of dextran sulfate at a final concentration of 50 μg/mL. When autoactivation was complete, as measured by the increase in chromogenic substrate activity (~90 min), the reaction mixture was applied to a 2.5 × 5.0 cm column of QAE-Sephadex equilibrated at 4 °C with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, 1 mM EDTA, and 1 mM benzamidine. The column was washed

with 100 mL of equilibration buffer and eluted with a 300-mL linear NaCl gradient up to a final level of 0.8 M. Two incompletely resolved peaks of chromogenic substrate activity were observed. SDS–PAGE demonstrated that the first, and largest, peak corresponded to α-XIIa while the second was β-XIIa. The α-form was pooled from the ascending limb of the first peak, free of the β-form.

Preparation of α-Factor XIIa-CK. One-tenth volume of 0.2 M Tris-HCl, pH 8.0, was added to a 0.4 mg/mL solution of α-factor XIIa in storage buffer containing 1 mM EDTA, at room temperature, and then a 50-fold molar excess of FFRCK was added. The loss of chromogenic substrate activity was monitored over 3 h until less than 0.05% active enzyme was present. The inactivated α-factor XIIa was then exhaustively dialyzed against storage buffer to remove excess FFRCK.

Autoactivation Experiments. All reactions were carried out at 25 °C in polystyrene tubes that had been coated overnight with a 1% solution of PEG 20000 and dried with nitrogen to prevent surface adsorption of the proteins (Lottenberg et al., 1983). Before use, commercial dextran sulfate was dissolved in 5 mM EDTA and 0.02% NaN_3, pH 7.0, at 10 mg/mL, dialyzed twice against 2000 volumes of the same EDTA solution, exhaustively dialyzed against deionized water, and then lyophilized. Factor XII autoactivation reactions were carried out in a pH 7.4 kinetics buffer consisting of 0.1 M HEPES, 0.1 M NaCl, and 0.1% PEG 6000 (with 1 mM EDTA when appropriate). To remove trace metal ions from the kinetics buffers that did not contain EDTA, they were stirred for 1 h with 1 g/L Chelex and Millipore filtered (0.22 μm). To initiate autoactivation, 125 μL of 125 μg/mL dextran sulfate in kinetics buffer was added to a mixture at 25 °C of 500 μL of factor XII that had been either diluted >6-fold in kinetics buffer from a stock solution in storage buffer or dialyzed against kinetics buffer, plus 13 μL of water or appropriate concentrations of divalent metal salts in water. For experiments examining the effect of Zn^{2+} on the solubility of dextran sulfate solutions, 535 μL of a 29 μg/mL dextran sulfate solution plus or minus 5.8 μM $ZnSO_4$ in kinetics buffer was preincubated for varying times up to 24 h at 25 °C and centrifuged in a Brinkmann microfuge for 10 min at room temperature, and then, after reequilibration at 25 °C, 90 μL of 1.7 μM factor XII in storage buffer was added to initiate autoactivation. Autoactivation progress was monitored by the increase in the initial rates of chromogenic substrate hydrolysis. Aliquots of the reaction mixtures were added to polystyrene cuvettes containing 0.9 mL of 200 μM S-2302 in kinetics buffer containing 50 μg/mL Polybrene, which was demonstrated to stop the autoactivation reaction. Initial velocities were measured by linear regression analysis of absorbance changes at 405 nm with a Cary 17C spectrophotometer thermostated at 25 °C and interfaced to a North Star microcomputer from OLIS (On Line Instrument Systems, Jefferson, GA). Linear changes in absorbance were obtained when the reactions were followed for 5 min with a total change of 0.01–0.1 absorbance unit. A molar extinction coefficient of 13000 at 316 nm was used to calculate the S-2302 concentration.

Data Treatment. The initial total concentration of factor XII was determined from the absorbance at 280 nm. The concentration of factor XIIa formed at any time during the reaction was determined from the initial velocity of chromogenic substrate hydrolysis by equating the maximum activity generated with the total factor XII concentration. Initial velocities for factor XII autoactivation were obtained according to the method of Tankersley and Finlayson (1984), where the initial slope of a plot of ln [XIIa] vs. time yields the XIIa turnover velocity (V_0) (the initial exponential increase in XIIa concentration implies a constant XIIa turnover velocity) and the extrapolated intercept at time zero represents the initial factor XIIa concentration. Initial velocities were determined over less than 20% of the total reaction amplitude. The dependence of initial velocities on factor XII concentration was fit by nonlinear least-squares analysis to the Michaelis–Menten equation to determine the apparent K_m and k_{cat} for the factor XIIa catalyzed activation of factor XII. When initial velocities increased linearly with factor XII concentration, the apparent k_{cat}/K_m was determined either from the linear least-squares slope or by analysis of complete progress curves by the method of Tans et al. (1983).

Dextran Sulfate–Agarose Chromatography of Factors XII and XIIa. A column of dextran sulfate–agarose (1 × 15 cm) was equilibrated at 4 °C with 0.1 M HEPES buffer, 0.1 M NaCl, 0.1% PEG 6000, 10 mM benzamidine, pH 7.4, and either 1 mM EDTA or 5 μM $ZnSO_4$. One-milliliter samples of various factor XII species were dialyzed against 200 volumes of the equilibration buffer prior to chromatography. Samples of factor XII were preincubated with a 10-fold excess of FFRCK for 2 h at room temperature before dialysis to inactivate any traces of XIIa. The sample was applied to the column and washed with 1 mL of equilibration buffer, and then the column was eluted at a flow rate of 10 mL/h with a 200-mL linear NaCl gradient up to 2.0 M in the equilibration buffer. Fractions of 2 mL were collected. Factor XII activity was measured by coagulation assays using factor XII deficient plasma (Bock et al., 1981) and factor XIIa by the initial rates of chromogenic substrate hydrolysis. The linearity of the gradient was established by measuring conductivities of fractions at room temperature. Conductivities were converted to ionic strengths by using measurements of the conductance of buffers containing known NaCl concentrations. In the experiments with factor XIIa-CK, fractions containing the protein were identified by SDS–PAGE under reducing conditions with silver staining (Bio-Rad) according to the manufacturer's directions.

Plasma Experiments. Samples of normal and deficient human plasmas (0.5 mL) were dialyzed in Polybrene-rinsed dialysis tubing against 100 mL of 0.1 M HEPES and 0.1 M NaCl, pH 7.4, buffer containing 6 g of Chelex 100. Aliquots (20 μL) of the dialyzed plasmas were added in a polystyrene cuvette to the same buffer without Chelex, containing 0.1% PEG 6000 and 200 μM S-2302 in a final volume of 1.0 mL. For reactions containing zinc, 10 μM $ZnSO_4$ was added prior to addition of the plasma. The reactions were initiated by addition of dextran sulfate at a final concentration of 1 μg/mL, and the absorbance at 405 nm was monitored continuously.

Results and Discussion

Effect of Divalent Metal Cations on Factor XII Autoactivation. The effect of 5 μM zinc sulfate on dextran sulfate induced activation of factor XII is demonstrated in Figure 1. Zinc sulfate enhanced the rate of activation as evidenced by the significantly shorter lag period as well as completion of the reaction in one-seventh the time. The maximum level of chromogenic substrate activity generated was the same for reactions in the presence and absence of zinc sulfate. Addition of 1 mM EDTA abolished the effect of zinc sulfate on the reaction rate without affecting the reaction in the absence of zinc sulfate, indicating that the metal cation was responsible for the rate increase. Zinc sulfate and zinc chloride resulted in comparable stimulatory effects, confirming the independence

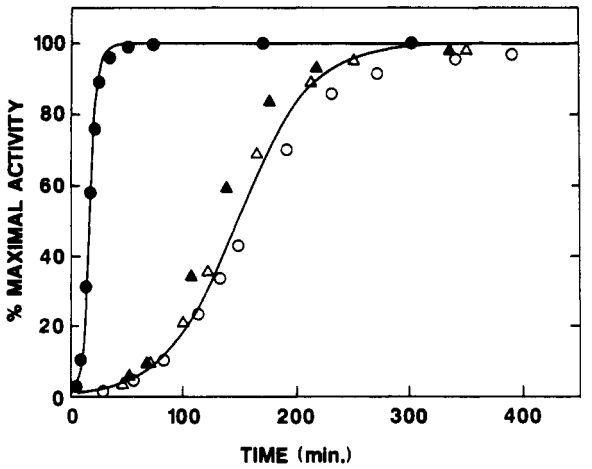

FIGURE 1: Effect of Zn^{2+} on factor XII autoactivation. Progress curves for factor XII autoactivation, measured as described under Materials and Methods, are shown for reaction mixtures that contained 0.20 μM factor XII (0.7% initial factor XIIa) and 25 μg/mL dextran sulfate (▲), plus 1 mM EDTA (△), 1 mM EDTA + 5 μM $ZnSO_4$ (○), or 5 μM $ZnSO_4$ (●) in μ = 0.15 kinetics buffer, pH 7.4 at 25 °C. The curves were calculated with kinetic parameters independently determined under the same conditions as described in the text and the equation published by Tans et al. (1983) for the data in the absence of Zn^{2+} or the iterative procedure of Tankersley and Finlayson (1984) for the reaction in the presence of Zn^{2+}.

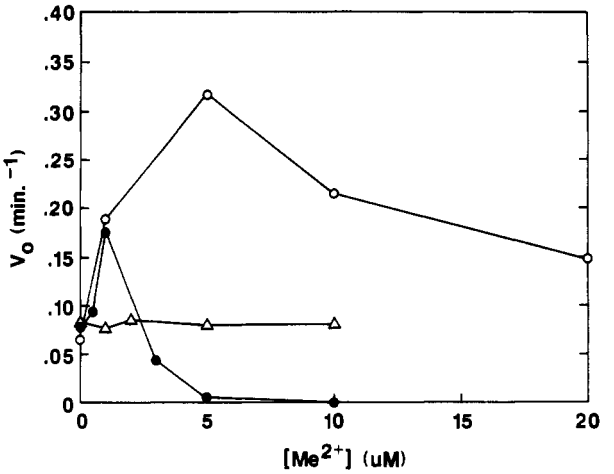

FIGURE 2: Dependence of rate of factor XII autoactivation on concentration of divalent cations. The initial turnover velocity (V_0) for autoactivation of 0.24 μM factor XII with 25 μg/mL dextran sulfate was determined as described under Materials and Methods as a function of the concentration of $ZnSO_4$ (○), $CuCl_2$ (●), or $CoCl_2$ (△) in μ = 0.15 kinetics buffer, pH 7.4 at 25 °C.

of the rate increase on the added anion. Activation of factor XII was completely dependent on the presence of dextran sulfate in both the absence and presence of zinc, with no detectable XIIa activity generated over 1 day when factor XII was incubated in kinetics buffer in the absence of dextran sulfate with or without 5 μM zinc sulfate.

Several control experiments demonstrated that the dextran sulfate induced activation of factor XII conformed to a true autoactivation process (Tans et al., 1983; Tankersley & Finlayson, 1984) in both the absence and presence of zinc. First, passage of factor XII through an SBTI–agarose column under conditions that bind kallikrein tightly (Bock et al., 1985), or inclusion of 1.5 μM SBTI in the reaction mixtures, had no effect on zinc-stimulated or unstimulated factor XII activation, indicating that activation was not due to traces of kallikrein. Second, analysis of the kinetics of factor XII activation as an autoactivation process provided second-order rate constants that were independent of the XIIa content of the preparation. Initial factor XIIa concentrations determined by extrapolation of the activation data to zero time (e.g., Figure 3A) were indistinguishable from those directly measured from the rate of chromogenic substrate hydrolysis, for all of the preparations studied (0.02–0.7% XIIa). Previously reported deviations of the initial portions of the progress curves from the behavior expected for an autocatalytic reaction (Tans et al., 1983) were not observed in these experiments. Finally, analysis of autoactivation reaction products by SDS–PAGE after full development of chromogenic substrate activity showed the complete loss of factor XII zymogen and the formation of α-XIIa as the primary product in both the presence and absence of zinc, while a slower conversion of α-XIIa to β-XIIa occurred over longer reaction times. This was consistent with the measured effect of zinc being on the rate of dextran sulfate mediated factor XII activation and not involving reactions that produce other enzymatically active forms of factor XIIa (Dunn et al., 1982). Centrifugation of dextran sulfate solutions, preincubated in the absence and presence of zinc for up to 24 h prior to initiation of factor XII autoactivation at the final concentrations employed in Figure 1, did not alter autoactivation progress curves, demonstrating that dextran sulfate was a soluble activator and that zinc did not promote autoactivation by forming insoluble aggregates as has been shown for ellagic acid (Bock et al., 1981 and unpublished data). These initial observations indicated that the effect of metal ions on the rate of generation of enzyme activity in the presence of dextran sulfate could be explained by metal ion dependent solution equilibria affecting the surface-induced autocatalytic activation of factor XII.

Other divalent cations were evaluated for their ability to enhance the rate of factor XII autoactivation. Figure 2 shows the dependence of the initial turnover velocity for the reaction on the concentration of zinc, copper, or cobalt in the presence of dextran sulfate. Zinc and copper showed optima at concentrations of approximately 5 and 1 μM, respectively, with inhibition of the rate of activation at higher concentrations of both cations. High concentrations of Cu^{2+} reduced the rate to values less than that obtained with dextran sulfate in the absence of metal ions. Cobalt had no measurable effect on autoactivation at concentrations up to 10 μM. Ultrapure calcium chloride at concentrations of 1–2 mM (physiological) also enhanced the initial rate of the reaction by 1.5–2.1-fold under the conditions of Figure 2 (not shown). Delineation of the metal ion specificity of these effects will require more extensive investigation. Because Zn^{2+} produced the largest rate increase and showed a relatively broad concentration optimum, it was chosen for further studies of the source of the metal ion enhancement of the autoactivation rate and its magnitude.

Effect of Zinc on Kinetic Parameters for Factor XII Autoactivation. Previous studies of surface-induced factor XII autoactivation (Silverberg et al., 1980; Tans et al., 1983; Tankersley & Finlayson, 1984; Griep et al., 1985) have led to the hypothesis that this reaction can be qualitatively described by the random formation of surface-bound enzyme–substrate (α-XIIa–XII) complexes that generate α-factor XIIa at the greatest rate. The nonspecific binding of enzyme (α-XIIa) and substrate (XII) to the surface and the undetectably slow rate of reaction in its absence, however, may result in complex dependencies of the initial reaction rate and full time course on the enzyme, substrate, and surface concentrations. The optimum in the rate observed as a function of Zn^{2+} or Cu^{2+} concentration and as a function of dextran sulfate concentration (unpublished results) indicated that a complex set of interdependent equilibria control the reaction rate in both

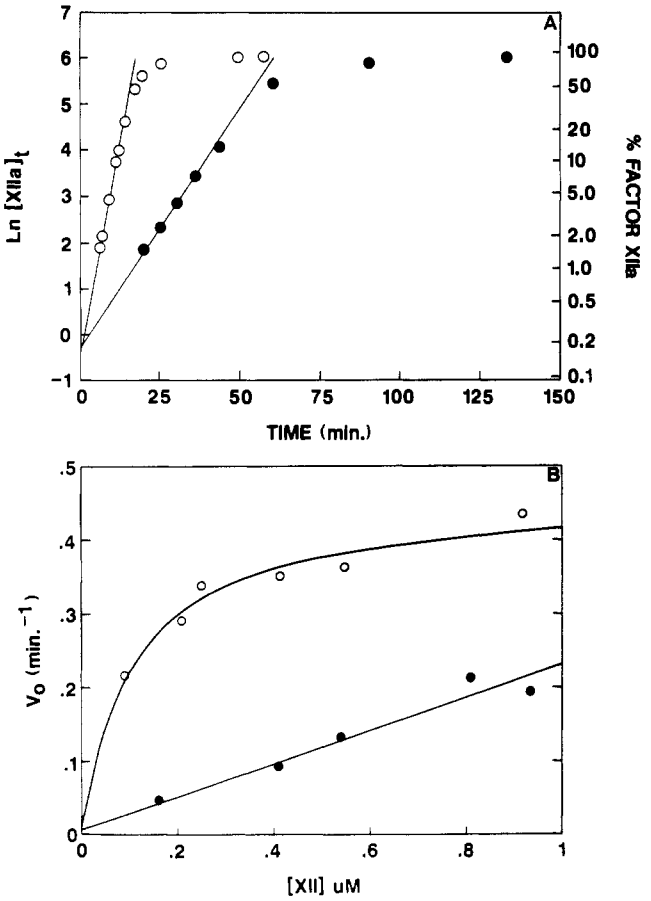

FIGURE 3: Kinetics of factor XII autoactivation at $\mu = 0.15$. (A) Typical plots of ln [factor XIIa] formed vs. time from which initial turnover velocities were obtained are shown for reactions containing 0.41 μM factor XII and 25 μg/mL dextran sulfate without added metal ions (●) and with 5 μM $ZnSO_4$ (○) in $\mu = 0.15$ kinetics buffer, pH 7.4 at 25 °C. (B) Dependence of the initial turnover velocity (V_0) on the total factor XII concentration, determined as illustrated in panel A, in the absence (●) and presence (○) of 5 μM $ZnSO_4$. The lines represent the linear and nonlinear least-squares fits calculated with the kinetic parameters listed in Table I.

the presence and absence of metal ions. In the presence of excess (inhibitory) concentrations of dextran sulfate, the initial rate of factor XII activation as a function of total factor XII concentration has been previously shown to be adequately described by the simplest model of enzyme-catalyzed autoactivation (Tankersley & Finlayson, 1984):

$$\alpha\text{-XIIa} + \text{XII} \underset{}{\overset{K_s \sim K_m}{\rightleftharpoons}} \alpha\text{-XIIa–XII} \xrightarrow{k_{cat}} \alpha\text{-XIIa} + \alpha\text{-XIIa}$$

Apparent kinetic parameters $K_{m,app}$ and $k_{cat,app}$ obtained by using this model are functions of the surface (Tans et al., 1983; Griep et al., 1985) and metal ion concentrations. In terms of this simple mechanism, the potentiating effect of zinc may be due primarily to enhancing the formation of productive α-XIIa–XII complexes, which would be reflected in $K_{m,app}$ for the reaction, and/or to enhancing the rate constants for α-XIIa cleavage of XII in these complexes, reflected in $k_{cat,app}$.

To gain insight into the mechanism by which metal ions enhance the rate of dextran sulfate induced factor XII autoactivation, the initial turnover rates of factor XII activation were measured as a function of total factor XII concentration by the method of Tankersley and Finlayson (1984) at a single concentration of dextran sulfate (in excess) in the presence and absence of the optimum zinc concentration. Typical data from which initial turnover velocities were obtained are shown in Figure 3A, while the dependence of the velocity on factor

Table I: Kinetic Parameters for Dextran Sulfate Induced Factor XII Autoactivation[a]

	$K_{m,app}$ (μM)	$k_{cat,app}$ (min^{-1})	$k_{cat,app}/K_{m,app}$ (μM^{-1} min^{-1})
$\mu = 0.15$			
1 mM EDTA			0.22 ± 0.04
5 μM $ZnSO_4$	0.11 ± 0.04	0.46 ± 0.05	4.2 ± 1.6
$\mu = 0.24$			
1 mM EDTA			0.0052 ± 0.0004
5 μM $ZnSO_4$	0.19 ± 0.04	0.52 ± 0.03	2.7 ± 0.6

[a] Kinetic parameters were determined by least-squares analysis of data in Figures 3B and 5 as described under Materials and Methods, with ±2 SE (95% confidence interval) given for the estimated error.

XII concentration in the presence and absence of Zn^{2+} is shown in Figure 3B. In the absence of zinc, the initial turnover rate varied linearly with factor XII concentration up to 0.9 μM, indicating that these factor XII concentrations were well below $K_{m,app}$. The slope of this linear plot, 0.22 μM^{-1} min^{-1}, therefore represented the apparent bimolecular rate constant for activation, $k_{cat,app}/K_{m,app}$. Since factor XII concentrations were well below $K_{m,app}$, analysis of complete progress curves by the method of Tans et al. (1983) provided an independent determination of $k_{cat,app}/K_{m,app}$, which yielded an indistinguishable value of 0.24 μM^{-1} min^{-1}. This behavior was qualitatively consistent with published values of 0.26 μM^{-1} min^{-1} for $k_{cat,app}/K_{m,app}$ and 7.5 μM for $K_{m,app}$ for this reaction, determined at lower ionic strength and higher temperature (Tankersley & Finlayson, 1984). In the presence of optimum Zn^{2+} concentrations (5 μM), initial velocities were enhanced over the same range of factor XII concentrations and exhibited saturation behavior (Figure 3B), indicating a substantial decrease in $K_{m,app}$ for the zinc-potentiated reaction. A nonlinear least-squares fit of this data to the Michaelis–Menten equation provided a $K_{m,app}$ of 0.11 μM and a $k_{cat,app}$ of 0.46 min^{-1}, indicating a 19-fold increase in $k_{cat,app}/K_{m,app}$ for the zinc-enhanced reaction under these conditions (Table I). Failure to observe evidence for saturation of the reaction rate in the absence of Zn^{2+} at measured velocities reaching approximately half the maximum achievable with zinc suggested that $k_{cat,app}$ may be decreased by Zn^{2+} and thus may not contribute to the rate increase under these conditions. The simplest interpretation of these results was that Zn^{2+} enhanced the rate of autoactivation by promoting the formation of productive α-XIIa–XII complexes.

Dependence of Zn^{2+}-Stimulated Autoactivation on Ionic Strength. The likely contribution of ionic protein–dextran sulfate interactions to $K_{m,app}$ for the reaction prompted us to investigate the effect of the ionic strength on the reaction rate and the rate enhancement produced by Zn^{2+}. Measurements of the initial turnover velocity in the presence and absence of 5 μM Zn^{2+} as a function of ionic strength between $\mu = 0.15$ and 0.30 suggested a differential effect (Figure 4). The reaction in the absence of Zn^{2+} was extremely sensitive to ionic strength between $\mu = 0.15$ and 0.24, while much smaller effects were observed over the same range in the presence of 5 μM Zn^{2+}. Between $\mu = 0.24$ and 0.30, both zinc-stimulated and unstimulated reactions decreased in rate with increasing ionic strength, with no detectable reaction observed over 1 day at $\mu = 0.30$ in the absence of zinc. To examine this effect further, the kinetics of the reaction were studied at an ionic strength of 0.24 (the highest ionic strength at which both reactions could be readily measured) and compared to the results previously obtained at the same Zn^{2+} and dextran sulfate concentrations at $\mu = 0.15$. Again, in the absence of zinc, a linear dependence of the initial autoactivation rate was observed (Figure 5) providing a $k_{cat,app}/K_{m,app}$ of 0.0052 μM^{-1} min^{-1},

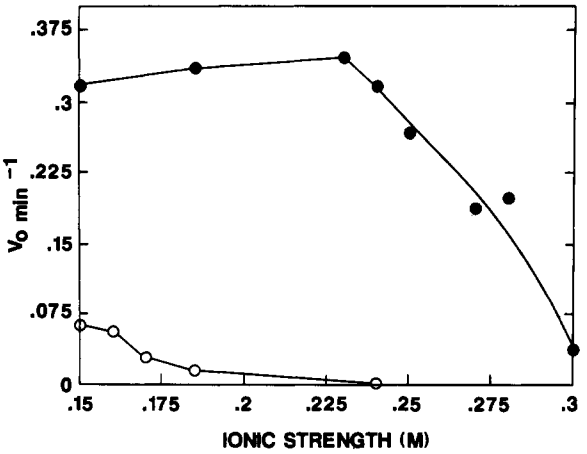

FIGURE 4: Effect of Zn^{2+} on the ionic strength dependence of the rate of factor XII autoactivation. The initial turnover velocity (V_0) of autoactivation was measured as a function of ionic strength. Reactions contained 0.24 μM factor XII and 25 μg/mL dextran sulfate in pH 7.4 kinetics buffer with NaCl added to vary the ionic strength, and either 5 μM $ZnSO_4$ (●) or 1 mM EDTA (○).

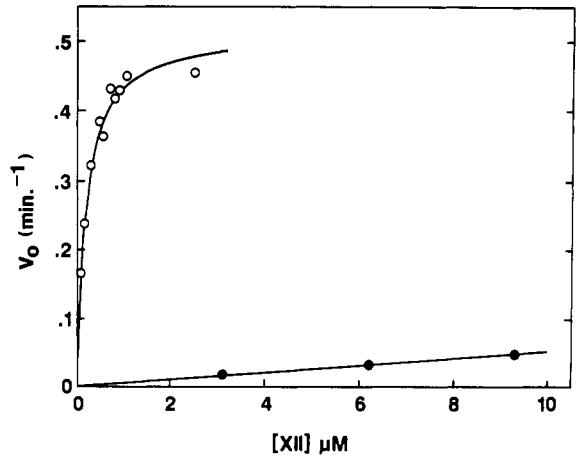

FIGURE 5: Kinetics of factor XII autoactivation at μ = 0.24. The initial turnover velocity (V_0) as a function of the total factor XII concentration was determined as described under Materials and Methods for reactions in μ = 0.24 kinetics buffer, pH 7.4, containing 25 μg/mL dextran sulfate without added metal ions (●) and with 5 μM $ZnSO_4$ (○). The lines represent the linear and nonlinear least-squares fits calculated with the kinetic parameters listed in Table I.

which represented a 42-fold decrease from the value at μ = 0.15. Zinc enhanced the rates of autoactivation to a much greater extent at this ionic strength, and they similarly approached a limiting velocity as the factor XII concentration was increased. From the hyperbolic fit of these data, a 1.7-fold greater $K_{m,app}$ of 0.19 μM and an essentially unchanged $k_{cat,app}$ value of 0.52 min^{-1}, relative to values determined at the lower ionic strength for the Zn^{2+}-stimulated reaction, were found (Table I). These parameters resulted in only a 1.6-fold decrease in $k_{cat,app}/K_{m,app}$ in the presence of zinc from that found at μ = 0.15, in sharp contrast to the 42-fold decrease in this parameter found in the absence of zinc. Because of this differential effect, the enhancement in $k_{cat,app}/K_{m,app}$ brought about by Zn^{2+} was a function of ionic strength. The enhancement increased greatly from 19-fold at μ = 0.15 to 520-fold at μ = 0.24 (Table I).

Effect of Zinc on the Affinity of Factors XII and XIIa for Dextran Sulfate. The large effect of zinc on $K_{m,app}$ for autoactivation and the differential effects of ionic strength suggested that increased binding of factors XII and α-XIIa to dextran sulfate in the presence of Zn^{2+} could be substantially

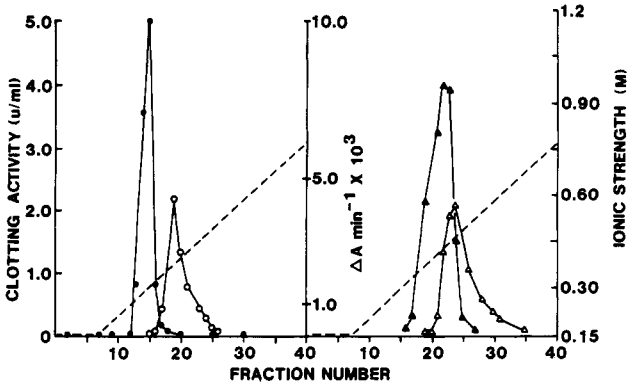

FIGURE 6: Chromatography of factors XII and α-XIIa on dextran sulfate–Sepharose. (Left panel) Factor XII (140 μg) was chromatographed on a column of dextran sulfate–Sepharose equilibrated with buffers containing 1 mM EDTA (●) or 5 μM $ZnSO_4$ (○) as described under Materials and Methods. The factor XII clotting activity measured in the fractions collected on elution with a linear NaCl gradient is shown. Ionic strengths of fractions shown by the dashed line were determined from measured conductivities. (Right panel) Elution of α-factor XIIa (40 μg) chromatographed under identical conditions in buffers containing 1 mM EDTA (▲) or 5 μM $ZnSO_4$ (△) was monitored by the chromogenic substrate activity in the fractions.

responsible for the increase in reaction rate. To investigate this possibility, the NaCl concentration required to elute the proteins from a dextran sulfate–agarose column was determined in the presence and absence of 5 μM Zn^{2+}. Autoactivation of factor XII during chromatography was prevented by pretreating it with FFRCK, running the column in the presence of 10 mM benzamidine at 4 °C, and completing the chromatography within 5 h. The elution profiles, obtained from independent chromatographic experiments under identical conditions, are shown in Figure 6 and demonstrate that factors XII and α-XIIa were eluted at a higher NaCl concentration in the presence of Zn^{2+}. The peaks of factor XII activity (average ± SD from at least two experiments) eluted at ionic strengths of 0.35 ± 0.01 M in the absence of Zn^{2+} and 0.43 ± 0.04 M in the presence of Zn^{2+}, while the values for α-factor XIIa were 0.43 ± 0.04 ($-Zn^{2+}$) and 0.51 ± 0.04 M ($+Zn^{2+}$). SDS–PAGE of the proteins eluted in these experiments showed that no detectable activation of factor XII or conversion of α-XIIa to β-XIIa had occurred during chromatography. Chromatography of β-factor XIIa resulted in its elution within a single column volume of starting buffer in either the absence or the presence of Zn^{2+}, indicating that an ionic interaction between immobilized dextran sulfate and the heavy-chain portion of the factor XII/α-XIIa molecule was responsible for the observed binding (Revak & Cochrane, 1976; Griep et al., 1985). These results were consistent with the conclusion that Zn^{2+}-promoted binding of factors XII and α-XIIa to dextran sulfate contributed significantly to the metal ion rate enhancement. The smaller relative increase in ionic strength required to elute α-factor XIIa as compared to that for factor XII in the presence of zinc suggested that zinc promoted factor XII binding to a greater extent than it did α-factor XIIa binding. Whether this increased affinity of factors XII and α-XIIa for dextran sulfate can account completely for the rate enhancement obtained with Zn^{2+} will require further investigation.

Unexpectedly, α-factor XIIa was eluted at higher ionic strengths than those for factor XII in both the presence and absence of Zn^{2+}, indicating that activation increased the affinity for dextran sulfate. To confirm this observation, mixtures of factors XII and α-XIIa-CK were chromatographed under the same conditions used in the previous experiments

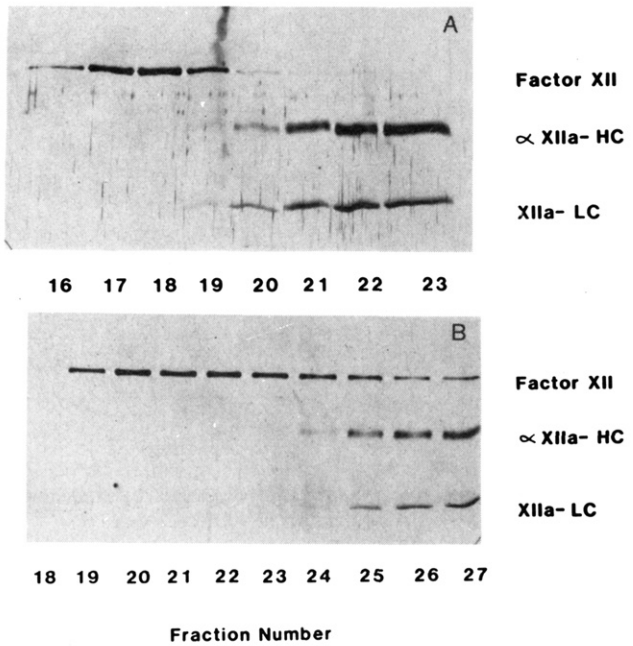

FIGURE 7: Chromatography of mixtures of factors XII and α-XIIa-CK on dextran sulfate–Sepharose. Mixtures containing equal amounts (300 μg) of factor XII and α-factor XIIa-CK were chromatographed on dextran sulfate–Sepharose and eluted with NaCl gradients as described under Materials and Methods. Equal-volume aliquots of the gradient fractions from experiments in the presence of 1 mM EDTA (A) or 5 μM $ZnSO_4$ (B) were subjected to 10% SDS–PAGE under reducing conditions, and the gels were silver stained. Peak fractions in these experiments, judged by the intensity of the stained bands, corresponded to ionic strengths given in the text as determined by conductivity measurements.

in the presence and absence of Zn^{2+} and their elution was detected by SDS–PAGE under reducing conditions. Factors XII and α-XIIa-CK in these mixtures eluted at ionic strengths either indistinguishable from or slightly higher than, respectively, those found when the proteins were chromatographed separately: XII + EDTA, 0.35 M; XII + Zn^{2+}, 0.43 M; α-XIIa-CK + EDTA, 0.47 M; α-XIIa-CK + Zn^{2+}, 0.56 M (Figure 7). The discrepancy between ionic strengths for elution of α-XIIa and α-XIIa-CK, although just outside the estimated experimental error, may be due to reaction of the enzyme with the peptide chloromethyl ketone. These results, nevertheless, confirmed that α-factor XIIa bound to dextran sulfate with a greater affinity than factor XII in both the absence and presence of Zn^{2+}. This apparently tighter binding of α-factor XIIa will affect the kinetics of factor XIIa surface-dependent reactions. Tighter binding of the product of autoactivation may inhibit this reaction under conditions where factors XII and α-XIIa compete for limited surface binding sites. Preferential surface binding of α-XIIa may also promote the formation of productive surface-bound complexes with factor XII and other factor XIIa substrates, such as prekallikrein and factor XI.

Zinc Effects on Contact Activation in Plasma. To determine whether zinc increased the overall rate of contact activation in normal human plasma, we evaluated the effect of Zn^{2+} on the rate of chromogenic substrate (S-2302) hydrolysis induced by dextran sulfate in diluted plasma that had been dialyzed against buffers containing Chelex. Evidence has been presented indicating that the increased hydrolysis of a similar chromogenic substrate observed when plasma is exposed to dextran sulfate is due primarily to kallikrein generation (Kluft, 1978; van der Graaf et al., 1982). Assuming relative concentrations of factors XIIa and XIa and kallikrein generated during contact activation equal to the relative plasma levels

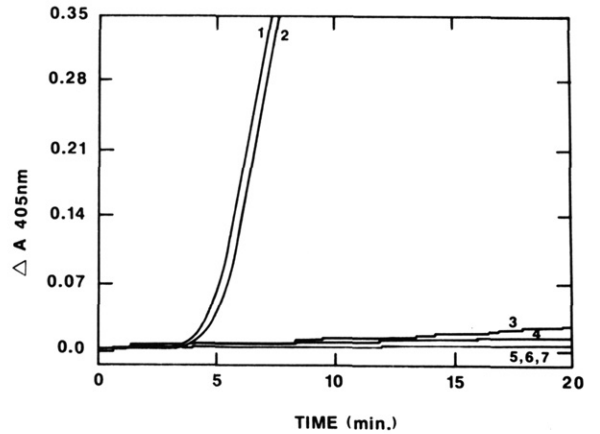

FIGURE 8: Effect of Zn^{2+} on dextran sulfate induced contact activation in plasma. The change in absorbance at 405 nm with time, due to hydrolysis of S-2302, is shown for 1-mL reaction mixtures in $\mu = 0.15$ kinetics buffer, pH 7.4 at 25 °C, that contained 1 μg/mL dextran sulfate and 20 μL of either factor XI deficient (1) or normal plasma (2) with 10 μM $ZnSO_4$, factor XI deficient (3) or normal (4) plasma with 1 mM EDTA + 10 μM $ZnSO_4$, and factor XII deficient (5), HMW-kininogen deficient (Fitzgerald) (6), or prekallikrein deficient (7) plasma with 10 μM $ZnSO_4$.

of their zymogens and using the K_m and k_{cat} values determined for these enzymes (unpublished results), kallikrein was calculated to account for about 95% of the observed S-2302 hydrolysis rate. The results shown in Figure 8 demonstrated that addition of dextran sulfate to normal and factor XI deficient plasmas in the presence of 10 μM Zn^{2+} resulted in generation of substantial kallikrein activity within 5–7 min (curves 1 and 2), while in the presence of EDTA, negligible generation of activity occurred over 20 min (curves 3 and 4). Plasma deficient in factor XII, prekallikrein, and HMW-kininogen, similarly dialyzed against buffers containing Chelex, showed no significant dextran sulfate stimulated generation of kallikrein activity over 20 min in the presence of 10 μM Zn^{2+} (curves 5–7). These experiments demonstrated an enhancement of the rate of dextran sulfate induced generation of enzyme activity in plasma at Zn^{2+} concentrations near the physiological concentration, which was dependent on the presence of the proteins of the contact system. Whether Zn^{2+}-stimulated factor XII autoactivation occurred in these plasma experiments could not be determined because of the relatively small contribution of factor XIIa to the observed S-2302 hydrolysis rate.

Factor XII autoactivation represents a model for other surface-dependent factor XII/XIIa reactions as well as a possible component reaction of contact activation in normal plasma. Our results indicate that metal ions may play a physiologically significant role in factor XII dependent contact activation reactions. Both Zn^{2+} and Cu^{2+} are present in plasma at concentrations between 10 and 25 μM (Woo & Cannon, 1984), levels that we have shown are sufficient to support their maximum effect on the rate of factor XII autoactivation. While most of the Zn^{2+} and Cu^{2+} ions are bound to plasma proteins (Whitehouse et al., 1983; Prasad & Oberleas, 1970; Woo & Cannon, 1984), factor XII and/or the physiological surface may effectively compete for the available Zn^{2+} or Cu^{2+} ions. Zinc enhancement of the rate of dextran sulfate induced contact activation reactions in plasma could thus involve enhanced factor XII autoactivation, but zinc may also affect other surface-dependent reactions that participate in contact activation. It has been demonstrated that zinc promotes the specific binding of HMW-kininogen to the activated platelet surface (Greengard & Griffin, 1984), and it may also affect binding of HMW-kininogen to less specific

surfaces. HMW-kininogen is clearly required for the effect of Zn^{2+} on dextran sulfate induced contact activation in plasma (Figure 8). However, studies of the effect of this cofactor protein on contact activation reactions in model systems with dextran sulfate or sulfatides as the surface have yielded only relatively small or inhibitory effects on the reaction rates (Rosing et al., 1985; Kurachi et al., 1980; Shimada et al., 1984). HMW-kininogen is known to form tight complexes with prekallikrein and factor XI and is thought to mediate the binding of these proenzymes to surfaces (Wiggins et al., 1977; Mandle et al., 1976; Thompson et al., 1977). Zinc may therefore play a role in prekallikrein and factor XI activation as well as factor XII activation by promoting the binding of prekallikrein, factor XI, or kallikrein, respectively, to surfaces through complexes with HMW-kininogen. Such a role for Zn^{2+} has been suggested for HMW-kininogen-mediated binding of factor XI to stimulated platelets (Greengard et al., 1986). Zinc may additionally enhance the rate of factor XIIa activation of prekallikrein and factor XI by promoting factor XIIa binding to a surface, as we have shown. The reported Zn^{2+} enhancement of sulfatide-dependent factor XII activation by kallikrein in the absence or presence of HMW-kininogen (Shimada et al., 1984) may also involve Zn^{2+} promoting factor XII–surface interactions. The combined effects of metal ions on factor XII and HMW-kininogen surface binding equilibria, studied under conditions where these interactions contribute significantly to the measured rate, may result in very large relative rate enhancements, comparable to those obtained for calcium and protein cofactors in other surface-dependent blood coagulation reactions (Jackson & Nemerson, 1980). These aspects of the effects of metal ions on contact activation reactions are currently being investigated in our laboratory.

From kinetic studies at a lower ionic strength than that used in our studies and in the absence of divalent cations, it was previously concluded that autoactivation induced by dextran sulfate was very much slower than activation of factor XII by kallikrein (Tankersley & Finlayson, 1984). It is possible that the divalent cation effect on autoactivation could increase the relative importance of this mechanism of factor XII activation, especially if this effect was selective for factors XII and α-XIIa as compared to kallikrein. In addition, the effect of HMW-kininogen on the reaction rates and therefore the preferred pathway of factor XII activation may be influenced by metal ions. The absolute magnitudes of zinc-enhanced bimolecular rate constants for dextran sulfate dependent factor XII autoactivation found in this study were about 20-fold greater than those previously measured at a lower ionic strength (Tankersley & Finlayson, 1984). Whether zinc enhances the rate constants for factor XII autoactivation induced by sulfatides, which are about an order of magnitude greater than those obtained with dextran sulfate (Tans et al., 1983; Griep et al., 1985), remains to be determined. The largest rate enhancement for autoactivation obtained in the model system of dextran sulfate and Zn^{2+} was observed at ionic strengths that are higher than that of normal plasma. However, the metal ion or ions that may affect this reaction and other contact activation reactions in plasma are not yet fully established, nor is the surface that serves to promote and localize these reactions in vivo. The properties of this surface, the presence of proteins that compete for surface and metal ion binding, and the concentrations of all the components in relation to the dissociation constants for these interactions will all contribute to the magnitude of the effect of divalent metal ions on these reactions. Evaluation of the magnitude of the effect of metal ions in model systems only at physiological ionic strength may therefore not be representative of their effect in plasma. In conclusion, our results suggest that metal ions play a role in the assembly of contact factors on surfaces and thereby affect the rates of surface-dependent contact activation reactions in both model systems and plasma. In this regard, it is of interest that our previous studies found that ellagic acid–divalent metal ion complexes were highly active in supporting contact activation reactions, with Zn^{2+} and Cu^{2+} complexes being the most effective (Bock et al., 1981).

Registry No. Zn, 7440-66-6; Cu, 7440-50-8; Ca, 7440-70-2; factor XII, 9001-30-3; factor α-XIIa, 77107-44-9; dextran sulfate, 9042-14-2.

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Lignin Peroxidase: Resonance Raman Spectral Evidence for Compound II and for a Temperature-Dependent Coordination-State Equilibrium in the Ferric Enzyme†

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ABSTRACT: Resonance Raman (RR) spectroscopy of lignin peroxidase (ligninase, diarylpropane oxygenase) from the basidiomycete *Phanerochaete chrysosporium* suggests two different coordination states for the native ferric enzyme. Evidence for a high-spin, hexacoordinate ferric protoporphyrin IX was presented by Andersson et al. [Andersson, L. A., Renganathan, V., Chiu, A. A., Loehr, T. M., & Gold, M. H. (1985) *J. Biol. Chem. 260*, 6080–6087], whereas Kuila et al. [Kuila, D., Tien, M., Fee, J. A., & Ondrias, M. R. (1985) *Biochemistry 24*, 3394–3397] proposed a high-spin, pentacoordinate ferric system. Because the two RR spectral studies were performed at different temperatures, we explored the possibility that lignin peroxidase might exhibit temperature-dependent coordination-state equilibria. Resonance Raman results presented herein indicate that this hypothesis is indeed correct. At or near 25 °C, the ferric iron of lignin peroxidase is predominantly high spin, pentacoordinate; however, at ≤2 °C, the high-spin, hexacoordinate state dominates, as indicated by the frequencies of well-documented spin- and coordination-state marker bands for iron protoporphyrin IX. The temperature-dependent behavior of lignin peroxidase is thus similar to that of cytochrome *c* peroxidase (CCP). Furthermore, lignin peroxidase, like horseradish peroxidase (HRP) and CCP, clearly has a vacant coordination site trans to the native fifth ligand at ambient temperature. High-frequency RR spectra of compound II of lignin peroxidase are also presented. The observed shifts to higher frequency for both the oxidation-state marker band ν_4 and the spin- and coordination-state marker band ν_{10} are similar to those reported for the compound II forms of HRP and lactoperoxidase and for ferryl myoglobin. These observations are consistent with a low-spin, hexacoordinate Fe(IV)=O structure for lignin peroxidase compound II.

Lignin is a complex, optically inactive, and random polymer that comprises 20–30% of woody plant tissue (Sarkanen, 1971; Crawford, 1981). Under secondary metabolic conditions the white rot fungus *Phanerochaete chrysosporium* produces at least two heme peroxidases (Gold et al., 1984; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985; Paszczynski et al., 1986) and efficiently degrades lignin to CO_2 and H_2O (Kirk et al., 1978; Gold et al., 1982). Lignin peroxidase (LiP,[1] ligninase, diarylpropane oxygenase) has been purified to homogeneity and shown to occur in multiple chromatographic forms (Gold et al., 1984; Renganathan et al., 1985; Kirk et al., 1986; Leisola et al., 1985), all of which contain a single iron protoporphyrin IX prosthetic group (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). Electronic absorption spectroscopy (Gold et al., 1984; Renganathan et al., 1985), EPR spectroscopy (Andersson et al., 1985), and

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[1] Abbreviations: LiP, lignin peroxidase; RR, resonance Raman; CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; Mb, myoglobin; metMb, metmyoglobin; Hb, hemoglobin; dp, depolarized; p, polarized; ap, anomalously polarized; Me_2SO, dimethyl sulfoxide; LPO, lactoperoxidase; EPR, electron paramagnetic resonance; PP, protoporphyrin IX.

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