Human Factor XII (Hageman Factor) Autoactivation by Dextran Sulfate

CIRCULAR DICHRORISIM, FLUORESCENCE, AND ULTRAVIOLET DIFFERENCE SPECTROSCOPIC STUDIES

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The first event leading to the activation of the plasma kallikrein-kinin system is the surface-dependent conversion of factor XII to an active enzyme. Factor XII autoactivation was investigated using dextran sulfate as a soluble activating surface, and the significance of aggregation and the nature of the conformational change were examined by ultraviolet difference spectroscopy, fluorescence and circular dichroism. Results indicate that DS500 (500-kDa dextran sulfate) induces aggregation of factor XII. Analysis of the binding data suggests that 165–192 factor XII molecules can bind to one DS500 chain, while a 1:1 stoichiometry is observed with 5-kDa dextran sulfate. The interaction of factor XII and dextran sulfate is a biphasic process. It is initiated by a fast contraction of the molecule upon binding, as revealed by an apparent increase in organized secondary structures, and then followed by a slow relaxation process during cleavage and subsequent activation. Overall, the results are consistent with a model in which factor XII undergoes conformational changes upon binding to the activating surface. The rapidity of autoactivation in the presence of DS500, as opposed to 5-kDa dextran sulfate, implies that aggregation provides a special mechanism whereby proteolytic cleavage is accomplished efficiently when factor XII molecules are bound side by side on the DS500 molecule.

Human blood coagulation factor XII (Hageman factor) is a single chain 80-kDa plasma glycoprotein. Concomitant with cleavage of a single peptide bond, factor XII is converted to a serine protease, factor XIIa, which initiates the intrinsic pathway of blood coagulation. The enzyme plays a role in the activation of the fibrinolytic system, in the production of kinins, in the initiation of cell-mediated inflammatory responses, and in the activation of the classical complement pathway (1–6). In vitro activation of factor XII occurs when the zymogen becomes bound to negatively charged "surfaces" such as kaolin, glass, dextran sulfate, and sulfatides (7–10). In vivo, contact of plasma with anionic components of the subendothelial basement membrane or cell surface may be responsible for the activation of factor XII (11, 12) but the specific component(s) have not been identified. Surface-bound factor XII is activated to factor XIIa and acquires enzymatic activity toward its protein substrates, prekallikrein and factor XI (5, 13), which are complexed in vivo, with the contact activation procofactor high molecular weight kinogen (14). Plasma kallikrein produced by the activation of prekallikrein by factor XIIa, in turn, cleaves more factor XII to factor XIIa. This reciprocal activation accounts for the rapid and amplified activation of the intrinsic pathway. To render factor XII susceptible to proteolytic cleavage by autoactivation or by enzyme activation, it has been hypothesized that a conformational change of the zymogen upon binding to a negatively charged activating substance must occur (15). Other investigators have also proposed a model in which a conformational change occurs upon activation (16–19). Earlier studies also indicated that autoactivation of factor XII in the presence of an insoluble surface, ellagic acid, was accompanied by aggregation (16). Although experimental verification of the conformational change has been reported, the exact nature of this change was not clearly evaluated, and the relevance of aggregation to autoactivation has not been investigated. In this report a comparative study of DS500 and DS5 was carried out since, unlike DS500, DS5 is a weak surface activator and does not cause aggregation of factor XII. Ultraviolet difference spectroscopy, fluorescence, and native electrophoretic analysis were used to determine the binding parameters of factor XII-DS500 and factor XII-DS5 interactions. By analysis of the spectral perturbations of aromatic amino acid residues, these techniques also provided new information on the nature of the surface binding site and the aggregation site in factor XII. The circular dichroism studies have identified some of the detailed features of the conformational change that accompanies the surface activation of factor XII.

EXPERIMENTAL PROCEDURES

Materials

The chromogenic substrate, S-2302 (H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride) was purchased from Helena Laboratories (Beaumont, TX). H-D-Phe-Phe-Arg-CMK was obtained from Calbiochem-Novabiochem Corporation, La Jolla, California.

1 The abbreviations used are: DS500, dextran sulfate, M, = 500,000; DS5, dextran sulfate, M, = 5,000; ΔM, molar difference absorption; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; S-2302, H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride; H-D-Phe-Phe-Arg-CMK, H-D-phenylalanyl-L-phenylalanyl-L-arginyl chloromethylketone.
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from Calbiochem Behring Corp. Dextran sulfates (DS9 and DS500), hexadimethrine bromide (polybrene), and the siliconizing agent Sigmacote were purchased from Sigma. Buffer strips and separation media used in PhastSystem Electrophoresis Unit were purchased from Pharmacia. Light scattering correction software (Interactive Microware, Inc., State College, PA) was employed. 1) Using a "LOGGEN" light scattering correction software alone.

Methods

Amidolytic Assay—The factor XII samples to be assayed were incubated at 25 °C in the buffer used in spectroscopic studies (50 mM NaCl, 1 mM sodium phosphate, pH 7.5), and amidolytic activity was measured using the chromogenic substrate, S-2302. Ninety μl of assay buffer (140 mM NaCl; 50 mM Tris-HCl, pH 7.8, 1 mM EDTA) containing a final substrate concentration of 0.7 mM was pipetted to a siliconized ultramicro cell. Ten μl of the sample to be assayed after necessary incubation was then added to the cuvette, and the absorbance change at 405 nm was recorded immediately using a 14-DS UV Visible spectrophotometer (AVIV Associates) to determine the initial velocity of the reaction.

Gel Electrophoresis—The "band shift" assay to demonstrate factor XII binding to DS500 was done under native conditions using a precast 12.5% homogeneous polyacrylamide gel (4.5% stacking gel) on the PhastSystem Electrophoresis Unit (Pharmacia). In these experiments, the samples are applied as in the PhastSystem Guide (Technique File No. 110) on a precast 10–15% gradient polyacrylamide gel. The gels were silver stained using the automated development unit (Separation Technique File No. 210). Densitometric analysis was performed using "IMAGE-PRO PLUS" image processing system software (Media Cybernetics, Silver Spring, MD).

Ultraviolet Difference Spectroscopy—All spectroscopic measurements were carried out on acid-cleaned (H3SO4-HN03) quartz cuvettes which have been coated with either polybrene (2 mg/ml) or Sigmacote. The UV difference spectra were recorded on a 14 DS UV Visible spectrophotometer using ultramicro cells (Hellma Cells). In order to investigate the extent of light scattering contribution to the UV difference spectrum of factor XII-DS500, three methods were employed: 1) Using a "LOGGEN" light scattering correction software (AVIV Associates): this program automatically subtracts the light scattering contribution based on the slope of the spectrum between 320 and 350 nm. 2) Using a modified 1-mm pathlength jacketed cylindrical fluorocell (21, 22) where the sample is surrounded by a solution of 0.1 M sodium salicylate. The difference between the spectrum taken in the fluorocell and standard cell is a direct measure of differential light scattering. 3) By comparing the UV difference spectra of factor XII-DS500 and factor XII-DS500, it is found to induce light scattering on factor XII while DS500 does not.

Circular Dichroism—The CD measurements were conducted on a Jasco J-500C Spectropolarimeter, and the CD difference spectra were obtained using the ADALAB-PC hardware and "ADAPT" computer software (Interactive Microwave, Inc., State College, PA). A solvent blank containing dextran sulfate (25 μg/ml), which gave a negligible CD (<5%) in the spectral region of interest, was subtracted from the CD spectra of the factor XII-DS500 complex. Sodium chloride was replaced by sodium fluoride in the solvent in order to improve the sensitivity and signal to noise ratio at lower wavelengths. The use of sodium fluoride did not affect the measured functional activities of factor XIIa. The mean residue molecular weight (Mr) of 106 (19) for factor XII is used in the ellipticity calculations.

In evaluating the contribution of light scattering to the CD spectrum of factor XII-DS500, the first method used involves varying the distance of the sample cell to the photomultiplier detector. Differential light scattering is indicated if the CD spectrum at 340 nm is a function of the distance from the detector. In addition, any deviation from the base line at 340 nm would indicate light scattering because proteins and polysaccharides do not have CD bands at this wavelength. The second method involves comparing the CD spectra of factor XII-DS500 at 340 nm with factor XII-DS500 and factor XII alone.

Fluorescence Spectroscopy—A Perkin-Elmer Cetus LS-5B luminescence spectrofluorometer is used to measure the protein intrinsic fluorescence. All measurements are performed at 25 °C in a 3-mm pathlength quartz microcuvette. The excitation wavelength is set at 280 nm, and the emission spectra are scanned from 300 to 500 nm. In all measurements, 200 μl/ml of factor XII is used in the presence of 50 mM NaCl, 1 mM phosphate, pH 7.5. The sensitivity scale is adjusted for an initial fluorescence above 80% full scale.

Analysis of Binding Parameters—In the derivation below, the following terms are used: [P], initial factor XII concentration; α, fraction of free [P]; (1–α), fraction of bound [P]; [D], initial DS500 concentration; β, fraction of free binding sites on DS500; (1–β), fraction of bound binding sites on DS500; n, number of binding sites/DS500 molecule, and Kd, dissociation constant.

\[
\alpha[P] + \beta[D] \rightarrow (1 - \alpha)[P] \tag{1}
\]

where

\[
(1 - \alpha)[P] = (1 - \beta)n[D] \tag{2}
\]

From Equation 1,

\[
\beta = 1 - \frac{(1 - \alpha)[P]}{n[D]} \tag{3}
\]

Substituting Equation 3 in Equation 2,

\[
K_d = \frac{\alpha}{1 - \alpha} - \frac{n[D]}{[P]} \tag{4}
\]

Rearranging,

\[
\alpha [P] = \frac{\alpha}{1 - \alpha} n[D] - K_d \tag{5}
\]

Plotting \(\alpha [P] \) versus \(\frac{\alpha}{1 - \alpha} [D] \) yields a straight line with a slope n and intercept \(-K_d\).

Equation 5 is particularly useful for the evaluation of binding parameters in which the concentration of free ligand is not measured directly but rather expressed in terms of the total ligand present in the system. The value of \(\alpha \) is calculated for every desired point on the titration curve of the molar difference absorption, \(\Delta_{m} \) versus total DS500 concentration, using the relationship

\[
\alpha = \frac{\Delta_{m} - \Delta_{max}}{\Delta_{max}} \tag{6}
\]

\(\Delta_{m} \) represents the molar difference absorption at a certain DS500 concentration and \(\Delta_{max} \) represents the maximum molar difference absorption. It is not possible to titrate DS500 with factor XII because the intensity of the corrected protein UV difference spectrum due to DS500 is not very large at low DS500 concentration. However, we find that similar binding parameters can be determined by titrating factor XII with DS500. Since the spectral parameter that is monitored originates from factor XII, the saturation of binding still corresponds to the saturation of the spectral change. (For a review of binding studies using this technique, see Ref. 25.)

In the evaluation of the binding parameters of factor XII-DS500 by fluorescence spectroscopy, the value of \(\alpha \) is calculated for every desired point in the titration curve using the relationship

\[
\alpha = \frac{F_{m} - F}{F_0 - F} \tag{7}
\]

where \(F \) represents the fluorescence intensity at a certain DS500 concentration, \(F_{m} \) represents maximum quenching of fluorescence upon saturation, and \(F_0 \) is the initial fluorescence intensity.

RESULTS

Studies of Dextran Sulfate-induced Factor XII Aggregation and Autoactivation Using Ultraviolet Difference Spectroscopy—The conformational aspects of factor XII autoactivation by insoluble surfaces such as glass and ellagic acid have been reported (19). Since it is not known whether the true
surface activator in vivo is soluble or not the present study was conducted in order to characterize the conformational and structural aspects of factor XII autoactivation by a soluble surface, dextran sulfate. We first determined if aggregation occurs in the presence of dextran sulfate. To this end, the technique of ultraviolet difference spectroscopy is used. When the UV difference spectrum of factor XII is examined in the presence of DS5 or DS500, striking differences are found. The factor XII spectrum in the presence of DS500 becomes positive immediately at 340 nm and thereafter rises exponentially at lower wavelength (data not shown). Since no sugars or amino acid residues absorb above 310 nm, this spectrum of factor XII in the presence of DS500 must contain contribution from light scattering due to aggregation. In order to interpret this spectrum, it is necessary to separate the light scattering contribution. This is accomplished by procedures outlined under "Experimental Procedures." The light scattering-corrected difference spectra (Fig. 1A) are obtained using the "LOGGEN" light scattering correction software and confirmed using the fluoroscat cell technique. In the presence of 25 μg/ml DS500 (0.05 μM), factor XII (2.4 μM) exhibits a positive difference spectrum with a ΔM of +2,500 ± 250 at 288 nm (dotted line). At higher protein concentration (5.1 μM factor XII), this spectral maximum is more "red-shifted" to 290 nm (dashed line). In the presence of 25 μg/ml DS5 (5 μM), no light scattering is observed, but the factor XII spectrum is negative (ΔM = −3,800 ± 300 at 280 nm) and independent of protein concentration (Fig. 1B). Fig. 1C shows the time-dependent changes in the protein UV difference spectra of factor XII in the presence of DS5 and DS500. Upon addition of DS500, an immediate positive absorption difference spectrum is observed followed by a gradual decrease in amplitude (open and closed squares). In the presence of DS5 (open and closed triangles), the initial change is also immediate, and the difference spectrum becomes more negative with time. The differences in both sign and magnitude of the spectral changes indicate that different perturbations of aromatic residues occur in each case.

Before evaluating the significance of these contrasting spectral changes to factor XII binding and autoactivation, we first need to demonstrate that they are not due to artifacts of the experimental conditions. The possibility that factor XIIa is released from dextran sulfate upon autoactivation can be ruled out because it has been demonstrated by previous studies that factor XIIa binds more strongly to dextran sulfate than factor XII (24). The light scattering is not due to a decrease in protein solubility because an absorbance measurement at 410 nm shows that the solution of factor XII-DS500 is more transparent than factor XII-DS5 (data not shown). We observed these contrasting difference spectra in polybrene-coated as well as in siliconized cuvettes (Fig. 2). This finding rules out the possibility of artifacts due to aggregation between the polybrene (polycation) present on the cuvette wall and dextran sulfate (polyanion).

The solvent condition used throughout these studies was 50 mM NaCl or 50 mM NaF, 1 mM sodium phosphate, pH 7.5. Low ionic strength could affect the binding and autoactivation reactions of factor XII. Therefore, we examined the ionic strength dependence of factor XII autoactivation under this condition using a reduced SDS-PAGE. The formation of the 52- and 28-kDa fragments resulting from factor XII autoactivation is found to be maximal at ionic strength around 50 mM (data not shown), and very little fragmentation occurs at high ionic strength. When the factor XII-DS500 complex is allowed to form at low ionic strength (50 mM) and then the ionic strength is raised to 0.5 M, the positive UV difference spectrum is reduced to the base line (Fig. 2, dashed-dotted line). This is presumably due to disruption of the protein-polyanion complex at high ionic strength.

We also examined the time-dependent cleavage patterns of factor XII from the uncleaved 80-kDa molecule to the cleaved activated molecule (52 and 28 kDa) by SDS-PAGE under reducing condition and the generation of amidolytic activity against the factor XIIa chromogenic peptide substrate S-2302. Both of these structural and functional tests showed that the time-dependent UV spectral changes (Fig. 1C) were accompanied by proteolytic cleavage of factor XII and the hydrolysis of S-2302 (data not shown). The data also confirmed the previous observation (25) that factor XII autoactivation is about 5-fold faster in the presence of DS500 as compared to DS5. We also observed slightly enhanced difference spectrum when factor XII is mixed with DS500 in the presence of factor
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**FIG. 2.** Light scattering corrected UV difference spectra of factor XII-DS500 under various conditions. Spectra taken in cuvettes coated with polybrene (---) or silicon (---) in the presence of 50 mM NaCl, 1 mM sodium phosphate, pH 7.4. Factor XII-DS500 spectrum taken in a silicon-coated cuvette after increasing NaCl concentrations to 0.5 M (--). Factor XII spectrum in the presence of 100 molar excess of factor XIIa inhibitor, H-D-Phe-Phe-Arg-CMK (---) to inhibit proteolysis and autoactivation. The spectra were all corrected for the respective base lines. In all cases, the final concentration of factor XII and DS500 were 2.5 μM and 25 μg/ml, respectively. The vertical lines represent the range of error based on three to four determinations.

XIIa inhibitor, H-D-Phe-Phe-Arg-CMK (Fig. 2, solid lines). This indicates that the UV difference spectrum may contain small components attributed to proteolytic cleavage and further suggests that the spectral contribution due to cleavage is a negative spectrum. It is now clear that the binding and cleavage of factor XII in the presence of dextran sulfate is accompanied by a negative UV difference spectrum as seen in the case of the low molecular weight DS5. In the case of DS500, the positive difference spectrum must therefore be the net effect of binding (which is negative) and aggregation. Therefore, it is concluded that aggregation of factor XII on DS500 is responsible for the positive difference spectrum.

The UV spectral changes observed as factor XII aggregates on DS500 are quantitative measure of factor XII binding and can be utilized to determine binding parameters. Since the UV difference spectrum of factor XII-DS500 complex has some negative spectral contribution due to cleavage the spectral titration to study the binding behavior is monitored in the presence of the factor XIIa inhibitor H-D-Phe-Phe-Arg-CMK. In the presence of this inhibitor, the spectrum is only due to binding and aggregation because catalysis cannot occur, and subsequent cleavage and autoactivation are suppressed.

To fixed amounts of factor XII (97.5 μl, 200 μg/ml) are added 2.5 μl of different stock solutions of DS500 to obtain the desired concentrations (0–120 μg/ml). The results shown in Fig. 3A (closed circles) indicate that the DS500-induced spectral change attain maximum between 6–10 μg/ml DS500, and the titration curve exhibits a hyperbolic behavior. However, as more DS500 is added, the spectrum diminishes and is abolished at high DS500 concentration (Δabs = 0 ± 100 at approximately 47 μg/ml DS500). Upon further titration, the spectrum becomes negative and is similar to that obtained in the DS5 titration. The abolition of the positive spectrum suggests that at high DS500 concentration the number of adjacent factor XII molecules bound to DS500 decreases as they redistribute among large number of DS500 chains. Under this condition, aggregation is diminished because the intermolecular interaction among factor XII molecules is decreased and so is the positive UV difference spectrum. To determine the binding parameters of the factor XII-DS500 interaction, the observed Δabs values from 0 μg/ml DS500 to 20 μg/ml DS500 are plotted against micromolar concentration of DS500, and the data are analyzed as shown in Fig. 3B according to Equation 5. From the intercept and slope of the straight line (closed circles), the apparent dissociation constant and the number of binding sites can be calculated. The Kd is 3.0 × 10^{-7} M (S.E. = 0.5 × 10^{-7}, n = 5) for a single site, and the number of factor XII-binding sites/DS500 chain is n = 192 ± 20 sites.

The titration of factor XII by DS500 was also examined by native gel electrophoresis (inset, Fig. 3A). This band shift assay was devised to visualize the binding of factor XII to...
DS500. When preincubated in varying amounts of DS500, the factor XII-DS500 complex migrates only a short distance within the stacking gel and does not enter the separation gel, presumably because of increase in size due to complex formation and aggregation. The unbound factor XII migrates to the separation gel as a light staining, diffused band. However, the amount of factor XII that is retained in the stacking gel depends on the DS500 concentration. Under the same conditions used in the spectral titration, all of factor XII is retained in the stacking gel (factor XII band disappears in the separation gel) when the concentration of DS500 reaches 10 μg/ml (lane 5), which corresponds to the maximum Δμμ in the spectral studies. The reappearance of protein staining at high DS500 concentration (lane 8) is possibly due to partial dissociation of the factor XII-DS500 complex as suggested by the loss of the positive UV difference spectra. When the binding parameters are evaluated from the band shift assay (Fig. 3A, open circles), the values obtained are \( K_D = 1.7 \times 10^{-7} \) M (S.E. = 0.4 × 10^{-7}, \( n = 5 \)) and \( n = 165 \pm 30 \) sites.

It is difficult to evaluate the binding parameters of factor XII-DS500 complex from the UV spectral data in Fig. 3A because the spectra at 280 nm did not show saturation and continue to become negative as more DS5 is added. To circumvent this problem, fluorescence spectroscopy is used by monitoring the quenching of the intrinsic fluorescence of factor XII as small amounts of DS5 are added. Fig. 4 shows the relative changes in the intrinsic tryptophan fluorescence of factor XII at 340 nm (excitation at 280 nm) as 2.5 μM of the protein is titrated with small increments of DS5. The fluorescence intensity decreases as DS5 is added and levels off when about 15% of the initial fluorescence is quenched at 2.4 μM DS5. Additional of more DS5 after the initial saturation is reached results in further quenching of fluorescence. When the data points from 0 to 2.4 μg/ml are plotted (inset, Fig. 4) according to Equation 5, the number of factor XII-binding sites/DS5 and apparent \( K_D \) are found to be \( n = 1.1 \pm 0.2 \) sites and \( K_D = 1.3 \times 10^{-7} \) M (S.E. = 0.2 × 10^{-7}, \( n = 5 \)), respectively. Fluorescence titration of factor XII in the presence of DS500 was also done to provide a more direct comparison with DS5, but the results were not interpretable due to fluorescence light scattering of aggregated factor XII.

Studies of Factor XII Conformation by Circular Dichroism—

The CD spectra of factor XII and factor XII in the presence of dextran sulfate were investigated in order to determine whether the anionic mucopolysaccharide has an effect on the secondary structure of the protein. To accomplish this we determined the possible contribution of light scattering to the CD spectrum of factor XII-DS500 as described under “Experimental Procedures.” First, we examined the effect on the CD of factor XII-DS500 of varying the distance of the sample cell from the photomultiplier detector in the Jasco J-500C spectropolarimeter. The CD scans between 195 and 350 nm were compared when the sample was placed 18.5 and 5.2 cm from the detector. It is found that the CD of factor XII-DS500 is independent of the distance from the detector and does not deviate from the base line at 340 nm (data not shown). It is also found that the CD spectrum of factor XII-DS500 is superimposable with the spectra of factor XII alone and factor XII-DS5 at 340 nm (data not shown). The same solution of factor XII-DS500 shows a large light scattering absorption when recorded in a regular spectrometer. Additionally, if light scattering contributed to the CD signal of factor XII-DS500, deviation from the base line at 340 nm would be observed as we examined the CD at increasing protein concentrations. No concentration dependence was observed for the CD of factor XII in the presence of either DS5 or DS500 over a factor XII concentration range of 0.3 to 5.1 μM (data not shown). These experiments indicate that although factor XII-DS500 has a large unpolarized scattering absorption, there is no detectable differential light scattering as determined by CD. From these data, we conclude that the aggregation of factor XII in the presence of DS500 must be scattering the left and right circularly polarized light with essentially equal efficiency so that the CD spectrum is solely due to differential CD absorption. This finding means that it is not necessary to correct the CD signal of factor XII-DS500 for light scattering.

The CD of factor XII in the absence of dextran sulfate is similar to previously reported studies (19) except for a higher ellipticity at 204 nm in the present studies (\( [\theta]_{220} = -4,500 \pm 250 \) degrees cm² dmol⁻¹) versus \( [\theta]_{220} = -900 \) degrees cm² dmol⁻¹).2 Overall, the CD spectra suggest that factor XII conformation is mostly of the random coil. A better fit was obtained using the data on synthetic polypeptides (27). The best fit corresponds to 0% α-helices, 26.5% β-sheet, and 73.5% random coil.

We examined closely the effect of dextran sulfate on the CD spectrum of factor XII in the far ultraviolet region between 225 and 195 nm. This region of the CD spectrum corresponds to the amide transitions and is very sensitive to changes in protein secondary structures. Fig. 5A shows representative scans of the time-dependent changes in direct CD spectra of factor XII in the presence of DS500. When DS500 is added to factor XII there is an immediate spectral change characterized by a 5 nm red-shift of the trough at 210 nm (curve b) to 207 nm (curve e). This is followed by a gradual “blue-shift,” and in 60 min (curve e) the trough shifts back to 204 nm and the amplitude is increased by 25%. The initial red-shift is the same for both DS5 and DS500 but the blue-shift occurs at a longer time frame in the case of DS5 (data not shown). These spectral shifts are also clearly demonstrated as CD difference spectra (Fig. 5B). Immediate enhancement of the difference CD signal (c-b) is observed in the 225-210-nm negative trough and 200-210-nm positive

![Fig. 4. Titration of factor XII with DS5 monitored by the quenching of protein intrinsic fluorescence and analysis of binding parameters. The data are represented as relative changes in fluorescence intensity as a function of DS5 concentration. Excitation wavelength was set at 280 nm and the emission wavelength at 340 nm. To a 150-μl factor XII (2.5 μM), 2-μl aliquots of different stock solutions of DS5 are added to obtain the desired DS5 concentration (0-30 μg/ml). The fluorescence was corrected for changes in protein concentrations due to dilution. The spectra are taken 30 s after the addition of DS5. Each data point is an average of four determinations, and all measurements were corrected for the baseline spectrum of the buffer solution. Buffer conditions are the same as in Fig. 1. Inset, linear least-square fit of the titration curve based on Equation 5. The five data points were taken between 0 and 2.4 μM DS5 using \( F_0 = 99% \) and \( F_{\text{min}} = 85.5% \). The slope and intercept correspond to \( n = 1.1 \pm 0.2 \) and \( K_D = 1.3 \times 10^{-7} \) M, respectively.](Image)
The origin of UV difference spectra of proteins have been well-documented (28-32). The spectral perturbation resulting in red-shift, indicated by a positive difference spectrum, arises when the aromatic chromophores (i.e., Trp, Tyr, Phe) experience more nonpolar environment and occurs when the chromophores become buried within the protein fold as a result of intermolecular/intramolecular interactions or masked from the solvent environment by ligands or substrate molecules. A blue-shift that is indicated by a negative spectrum is generally interpreted as due to increased polarity of the chromophoric environment and can be due to increases in exposure of the chromophores to the solvent as a result of a conformational change or by direct interaction with molecules more polar than the solvent environment.

The negative UV difference spectrum of factor XII in DS5 suggests that the chromophores are in a more polar environment when factor XII is bound to DS5. Since no aggregation is observed in the presence of DS5, this negative spectrum must be the result of direct interaction of factor XII with highly anionic DS5. The positive UV difference spectrum of factor XII in DS500 suggests increased hydrophobicity of the aromatic environment when factor XII is bound to DS500. Since DS500 causes aggregation of factor XII we interpret this to mean that, in addition to direct interaction with DS500, part of the contribution to the spectrum arises from perturbation of aromatic chromophores located at the aggregation site, the interface between adjacent factor XII molecules bound to neighboring sites in the DS500 chain. This is possible only if factor XII molecules aggregate on the negative surface, and this accounts for the positive difference spectrum. The longer red-shift at higher concentrations of factor XII (5.1 μM factor XII in 25 μg/ml DS500, Fig. 1A) is an additional indication of larger contributions from this type of perturbation.

The number of factor XII-binding sites in the DS500 chain was previously reported to be about 88-100, based on the assumption that the average size of a globular factor XII molecule is equivalent to the size of a linear 5-kDa dextran sulfate (25, 33). This assumes a single linear chain of factor XII molecules along the DS500 chain. The result of the present titration studies indicates that about twice this number of sites could be present in the DS500 chain (n = 165-192 sites). This suggests the possibility that aggregation of factor XII in DS500 may be more compact as depicted in the model below.
In this model, factor XII is visualized as aggregating on either side of DS500 such that two linear chains of factor XII wrap around the DS800 chain. The closed circles represent the aromatic chromophores which are located at the factor XII interface (aggregation site). The positive UV difference spectrum arises when these chromophores, which are accessible to the solvent in free factor XII, are masked by the hydrophobic environment at the factor XII interface in the factor XII-DS500 complex. In the case of DS5, a 1:1 stoichiometry with factor XII was demonstrated by fluorescence spectroscopy at low DS5 concentration (Fig. 4). However, both fluorescence and UV difference spectroscopic titrations show further spectral changes after the stoichiometric amount of DS5 is reached. We cannot provide an unequivocal interpretation of this behavior, but it is reasonable to speculate that factor XII may have multiple binding sites for DS5.

Overall the results of both UV difference spectroscopy and circular dichroism studies demonstrate that autoactivation of factor XII in the presence of dextran sulfate is a biphasic process. The first rapid phase is associated with binding and aggregation and occurs concurrently with small but significant changes in the protein secondary structures. The second slow phase is associated with proteolytic cleavage and is also accompanied by conformational alteration of the factor XII molecule. Our interpretation of this observation is that when factor XII binds to dextran sulfate, the protein molecule tightens up transiently resulting in formation of small amount of organized elements of protein secondary structure such as α-helix or β-sheets. After cleavage during subsequent autoactivation, the factor XII molecule gradually relaxes back to its mostly random conformation.

Since the biphasic behavior of factor XII autoactivation has been demonstrated also in factor XII-sulfate complex (34) and aggregation of factor XII has been observed in factor XII-ellagic acid complex (10), the same mechanism may mimic factor XII autoactivation in vivo. Whether or not the fast initial conformational change of factor XII, as demonstrated by the present studies, is the key step that triggers the first event in contact activation can only be proven when the natural surface activator is identified. There are numerous physiologically relevant activating surfaces, such as polyanionic glycosaminoglycans, acidic phospholipids, glycolipids, and subendothelial basement membrane components, but direct demonstration of their role in surface activation of factor XII has been elusive. Heparin and collagen do not even activate purified factor XII in vitro (35, 36), acidic phospholipids are located in the cytosolic face of cell membranes, and the concentration of sulfatides are below the levels at which they could facilitate factor XII activation in vivo. Our understanding of dextran sulfate autoactivation of factor XII should provide some information on the expected properties and possible mechanism of action of the natural activator. These studies will be extended to other known activators as we learn more about the nature of the biological surface responsible for the activation of factor XII. At any rate, the use of soluble DS5 and DS500 proved valuable in explaining previously puzzling features of factor XII autoactivation.

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MODEL I. Factor XII-DS500 complex.