Ca^{2+}-Dependent Structural Changes in Bovine Blood Coagulation Factor Va and Its Subunits†

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ABSTRACT: The calcium dependence of the structures of bovine blood coagulation factor Va and its subunits (Vh and Vl) has been examined spectroscopically in order to characterize the conformational changes which accompany the binding of Ca^{2+} to Vh and Vl to form factor Va. The far-UV CD spectra of the isolated subunits indicate that the secondary structures of both Vh and Vl are predominantly β-sheet (>45%), with little α-helix content (<15%). No change in the far-UV CD spectrum was observed when factor Va was formed by the addition of Ca^{2+} to an equimolar mixture of Vl and Vh. Hence, no detectable change in secondary structure occurs during the formation of factor Va. In contrast, the addition of Ca^{2+} to an equimolar mixture of Vh and Vl caused a small (2%) increase in the total intrinsic fluorescence intensity and a blue shift in the emission spectrum that resulted from a tertiary structural change and/or the association of nonpolar surfaces at the subunit interface. This fluorescence change correlated closely with the appearance of functional factor Va, since the rate of the spectral change was the same as the rate of recovery of cofactor activity, and since both were half-maximal near 50 μM Ca^{2+}. This fluorescence change required both subunits, was reversed by the addition of EDTA, and was observed only with metal ions that can substitute for Ca^{2+} in reconstituting factor Va activity from Vh and Vl (Mn^{2+} and Tb^{3+}; not Mg^{2+}). When a sample containing ANS (8-anilino-1-naphthalenesulfonate) and an equimolar mixture of calcium-free Vh and Vl was titrated with Ca^{2+}, the ANS emission intensity decreased by about 30%, most likely because the association of Vl and Vh caused nonpolar regions at the subunit–subunit interface to become inaccessible for ANS binding. The calcium dependence of this spectral change yielded a K_d of 51 ± 2 μM, and the rate of the decrease in ANS fluorescence occurred at nearly the same rate as the recovery of factor Va activity. Thus, both intrinsic and extrinsic fluorescence data, as well as other data, indicate that the calcium binding site in factor Va has an apparent K_d of 50 μM under our conditions and that the calcium-mediated binding between Vl and Vh involves hydrophobic interactions between the subunits. The tight binding of Tb^{3+} ions to a Ca^{2+}-specific site(s) on Vh and on Vh + Vl was detected by the sensitivity of some of the protein-dependent terbium fluorescence to competition with Ca^{2+}, but not Mg^{2+}, ions. No tight binding site for Tb^{3+} was observed on Vl, and no new Ca^{2+}-specific binding site appeared to be created during the association of Tb^{3+} with Vh and Vl to form an active protein. These data are consistent with the single functionally important Ca^{2+} binding site on factor Va being located primarily on Vh. However, both subunits are required, either directly or indirectly, for the tight binding of Ca^{2+} and the subsequent formation of active factor Va. Finally, the data presented here and elsewhere demonstrate that the high activation energy required to form active factor Va from Vh, Vl, and Ca^{2+} does not result from an extensive, calcium-mediated change in polypeptide secondary, tertiary, or hydrodynamic structure.

The prothrombinase complex converts prothrombin to thrombin in the penultimate step of the blood clotting cascade [reviewed in Jackson and Nemerson (1980) and Mann et al. (1988)]. This membrane-bound procoagulant complex consists of a serine protease (factor Xa), a protein cofactor (factor Va), calcium ions, and a phospholipid surface, each of which is required to obtain maximal activity (Nesheim et al., 1979; Rosing et al., 1980). The functional role of the nonenzymatic factor Va molecule in this multicomponent complex is to increase the rate of prothrombin activation by factor Xa to a physiologically relevant level, and it appears to accomplish this by directly altering the structure and membrane binding properties of both the enzyme and the substrate. Specifically, factor Va increases the affinity of factor Xa for the membrane surface (Nesheim et al., 1981; van de Waart et al., 1984), allosterically alters the active-site conformation of the enzyme (Husten et al., 1987), and raises the active site of the enzyme to the correct distance above the membrane surface for prothrombin activation (Husten et al., 1987). In addition, the cofactor increases the affinity of prothrombin for the phospholipid surface (van de Waart et al., 1984) and for the complex (Esmon et al., 1973; Guinto & Esmon, 1984).

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Factor Va contains two nonidentical subunits, designated Vl and Vh, that associate noncovalently (Esmon, 1979), and a single tightly bound calcium ion (Guinto & Esmon, 1982). In the absence of calcium, factor Va is inactive; the exact nature of the structural change(s) which causes the loss in functional activity is unclear but may involve subunit separation (Esmon, 1979; Laue et al., 1984), polypeptide conformational changes, or both. In the presence of calcium, neither Vl nor Vh, by itself, is active in promoting prothrombin activation (Esmon, 1979). Also, neither subunit exhibits measurable affinity for Ca^{2+} (Guinto & Esmon, 1982), and the subunits do not associate significantly in the absence of Ca^{2+} (Laue et al., 1984). Thus, it appears that subunit association, the binding of a single calcium ion by the protein, and the appearance of functional activity are strongly linked for factor Va.

We have observed, as have others, that the association of purified Vl and Vh subunits in the presence of Ca^{2+} to form active factor Va is a very slow process (Esmon, 1979; Suzuki et al., 1982; data herein). In view of the Ca^{2+} requirement for the interaction between the subunits and the slow kinetics of the process, one might reasonably expect that the Ca^{2+} ion nucleates large conformational changes in the Vl and/or Vh polypeptides and that these changes are required to reconstitute the functional protein from Ca^{2+}, Vh, and Vl. Since we found that the sedimentation velocities of Vl and of Vh were the same in 2 mM EDTA and in 10 mM Ca^{2+} (Laue et al., 1984), it appears that Ca^{2+} does not elicit a major change in the overall hydrodynamic shape of either of the subunits individually. However, these experiments did not eliminate the possibility that a large conformational change occurs and that it requires an intersubunit interaction to be effected or stabilized.

In order to clarify the role of the single required calcium ion in the assembly of factor Va, we undertook to examine the nature of the metal ion–protein interaction using a variety of spectroscopic techniques. In particular, since an examination of the sensitivity of Vl and Vh structure to calcium might reveal the nature of the rate-limiting step in factor Va reconstitution, and possibly even localize the calcium ion binding site in the protein, we set out to characterize the metal ion dependence of subunit conformation and of subunit interactions.

Experimental Procedures

Proteins. Bovine factors Va, Vl, and Vh were isolated as described by Esmon (1979) and stored at 4 °C in the presence of 5 mM benzamidine. In some cases, factor Va was further purified by using an FPLC chromatography system (Pharmacia) equipped with a Mono-Q column, from which factor Va eluted near 0.35 M NaCl in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)[1] (pH 7.5) and 5 mM $CaCl_2$. For some experiments, Vl and Vh were prepared by dialyzing factor Va into buffer A (0.1 M NaCl and 0.02 M Tris-HCl, pH 7.5) containing 2 mM EDTA for 10–24 h at 4 °C and then separating the subunits on a Mono-Q column using a linear NaCl gradient in buffer A plus 2 mM EDTA (Vl eluted in the void volume, while Vh eluted near 0.35 M NaCl). The purification of Vl was then completed by chromatography over a Mono-S column in buffer A plus 2 mM EDTA (Vl eluted at 0.5 M NaCl). Protein homogeneity was evaluated by polyacrylamide gel electrophoresis in 0.1% (w/v) sodium dodecyl sulfate using a 7.5% resolving gel, either as described by Laemmli (1970) or with a Phast-Gel System (Pharmacia). Factor Va activity was routinely assessed by using the one-stage clotting assay (Esmon, 1979). At least two separate preparations of factor Va and subunits were examined with each spectral technique described in this paper, and in very case, different protein preparations gave quantitatively similar results.

Prior to use, proteins were dialyzed extensively against the desired buffer at 4 °C in polypropylene containers, always in the presence of Chelex 100 (Bio-Rad). Thereafter, prior to intentional exposure to metal ions, proteins were exposed only to solutions that had been treated with Chelex.

Protein concentrations were determined by using the following $E^{1\%}_{1cm}$ extinction coefficients at 280 nm for Vl, Vh, and factor Va, respectively: 18.7, 12.9, and 15.1 (Guinto & Esmon, 1982). Absorbance measurements were made on either a Cary 118 or a Varian DMS 100 spectrophotometer. The molecular weights for factor Va, Vl, and Vh were taken to be 174 000, 82 500, and 92 300, respectively (Laue et al., 1984).

Circular Dichroism. For CD measurements, protein solutions were dialyzed into buffer B (a 0.1 M NaCl, 1 mM Tris-HCl, and 3 μM EDTA solution with a measured pH of 7.3 at 25 °C). Protein samples were then kept at 4 °C or on ice until use, which was always within 24 h after dialysis. All solutions were degassed by aspiration just prior to spectral analysis. After the Ca^{2+}-free CD spectra of samples were recorded, buffer B containing 1 M $CaCl_2$ was added to obtain a final Ca^{2+} concentration of 1.2 mM, and samples were then incubated for at least 40 min at 37 °C prior to recording the CD spectra in the presence of Ca^{2+}. Degassing in the presence of Ca^{2+} often led to concentrated Vl and Vh solutions (>1 mg/mL) becoming turbid. Particulate matter was therefore removed from these samples by sedimentation in a microfuge prior to analysis by CD. The removal of this turbidity reduced the observed A_{280} by about 5%. Protein concentration, solution pH, and factor Va activity were determined both before and after the CD measurements.

Spectra were recorded on a Cary 61 spectropolarimeter in a jacketed cell holder maintained at 35 °C by a circulating water bath. Data were accumulated using a time constant of 10 s and a scanning speed of 0.1 nm/s. Far-UV (195–250 nm) spectra were obtained in a 2-mm path-length cell using samples with absorbances between 0.1 and 0.2 at 280 nm. Near-UV (245–320 nm) spectra were obtained by using a 1-cm path-length cell and samples with absorbances between 1.0 and 2.2 at 280 nm. Three or four scans of each sample and of its protein-free blank were recorded, and the far-UV protein CD spectra shown in Figure 1 were obtained by subtracting the appropriate average blank signal from the average sample signal at 3-nm intervals. Mean residue ellipticities were calculated from the far-UV data by using average amino acid molecular weight values of 116 for Vl, 115 for Vh, and 115.5 for factor Va, based on the amino acid composition data of Guinto and Esmon (1982). Estimates of the helical content of the proteins were calculated by using the algorithms and basis spectra of Chen et al. (1972, 1974).

Fluorescence Spectroscopy. Fluorescence measurements were made in 1 cm × 1 cm quartz cuvettes using either a Spex Fluorolog I or a SLM 8000 spectrofluorometer. Each is a photon-counting instrument equipped with a 450-W xenon lamp and double monochromators in the excitation light path. The Spex instrument also has two monochromators in the emission light path. Operational control, data collection, correction of emission intensities, and signal integration were

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; CD, circular dichroism; ANS, 8-anilino-1-naphthalenesulfonate.

provided by a Tektronix 31 programmable calculator interfaced to the Spex or an Apple IIe computer interfaced to the SLM. Emission correction factors for the Spex were determined by using a standard lamp (Optronics). Temperature control was maintained by using a refrigerated Lauda K2R circulating bath attached to the cell holder. Shutters were kept closed except during scans to avoid photodegradation of the sample. In all experiments, background signal due to solvent was subtracted.

Intrinsic Protein Fluorescence. Intrinsic emission experiments were carried out at 37 °C in buffer C (0.1 M NaCl and 0.05 M Tris-HCl, pH 7.6). Samples were excited at 280 nm, and a 10-nm band-pass was used on both excitation and emission. Emission spectra were recorded at 1-nm intervals from 300 to 400 nm, and the collected emission intensity data were then corrected. Intensities were quantified by integration of the corrected data at 1-nm intervals both over the entire emission scan and also over each 10-nm interval of the spectrum.

Titrations. Samples of Vl, of Vh, of Vl + Vh, and of a protein-free blank were initially incubated for at least 1 h to establish an equilibrium, metal-free emission intensity (F_0) prior to adding any metal ions. Samples were then titrated in parallel by the sequential addition of metal ions to each cuvette. The total volume of titrant added to a sample never exceeded 3% of the original sample volume (2.2 mL). In all titrations, intensity data were corrected for dilution due to the addition of titrant. The affinity of the proteins for metal ions was determined by using linear least-squares regression analysis of titration data presented in a double-reciprocal plot to estimate K_d values [cf. Johnson et al. (1983)].

ANS-Containing Titrations. After dialysis in EDTA to remove Ca^{2+} and then further dialysis to remove EDTA, samples of calcium-free factor Va (=Vl + Vh; 1.1–1.6 μM) in buffer A were made 3.4–10.2 μM in 8-anilino-1-naphthalenesulfonate (ANS) and titrated with either $CaCl_2$ or $MgCl_2$ at 25 °C. In some experiments, purified Vh and Vl polypeptides were added, each to a final concentration of 1.2 μM, to the protein-containing cuvette instead of EDTA-treated factor Va. After each addition of metal ions in all of these titrations, a sample was mixed and incubated for at least 60 min before the emission intensity of the sample was measured. Parallel titrations were carried out on samples that contained ANS, but contained no protein. Protein-dependent emission intensity was obtained by subtracting the signal of the protein-free sample from the signal of the protein-containing samples. At each titration point, both the emission intensity and the clotting activity of the sample were determined.

The rate of the ANS emission change was examined by using calcium-free samples prepared as above and initiating the experiment by adding calcium, to a final concentration of either 1 mM or 50 μM, to a sample. Both fluorescence intensity and factor Va activity, as measured in a one-stage clotting assay, were then monitored as a function of time.

ANS-containing samples were excited at 390 nm with a band-pass of 4 nm, and emission intensities were monitored at 475 nm with a band-pass of 8 nm. ANS concentration was determined by using an extinction coefficient of 6240 M^{-1} cm^{-1} at 351 nm (Ferguson & Cahnmann, 1975).

Terbium Ion Titrations. Terbium titrations and their controls were carried out at 37 °C in buffer D (0.02 M MOPS, pH 6.5, and 0.1 M NaCl). Samples were excited at 280 nm with a band-pass of 4 nm, and emission intensities were measured at 545 nm using a band-pass of 16 nm. A UV cutoff filter (Oriel 5215) was inserted in the emission light path to remove signal arising from diffraction grating second-order effects. Protein concentrations were 20–35 nM. After each addition of metal ions, a sample was mixed and then incubated for at least 30 min at 37 °C before measuring its emission intensity. At each Tb^{3+} concentration, the emission of the protein-free blank was subtracted from the emission of a sample containing protein to give the protein-dependent terbium emission intensity of the sample.

Results

Circular Dichroism. In order to assess the effect of Ca^{2+} on the secondary structure of factor Va and its individual subunits, their CD spectra were recorded in the presence and absence of Ca^{2+}. The far-UV CD spectra of factor Va and its subunits in the presence of calcium are shown in Figure 1A. All three spectra exhibit a single distinct, but relatively weak, minimum of molar ellipticity near 219 nm, a spectral feature exhibited by polypeptides predominantly in a β-sheet conformation. Using the algorithms and basis spectra of Chen et al. (1972, 1974), the α-helix content was estimated to be less than 15% in factor Va, in Vh, and in Vl, while the β-sheet content was estimated to be in excess of 45% in each case. Although the CD spectra do not provide firm numbers for the α-helical and β-sheet contents of the proteins, the similar molar ellipticities of Vh, Vl, and factor Va indicate that the extent of α-helix and β-sheet formation is similar in each protein.

The calcium-free spectra for Vl and for Vh were identical with the corresponding spectra in Figure 1A (data not shown). Hence, neither subunit, by itself, experienced a significant alteration in CD-detected secondary structure upon the addition of Ca^{2+}.

When an equimolar mixture of Vl and Vh was examined, identical spectra were obtained in the absence and presence of 1.2 mM Ca^{2+} (Figure 1B). This demonstrates that the association of Vl and Vh in the presence of Ca^{2+} to form factor Va does not elicit a significant change in the CD-detected secondary structure of the polypeptides. The factor Va formed from the isolated subunits in this experiment was fully functional in the one-stage clotting assays, while the clotting activity of the subunit mixture in the absence of calcium was less than 5% of that in the presence of calcium.

The near-UV CD spectra for Vl, Vh, and factor Va exhibit a single positive maximum near 295 nm and a complex negative trough around 280 nm (Figure 2). After subtraction of the solvent blank signals from the appropriate sample signals, positive molar ellipticity extrema were found at 296 nm for Vl (43 000 deg·cm^2/dmol) and at 293 nm for Vh (46 000 deg·cm^2/dmol). Again, only small differences in the near-UV CD signal were observed in the presence and absence of Ca^{2+} for the individual subunits. In contrast, equimolar mixtures of Vl and Vh exhibited a positive molar ellipticity maximum at 294 nm that decreased from 36 000 to 30 000 deg·cm^2/dmol upon the addition of Ca^{2+}. This indicates that the association of the subunits to form factor Va resulted in an alteration in the asymmetry of the environments of some tryptophans in Vl and/or Vh. Although the magnitude of this spectral change is small, its existence suggests that some tryptophans are sensitive to the tertiary and/or quaternary structural changes that accompany the formation of factor Va.

Calcium Dependence of Intrinsic Fluorescence. The effect of Ca^{2+} on the intrinsic fluorescence of factor Va is shown by comparing the emission spectra of an equimolar mixture of Vl and Vh obtained in the presence and absence of Ca^{2+} (Figure 3). The addition of Ca^{2+} caused both a slight increase (2%) in the total emission intensity and a shift in the emission

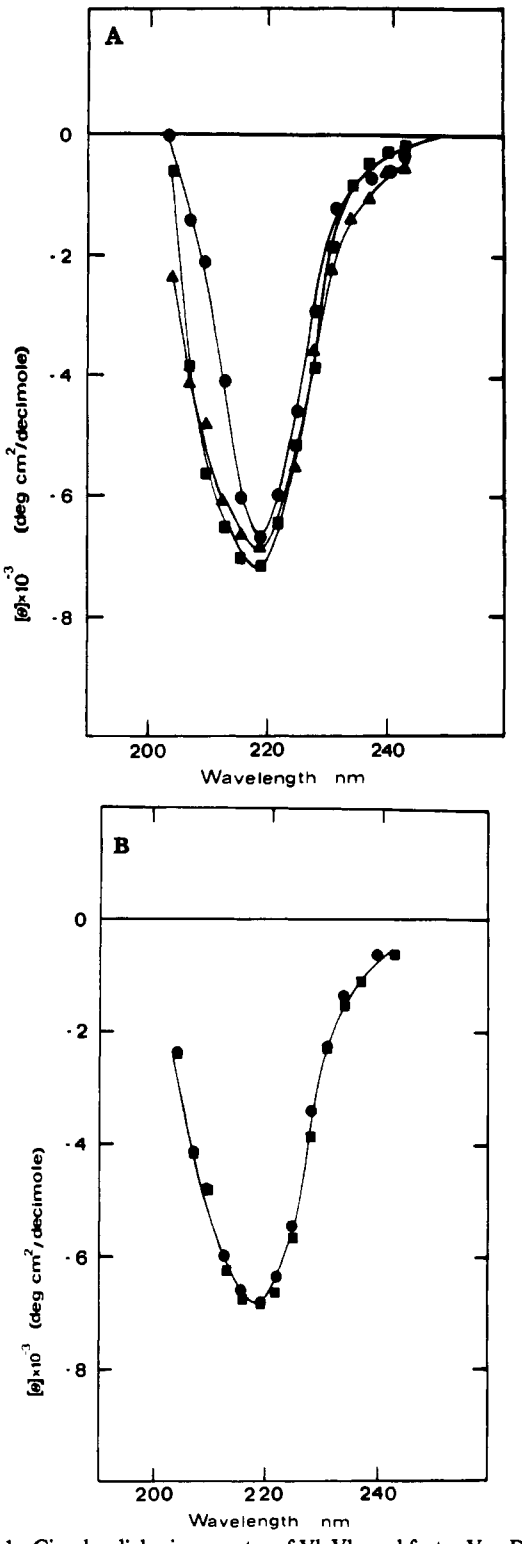

FIGURE 1: Circular dichroism spectra of Vl, Vh, and factor Va. Details are given under Experimental Procedures. (A) CD spectra for 0.11 mg/mL Vl (●), 0.18 mg/mL Vh (■), and 0.15 mg/mL factor Va (▲) in buffer B containing 1.2 mM $CaCl_2$. (b) Spectra for an equimolar mixture of Vl and Vh (total protein concentration of 0.15 mg/mL) in buffer B in the absence (●) and in the presence (■) of 1.2 mM Ca^{2+}.

spectrum toward the blue. This spectral shift is clearly evident when the calcium-dependent emission intensity changes are shown as a function of emission wavelength (inset, Figure 3). Although both the intensity increase and the spectral shift were small, they were reproducible: identical changes were observed in 11 experiments performed with 4 different preparations of Vl and Vh.

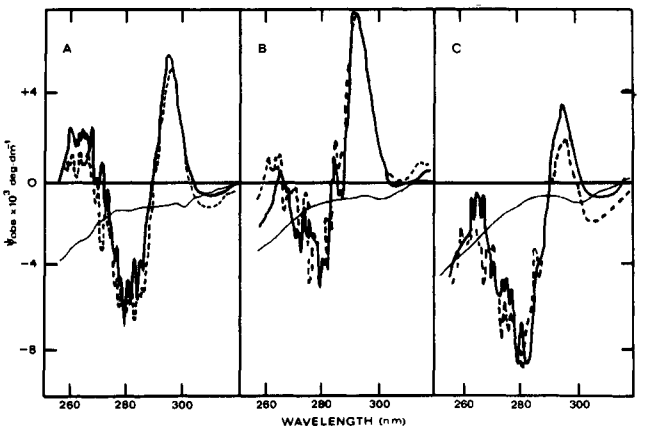

FIGURE 2: Near-UV circular dichroism spectra. Spectra were obtained in buffer B as described under Experimental Procedures in the absence (thicker solid line) and in the presence (dashed line) of 1.2 mM Ca^{2+}. The thin solid line is the spectrum obtained with the solvent blank. (A) Vl, 1.1 mg/mL; (B) Vh, 1.8 mg/mL; (C) an equimolar mixture of Vl and Vh, 1.5 mg/mL total protein concentration.

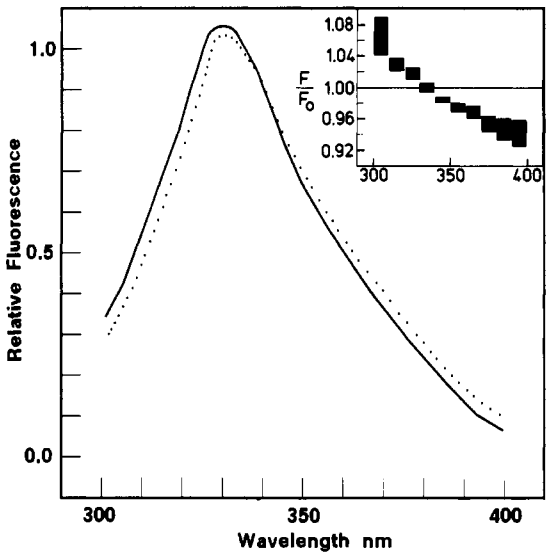

FIGURE 3: Calcium dependence of intrinsic fluorescence. Corrected emission spectra were obtained in buffer C at 37 °C as described under Experimental Procedures for an equimolar mixture of Vl and Vh (total protein concentration of 28 μg/mL) in the absence (dotted line) or presence of 1.2 mM Ca^{2+} (solid line). After the addition of calcium, samples were incubated in the cuvette for at least 40 min at 37 °C before further data acquisition. The inset depicts the ratio of the protein emission intensities in the presence and absence of Ca^{2+} for each 10-nm region of the emission spectra. In this plot, F is the fraction of the total corrected fluorescence intensity in a calcium-containing sample that emits in a particular 10-nm window, while F_0 is the corresponding fraction of the total intensity of that sample measured in the absence of calcium. The range of values shown for F/F_0 in each 10-nm region represents the 67% confidence interval calculated from 11 replicate experiments.

Neither of the isolated subunits exhibited any change in intrinsic emission upon the addition of calcium (data not shown). Hence, the Ca^{2+}-dependent change in protein fluorescence shown in Figure 3 requires both Vl and Vh, either for binding the calcium ion and/or for stabilizing the metal ion dependent structure.

The calcium-dependent fluorescence change was also shown to be fully reversible by the sequential addition of first Ca^{2+} and then EDTA to an equimolar mixture of Vl and Vh (data not shown).

Metal Ion Specificity. The metal ion specificity of the blue shift shown in Figure 3 was examined by adding Ca^{2+}, Mn^{2+}, or Mg^{2+} ions to an equimolar mixture of Vl and Vh. The

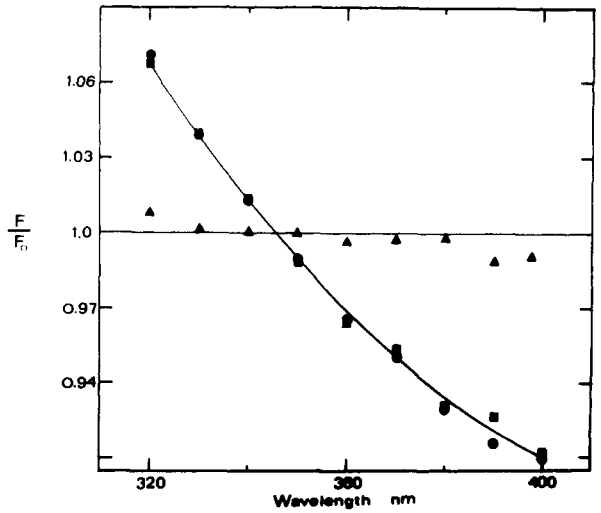

FIGURE 4: Metal ion dependence of intrinsic protein fluorescence. Corrected emission scans of parallel samples containing an equimolar mixture of Vl and Vh (total protein concentration 30 μg/mL) in buffer C were obtained at 37 °C as described under Experimental Procedures. After the addition of 1 mM Ca^{2+} (●), 1 mM Mn^{2+} (■), or 1 mM Mg^{2+} (▲), samples were incubated for at least 30 min at 37 °C prior to the acquistion of further emission data. F_0 represents the fraction of the total corrected fluorescence intensity of the initial metal ion free sample that emits in a particular 10-nm region of the emission spectrum. F is the corresponding value obtained after incubation of the sample with a metal ion.

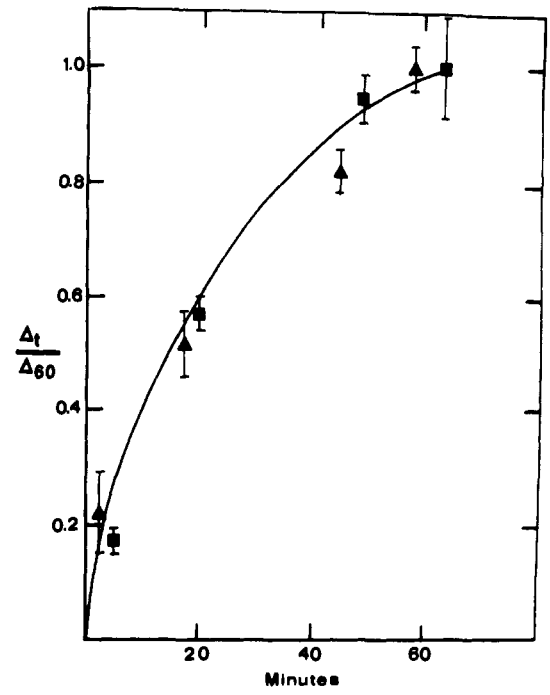

FIGURE 5: Time dependencies of the fluorescence-detected structural change and of the recovery of activity. After initial fluorescence scans and activity assays, an equimolar solution of Vl and Vh (total protein concentration 30 μg/mL) in buffer C was brought to 5 mM Ca^{2+}, mixed, and incubated in the fluorometer at 36 °C. Emission intensity was measured (▲), and one-stage clotting assays were done (■) at the time points indicated. Data are graphed as a fraction of the 60-min values for emission intensity and clotting activity (Δ_t/Δ_{60}). The fluorescence measurements are presented as the mean and the standard deviation of the fractional change determined at each of the ten 10-nm wavelength regions (described in legend to Figure 3). Activity assay data are presented as the mean and standard deviation for triplicate measurements at each time point. In this experiment, the activity at 60 min was 290 units/mg of factor Va (see legend to Figure 6).

resultant spectral changes are shown in Figure 4. Ca^{2+} and Mn^{2+} ions elicited identical fluorescence changes in parallel samples of Vl + Vh, which suggests that the association of Vl and Vh with a Mn^{2+} ion yields a structure that is the same, or nearly the same, as that of native factor Va. This conclusion is supported by the observation that the Ca^{2+}- and Mn^{2+}-containing samples were equally active in the one-stage clotting assay.

In contrast, incubation of Vl and Vh with Mg^{2+} did not result in a change in protein emission (Figure 4), and activity assays showed that the Mg^{2+}-containing samples of Vl and Vh did not support clotting. Moreover, the presence of Mg^{2+} did not block the Ca^{2+}-dependent structural changes: when 1 mM Ca^{2+} was added to solutions that already contained 1 mM Mg^{2+}, the recovery of factor Va activity and the Ca^{2+}-dependent blue shift in protein fluorescence were both found to occur (data not shown).

Fluorescence-Detected Affinity of Vl and Vh for Ca^{2+}. When equimolar mixtures of Vl and Vh were titrated with Ca^{2+}, the intrinsic fluorescence change was found to be half-maximal at a Ca^{2+} concentration near 50 μM (data not shown). This indicates that the K_d value for the interaction between Ca^{2+} and the factor Va subunits is near 50 μM. A more precise number could not be extracted from these data because the small magnitude of the intrinsic emission intensity change led to a relatively large uncertainty in the estimated K_d values. The calcium dependence of this fluorescence change is compatible with the Ca^{2+} binding determined by equilibrium dialysis (K_d = 24 μM; Guinto & Esmon, 1982) when the protein concentration differences in the two techniques are taken into account.

Kinetics of the Change in Intrinsic Protein Fluorescence. After the addition of Ca^{2+} to a calcium-free equimolar mixture of Vl and Vh, several minutes of incubation at 37 °C were required for the fluorescence change to reach completion. The recovery of activity was also slow. The time required for complete recovery of activity was dependent upon the subunit concentration, and also varied with the subunit preparation, but was generally between 60 and 75 min in our experiments. The rate of activity regain was independent of the calcium ion concentration between 0.1 and 10 mM (data not shown).

In order to compare the rate of the fluorescence intensity increase and blue shift with the rate of recovery of factor Va activity, we simultaneously monitored, in a single sample, the time dependencies of both the spectral change and the activity of the protein. For the latter, aliquots of the sample were removed and evaluated by using the one-stage clotting assay. As can be seen in Figure 5, the rate of the fluorescence-detected structural change is very similar to that of the functional change, which suggests that each is governed by the same rate-limiting step.

Metal Ion Dependent Changes in ANS Fluorescence. ANS exhibits a large increase in fluorescence intensity when it binds noncovalently to nonpolar sites on proteins (Weber & Laurence, 1954; Stryer, 1965). When factor Va was added to a solution of ANS, the intensity of ANS fluorescence immediately increased severalfold, and the wavelength of maximum emission shifted from 530 to 480 nm (data not shown). This indicates that factor Va has nonpolar regions on its surface to which the ANS molecule binds, a conclusion that is fully consistent with our previous observation of nonpolar surfaces on factor Va using lipophilic photoreagents (Krieg et al., 1987).

When a sample containing ANS and equimolar amounts of purified Vl and Vh subunits was titrated with Ca^{2+}, the ANS fluorescence intensity at 475 nm decreased by a total of 30% (Figure 6A). This spectral change was calcium specific, because no change in ANS fluorescence was observed when a parallel sample was titrated with Mg^{2+} (Figure 6A).

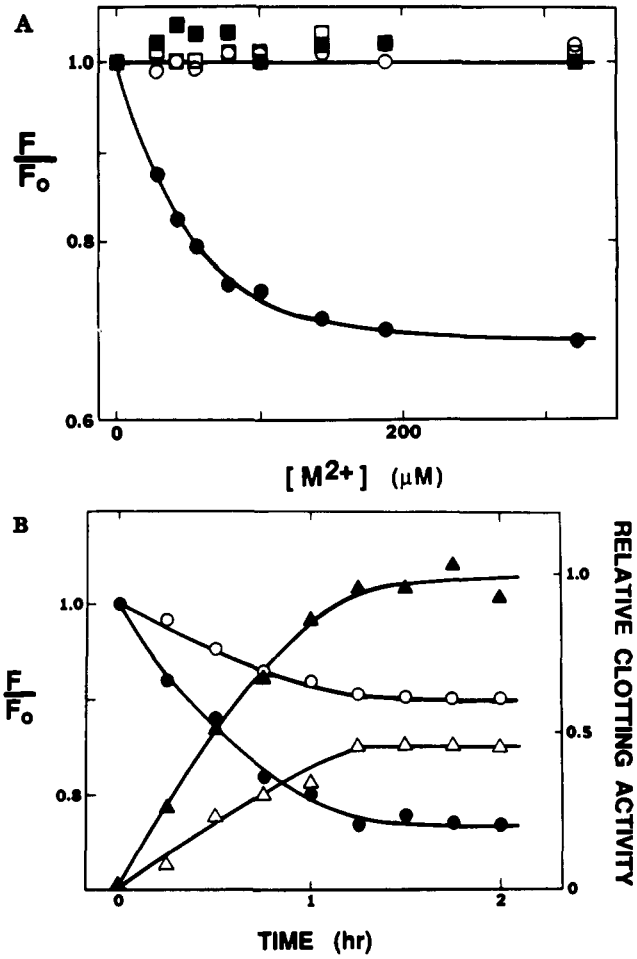

FIGURE 6: ANS fluorescence-detected formation of functional factor Va. (A) Samples containing ANS in buffer A were titrated with Ca^{2+} (●, ○) or Mg^{2+} (■, □) as described under Experimental Procedures. In the experiments shown here, protein samples (●, ■) initially contained factor Va (1.15 μM) that had been dialyzed extensively to remove calcium. Data obtained from protein-free samples are shown with open symbols. The initial uncorrected emission intensity for each sample is given by F_0, while F represents the dilution-corrected intensity at a particular metal ion concentration. (B) Calcium was added to calcium-free samples as in (A) to a final concentration of either 1 mM (●, ▲) or 50 μM (○, △), and the resulting protein-dependent ANS emission intensity (●, ○) and clotting activity (▲, △) are shown as a function of time at 25 °C. Activity was determined by using the one-stage clotting assay (Esmon, 1979) and is expressed in units per milligram of protein, where bovine plasma is defined as having 1 unit of factor V/mL. The 100% activity values ranged from 200 to 300 units/mg of factor Va in different experiments and were determined in each case after incubating an aliquot of the protein sample in buffer A plus 2 mM calcium for 60 min at 37 °C.

The fluorescence change was also observed only in the presence of protein (Figure 6A). Double-reciprocal analysis of the calcium dependence of this spectral change yielded an apparent average K_d value of 51 ± 2 μM in four experiments. The calcium dependence of the change in protein-dependent ANS fluorescence is therefore very similar to the calcium dependence of the intrinsic fluorescence change. This suggests that the ANS probe may also be sensitive to functionally important changes in protein structure.

Kinetics of the Change in ANS Fluorescence. We therefore examined the kinetics of both the spectral change and the recovery of factor Va activity by adding Ca^{2+} ions to a calcium-free sample of Vl and Vh containing ANS and then monitoring the ANS emission and the one-stage clotting activity as a function of time. (Control experiments showed that the clotting times were unaffected by the low concentrations of ANS in these samples.) As shown in Figure 6B, the fluorescence decrease and the activity recovery occurred at about the same rate at two different calcium ion concentrations. The slow, but equivalent, rates of the spectral and activity changes, as well as their calcium dependence, are consistent with the intrinsic emission data (Figure 5 and above) and independently confirm the slow kinetics of a functionally important protein structural change. The ANS-detected structural change therefore appears to constitute a spectral indicator of the conversion of the isolated subunits into an active factor Va molecule.

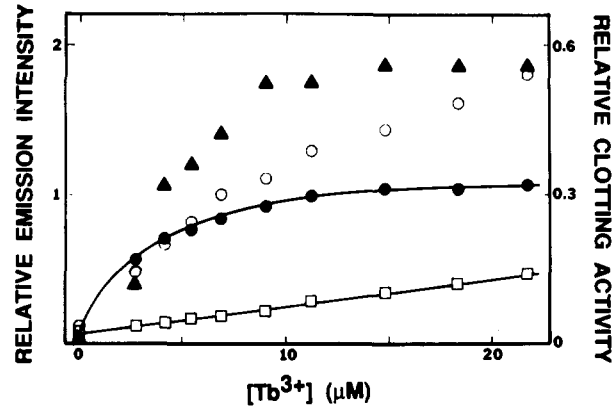

FIGURE 7: Protein-dependent terbium fluorescence. Calcium-free samples of Vh (0.29 μM; ●), Vl (0.28 μM; □), and Vh + Vl (0.29 and 0.28 μM, respectively; ○) in buffer D were titrated with Tb^{3+} as detailed under Experimental Procedures. Only the protein-dependent emission intensity is shown. The clotting activity (▲) of the sample containing both Vh and Vl at pH 6.5 was determined by using the one-stage clotting assay and is expressed relative to the clotting activity of a parallel sample of Vh and Vl that had been reconstituted in the presence of Ca^{2+} at pH 7.5 (180 units/mg of protein, defined as in Figure 6).

A limitation in using noncovalent extrinsic dyes as probes of protein structure is the possible binding of the dyes to several different sites on the protein. Since the observed fluorescence intensity of a sample will depend upon the extent of occupancy of each type of site and the emission properties of the ANS in each site, the magnitude of the observed change in ANS emission which accompanies a specific structural alteration will vary, depending upon, among other things, the total ANS concentration in the sample and its temperature. Hence, we chose to use a low ANS concentration in order to reduce the heterogeneity of the probe locations in the sample. Results similar to those of Figure 6A were obtained by using higher concentrations of ANS, but the fractional change in intensity was lower, presumably due to ANS binding to additional sites on the proteins at the higher ANS concentrations.

Terbium Binding to Vh and Vl Polypeptides. Terbium has frequently been employed to investigate the calcium ion binding sites on proteins by using the protein-dependent terbium emission that results from singlet–singlet energy transfer from Trp and Tyr residues [human factor Va contains 67 tyrosines and 32 tryptophans (Jenny et al., 1987)] to protein-bound terbium [e.g., see Martin and Richardson (1979), Johnson et al. (1983), and Morita et al. (1984)]. When terbium was added to a mixture of separated Vl and Vh subunits, functional factor Va was generated (Figure 7) at a rate that was similar to that observed upon calcium addition. Since factor Va activity is metal ion dependent (Esmon, 1979), these results show that Tb^{3+} must bind to and successfully fill the single high-affinity Ca^{2+} binding site in factor Va. Therefore, it seemed reasonable to examine whether protein-dependent terbium emission could be used to determine which, if either, of the subunits contained the high-affinity calcium ion binding site.

All terbium experiments were done at pH 6.5 to avoid terbium-dependent precipitation of the protein. However, the lower pH in the samples did not appear to significantly alter the protein structure. First, complexes of Vl and Vh formed in the presence of Tb^{3+} were functionally active (Figure 7). Second, the intrinsic protein emission changes observed when Tb^{3+} was titrated into an equimolar mixture of Vl and Vh (data not shown) were identical with those observed when titrations were carried out with either Ca^{2+} or Mn^{2+} (Figure 4). These results suggest that the factor Va reconstituted with terbium is very similar to, if not identical with, the factor Va reconstituted with calcium.

When Vl was titrated with Tb^{3+}, a small increase in emission intensity was observed that was approximately directly proportional to the terbium concentration (Figure 7). This emission therefore appeared to result from a weak, and possibly nonspecific, association of Tb^{3+} with Vl. In contrast, when Vh was titrated with terbium, the shape of the emission intensity versus $[Tb^{3+}]$ plot was hyperbolic (Figure 7), suggesting that terbium ions were binding specifically to a site(s) on Vh. The apparent dissociation constant for the fluorescence-detected terbium binding to Vh, determined from the double-reciprocal plots of the spectral data, averaged 4.1 ± 2.8 μM over six experiments. Titration of an equimolar mixture of Vl and Vh with terbium yielded an emission intensity versus $[Tb^{3+}]$ profile that was similar to the sum of the profiles observed with the two subunits individually (Figure 7). This suggests that the changes in terbium fluorescence observed with the separated subunits are largely independent of each other.

Since terbium ions bound to each subunit, we asked whether the preincubation of one of the subunits with Tb^{3+} would increase the rate of recovery of factor Va activity when separated subunits were mixed. Vh (or Vl) was incubated with 10 μM Tb^{3+} at 37 °C for up to 90 min prior to adding Vl (or Vh) and then assayed for activity by the one-stage clotting assay. Neither preincubation stimulated the slow rate of factor Va formation (data not shown). Hence, both subunits must be present to stabilize or create the rate-limiting and metal ion dependent structural change that results in functional factor Va. This is true whether the required metal ion is Ca^{2+} (Suzuki et al., 1982) or Tb^{3+}.

Competition of Ca^{2+} and Tb^{3+} for Binding to Proteins. In order to determine whether the observed terbium emission resulted from the binding of Tb^{3+} to a Ca^{2+} binding site, the terbium titrations of Vh, of Vl, and of Vh + Vl were repeated in the presence of a high molar excess of Ca^{2+} (2–50 mM). Control titrations were done in the presence of the same concentration of Mg^{2+} in order to determine to what extent any observed competition was ion specific. As shown in Figure 8, the presence of Ca^{2+} reduced the total Vh-dependent terbium fluorescence by 14%, which indicates that Ca^{2+} ions competed with, and replaced, a fraction of the Tb^{3+} ions that were bound to Vh. Furthermore, the presence of Ca^{2+} substantially increased the apparent K_d for the Vh–Tb^{3+} complex (Figure 8), which indicates that calcium ions filled the site(s) on Vh that has (have) the highest affinity for terbium ions. These effects upon terbium fluorescence were ion specific, because Mg^{2+} ions did not compete with Tb^{3+} for binding to Vh (Figure 8). Similar results were obtained with samples containing Vh + Vl, except that Ca^{2+} and Mg^{2+} at 5 mM decreased the total terbium fluorescence by 25% and 10%, respectively, to give a Ca^{2+} ion specific decrease in terbium fluorescence of 15% (data not shown). No calcium-specific changes in terbium emission were observed in samples containing only Vl (data not shown).

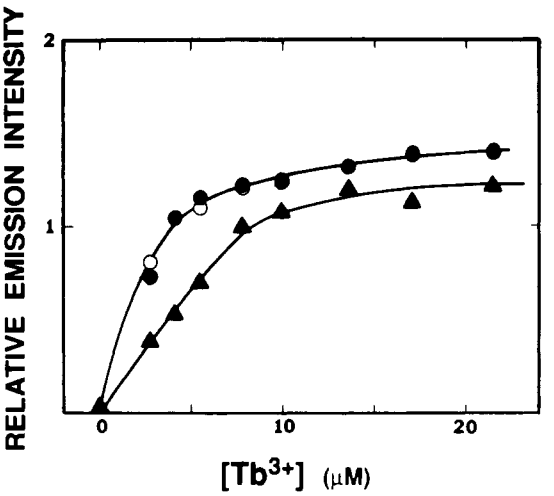

FIGURE 8: Fluorescence-detected competition of Ca^{2+} and Mg^{2+} for Tb^{3+} binding sites on Vh. Parallel samples contained Vh (0.33 μM) in buffer D (●), in buffer D plus 50 mM $CaCl_2$ (▲), or in buffer D plus 50 mM $MgCl_2$ (○) and were titrated with Tb^{3+} as described under Experimental Procedures. Only the protein-dependent terbium emission intensity is shown.

Since Ca^{2+} ions did not totally eliminate protein-dependent terbium fluorescence in the Vl + Vh samples, and hence did not completely block Tb^{3+} binding to the protein, it was necessary to ascertain that Ca^{2+} ions did in fact bind to the high-affinity calcium site on factor Va at pH 6.5. The one-stage clotting assay was used to demonstrate that the factor Va activity was the same when Vh and Vl were incubated (37 °C, 90 min) in the presence of 1 mM (or 50 μM) Ca^{2+} at either pH 7.5 or pH 6.5 (data not shown). Hence, Ca^{2+} does fill the factor Va site at pH 6.5 in our terbium experiments, and the Ca^{2+}-sensitive terbium emission observed with Vh and with Vh + Vl may therefore represent the binding of Tb^{3+} to the calcium ion binding site that is required for function. If so, the data are consistent with this site being located on the Vh subunit, because the magnitudes of the Ca^{2+}-dependent intensity changes were very similar for Vh and for Vh + Vl. However, this conclusion is not unambiguous, as discussed below.

Discussion

A primary goal of this study was to identify the nature of the structural change which accompanies the association of Ca^{2+} with Vl and Vh. Thus, a variety of spectroscopic techniques were employed to probe the Ca^{2+}-dependent changes in protein conformation, including techniques sensitive to changes in secondary structure (CD), in tertiary and quaternary structure (intrinsic fluorescence), in nonpolar surface area (ANS emission), and in metal ion binding to proteins (protein-dependent terbium emission). Taken together, the spectroscopic data reported here and the sedimentation results published earlier (Laue et al., 1984) indicate that the association of the single calcium ion with Vh and Vl to form functional factor Va does not elicit a major restructuring of either of the polypeptides but does elicit a change in quaternary and possibly tertiary structure that can be detected spectroscopically. Furthermore, this spectroscopically detected structural change(s) appears to be required for function, since its occurrence correlated directly with the recovery of factor Va activity.

These conclusions are justified by the following observations. First, the circular dichroism spectra of Vl and of Vh were the same in the presence and absence of calcium, and no significant

change in CD signal was observed when the two subunits associated to form factor Va (Figure 1). Hence, there is no apparent change in the secondary structure of the subunits when they are exposed to Ca^{2+} and associate with each other. Also, the sedimentation velocities of Vl and Vh were the same in the presence and absence of calcium (Laue et al., 1984), which indicates that the overall shape of the individual subunits is not significantly altered by calcium. Thus, our CD and sedimentation data appear to rule out the possibility that Ca^{2+} nucleates major polypeptide rearrangements. On the other hand, the intrinsic fluorescence emission was blue shifted and the total emission intensity was slightly reduced when an equimolar mixture of Vl and Vh was titrated with Ca^{2+} (Figure 3). In addition, the near-UV CD spectrum was altered when factor Va was formed from the individual subunits by the addition of calcium (Figure 2). The fluorescence change was half-maximal near 50 μM and was reversible upon addition of EDTA. This spectral change required both subunits (data not shown) and was also ion specific, because Mg^{2+} did not elicit any fluorescence change, while Mn^{2+} elicited changes in intrinsic fluorescence equivalent to those of Ca^{2+} (Figure 4). Thus, the association of Ca^{2+} with Vl and Vh to form factor Va elicits a structural change that alters the environment of one or more of the tryptophans and tyrosines of factor Va.

A blue shift in intrinsic protein emission typically results from the movement of a tryptophan(s) and/or a tyrosine(s) to a more nonpolar environment. Thus, it would appear that the formation of factor Va causes a change in either tertiary or quaternary structure that reduces the exposure of tryptophans and/or tyrosines to the aqueous medium. A likely explanation for the blue shift in emission is that Trp and/or Tyr residues are located on the subunit surface at the Vl–Vh interface and are buried when the subunits associate in the presence of Ca^{2+} to form factor Va.

This interpretation is supported by two different types of experiments. First, the extent of photo-cross-linking of lipophilic photoreagents to the subunits was substantially higher in the absence of calcium (when subunits were dissociated) than in the presence of calcium (when subunits were associated) (Krieg et al., 1987). This indicates that the subunits had more nonpolar surface area exposed when they were dissociated than when they were associated. Second, the intensity of ANS emission was reduced when calcium ions were added to a sample to form factor Va from separated Vl and Vh subunits (Figure 6). Since the fluorescence intensity of ANS is much higher when it is bound to a nonpolar site on a protein than when the dye is free in solution (Weber & Laurence, 1954; Stryer, 1965), the total aqueous-exposed nonpolar surface area of Vh and Vl appears to be reduced when the two subunits associate to form factor Va. It therefore seems likely that the structural change monitored by the ANS is the calcium-dependent association of the factor Va subunits and that the decrease in ANS emission intensity results from the loss of ANS binding sites when subunit association causes hydrophobic regions located at the subunit–subunit interface to become inaccessible for ANS binding. Thus, three independent sets of data indicate that nonpolar regions on the surfaces of the subunits are buried when the subunits associate, and hence strongly suggest that hydrophobic interactions between Vl and Vh are involved in the formation of factor Va.

Of course, the above data and rationale do not rule out the possibility that the blue shift in intrinsic emission and/or the loss of ANS binding sites/emission intensity results primarily, or even solely, from a change in tertiary structure. Also, even though the 30% reduction in ANS fluorescence has the same calcium dependence (half-maximal near 50 μM) as the change in intrinsic fluorescence, it is conceivable that the two spectral techniques are monitoring different aspects of the same process.

It is important to emphasize that the structural change(s) detected by the change in both intrinsic and extrinsic fluorescence appeared to be directly related, both in its kinetics and in its dependence upon $[Ca^{2+}]$, to the structural change(s) required to obtain an active factor Va (Figures 5 and 6). Both the activity and the spectral changes were half-maximal at a Ca^{2+} concentration near 50 μM, and the rates of the intrinsic and ANS emission changes were very similar to the rates of recovery of factor Va activity (Figures 5 and 6B). Furthermore, the metal ion dependencies of the intrinsic and extrinsic fluorescence changes (Figures 4 and 6A) correlate with the metal ion dependence of the activity (Esmon, 1979), since Vl and Vh can be reconstituted into active factor Va using either Mn^{2+} or Ca^{2+}, but not Mg^{2+}. Hence, the changes in both intrinsic and extrinsic fluorescence appear to coincide with the formation of active factor Va molecules.

Another issue examined in this study was the nature of the secondary structure of factor Va and its subunits. The CD spectra of Vl, Vh, and intact factor Va (Figure 1) showed that the predominant folding pattern of the polypeptide chains in functional factor Va, as well as the individual subunits, is that of a β-sheet. The α-helix content of each of these proteins was low (<15%). It should be noted that ceruloplasmin, which has some sequence similarity to factor Va (Church et al., 1984), has also been shown to have a high percentage of β-sheet structure (Hibino et al., 1968, 1969).

A question that remains unanswered is where the single tight calcium ion binding site is located in the factor Va molecule. The similar sizes and coordination properties of Tb^{3+} and Ca^{2+} have previously permitted the use of protein-dependent emission from terbium ions as a means of monitoring terbium ion binding to calcium ion binding sites [e.g., see Martin and Richardson (1979), Johnson et al. (1983), and Morita et al. (1984)]. Since terbium can form active factor Va in the absence of calcium (Figure 7), the Tb^{3+} ion provided a promising approach to investigate metal ion binding to factor Va. In particular, because the binding of the triply charged terbium ion to the protein can be tighter than that of the divalent calcium ion [e.g., see Johnson et al. (1983) and Morita et al. (1984)], we were interested to see whether either subunit of factor Va would exhibit protein-dependent terbium fluorescence, in hopes of identifying which subunit contained the Ca^{2+} binding site.

The binding of Tb^{3+} to Ca^{2+}-specific sites was observed both with Vh and with Vh + Vl. This was shown both by the reduction in total protein-dependent terbium emission and by the decrease in apparent affinity for terbium that was caused by the addition of Ca^{2+}, and not by the addition of Mg^{2+}, to the sample (Figure 8; data not shown). However, since only 15% of the total protein-dependent terbium emission was sensitive to the presence of Ca^{2+}, it is clear that there are other binding sites for terbium ions on the proteins. It is not clear whether the small magnitude of the calcium-sensitive terbium emission is due to this Ca^{2+} binding site on Vh and factor Va being located relatively far from tryptophans or tyrosines [compared to the other terbium binding site(s)] or to there being many more calcium-insensitive than calcium-sensitive binding sites for terbium ions on the proteins. It is also interesting to note that the calcium-specific terbium emission intensity did not increase markedly upon association of Vl and Vh to form factor Va (Figure 7). This suggests either that no new binding site for Tb^{3+} was created by the association

of Vh and Vl or that such a site was located too far from Trp or Tyr residues for significant energy transfer to occur.

An important question is whether the calcium-sensitive terbium binding that we have observed spectroscopically represents Tb^{3+} binding to the functionally important Ca^{2+} site or to another site(s) that binds Ca^{2+} but not Mg^{2+}. Because the large calcium-insensitive terbium fluorescence intensity obscures the calcium-sensitive signal, we have not been able to show a direct correlation between the functional state of factor Va and the fluorescence-detected binding of Tb^{3+} to a Ca^{2+} binding site on the protein. Thus, the terbium data are consistent with the single high-affinity Ca^{2+} site in factor Va being located largely on Vh, but this interpretation is complicated by the large amount of Ca^{2+}-insensitive terbium binding and by the absence of a Vl-dependent increase in Ca^{2+}-sensitive terbium emission.

The inability of equilibrium dialysis (Guinto & Esmon, 1982), sedimentation (Laue et al., 1984), and various spectroscopic techniques (this paper) to detect the binding of Ca^{2+} to Vh or Vl individually shows that both subunits are required, either directly or indirectly, to create or stabilize the single high-affinity Ca^{2+} binding site of factor Va. One possible explanation for these data is that each subunit possesses part of the metal ion binding site and that the single high-affinity binding site for calcium is located between Vl and Vh. If this is the case, the terbium data suggest that the metal ion is coordinated primarily to the Vh subunit. An alternative possibility is that one subunit does not interact directly with the Ca^{2+} ion but is required to stabilize or induce a conformational change in the other subunit that increases its affinity for the calcium ion. However, Vl appears to have little affinity for Vh in the absence of Ca^{2+} (Laue et al., 1984), and no significant Ca^{2+}-dependent secondary structure changes were detected by CD when factor Va was formed from Vl and Vh (Figure 1). Thus, it would appear that the high-affinity metal ion binding site is created by the appropriate juxtaposition of Vh and Vl and that the strongly bound Ca^{2+} ion bridges the two subunits. Such an arrangement is not unprecedented, for recent results indicate that the binding of protein C to thrombomodulin involves a calcium ion bridge (Galvin et al., 1987; Kurosawa et al., 1987). Such an arrangement may also explain why the two subunits do not associate appreciably in the absence of calcium (Laue et al., 1984), even though hydrophobic (and presumably Ca^{2+}-independent) interactions are involved in subunit association (Figure 6; Krieg et al., 1987). We speculate that negatively charged moieties on each subunit are involved in forming the calcium ion binding site and that, in the absence of calcium, the electrostatic repulsion between these groups destabilizes the Vh–Vl association. On the other hand, we note that high Ca^{2+} concentrations did not inhibit subunit association (Guinto & Esmon, 1982) and that the Ca^{2+}-specific terbium emission did not increase upon subunit association (above), so not all of the data fully support a two-subunit metal ion binding site in factor Va.

No matter what model of Ca^{2+} binding to Vh and/or Vl is favored, it is difficult to explain the slow kinetics of the Ca^{2+}-dependent recovery of active factor Va from Vh and Vl. The data presented here have ruled out the most likely explanation for the slow kinetics: we were unable to detect any major polypeptide structural rearrangements that accompanied the association with Ca^{2+}. Thus, it appears that the high activation energy barrier does not result from a substantial intra- or intermolecular structural change that is nucleated in Vh and/or Vl by the binding of a Ca^{2+} ion. Instead, the rate-limiting step appears to be a more subtle rate-limiting conformational change that is required upon, or prior to, subunit association, based upon the strong correlation between the recovery of activity and the intrinsic and extrinsic fluorescence changes (Figures 5 and 6). Since the magnitudes of the spectroscopic changes that accompany the metal ion dependent structural change are small, it would appear that the critically important conformational change either is small in magnitude and/or occurs in a spectroscopically silent region or domain of the protein.

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Inhibition of Eukaryotic Translation by Nucleoside 5′-Monophosphate Analogues of mRNA 5′-Cap: Changes in N7 Substituent Affect Analogue Activity†

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ABSTRACT: Nucleotide cap analogues of 7-methylguanosine 5′-monophosphate (m^7GMP) were synthesized in which the 7-methyl moiety was replaced with 7-ethyl (e^7), 7-propyl (p^7), 7-isopropyl (ip^7), 7-butyl (b^7), 7-isobutyl (ib^7), 7-cyclopentyl (cp^7), 7-(carboxymethyl) (cm^7), 7-benzyl (bn^7), 7-(2-phenylethyl) [7-(2-PhEt)], and 7-(1-phenylethyl) [7-(1-PhEt)]. These derivatives were assayed as competitive inhibitors of capped mRNA translation in reticulocyte lysate. We observed that N7 alkyl and alicyclic substituents larger than ethyl significantly decreased the inhibitory activity of these cap analogues presumably by decreasing their affinity for cap binding proteins, which participate in the initiation of translation. This result defined a maximum size for this class of N7 substituents in the nucleotide binding domain of cap binding proteins. Like m^7GMP, the N7-substituted GMP derivatives synthesized in this study were found to be predominantly in the anti conformation as determined by proton NMR analyses. However, bn^7GMP and 7-(2-PhEt)GMP, which have aromatic N7 substituents, were more effective than m^7GMP as competitive inhibitors of translation. The increased affinity of bn^7GMP for cap binding proteins was further examined by synthesis of β-globin mRNA containing 5′-bn^7G, 5′-m^7G, or 5′-e^7G cap structures. These modified mRNAs were tested as translation templates. Messenger RNA capped with bn^7G was observed to increase the translation activity of the template 1.8-fold relative to that of its m^7G-capped mRNA counterpart. By contrast, e^7G-capped mRNA was 25% less active than m^7G-capped mRNA. UV photo-cross-linking of m^7G-capped mRNA to cap binding proteins was also inhibited to a greater extent by bn^7GMP than by m^7GMP or e^7GMP. Thus, from these data the inhibitory effect of bn^7GMP was due to its increased affinity for cap binding proteins and not by inhibition at another step of initiation.

One role of the 5′-cap structure [m^7G(5′)ppp(5′)N][1] found in all eukaryotic mRNAs is to facilitate ribosome binding during the initiation phase of translation (Shatkin, 1985; Banerjee, 1980). In eukaryotic translation, several key interactions between the 5′-cap of mRNAs and cap binding proteins are required for optimum binding of 5′-caps to these ligand binding proteins [Adams et al., 1978; Shatkin, 1985; Darzynkiewicz et al., 1981, 1985, 1987; for review of initiation, see Pain (1985)]. Individually, these proteins have been identified as eIF-4A (Grifo et al., 1982; Edery et al., 1983), eIF-4B, (Grifo et al., 1982), eIF-4E or CBP I (Sonenberg et

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[1] Abbreviations: m^7G, 7-methylguanosine; m^7GMP, 7-methylguanosine 5′-monophosphate; e^7GMP, 7-ethylguanosine 5′-monophosphate; p^7GMP, 7-propylguanosine 5′-monophosphate; a^7GMP, 7-allylguanosine 5′-monophosphate; ip^7GMP, 7-isopropylguanosine 5′-monophosphate; b^7GMP, 7-butylguanosine 5′-monophosphate; ib^7GMP, 7-isobutylguanosine 5′-monophosphate; cp^7GMP, 7-cyclopentylguanosine 5′-monophosphate; cm^7GMP, 7-(carboxymethyl)guanosine 5′-monophosphate; bn^7GMP, 7-benzylguanosine 5′-monophosphate; 7-(2-PhEt)-GMP, 7-(2-phenylethyl)guanosine 5′-monophosphate; 7-(1-PhEt)GMP, 7-(1-phenylethyl)guanosine 5′-monophosphate; CBP, cap binding protein; eIF, eukaryotic initiation factor; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; TEAB, triethylammonium bicarbonate; TLC, thin-layer chromatography.

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