Molecular Weight of Undegraded Plasma Factor V†

Kenneth G. Mann,* Michael E. Nesheim, and Paula B. Tracy

ABSTRACT: The molecular size and immunochemical properties of the unfractionated factor V present in plasma collected by venipuncture into a broad-spectrum anticoagulant and platelet-inhibited mixture were compared with those reported for the isolated, single-chain factor V molecule of 330 000 daltons. The anticoagulant–plasma mixture included 0.28% trisodium citrate, 1 mM benzamidine hydrochloride, 0.02% soybean trypsin inhibitor, 2.0 mM diisopropyl phosphorofluoridate, 10 μM dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide, and 5 μM prostaglandin E_1. The Stokes radius of unfractionated factor V present in highly inhibited plasma (93 Å) is virtually identical with the Stokes radius predicted from the hydrodynamic data for the highly asymmetric, single-chain factor V molecule (91 Å). With an expression which relates the Stokes radius and the sedimentation coefficient to the molecular weight of hydrodynamic units, the molecular weight obtained for factor V, using gel filtration data, is 336 000, in good agreement with the molecular weight determined from the sedimentation equilibrium, 330 000. In contrast, the Stokes radius for the factor Va present in serum is significantly smaller (50.5 Å) and equivalent to the Stokes radius obtained upon activation of isolated factor V with thrombin. Immunochemical comparisons of the factor V present in the inhibited plasma and isolated factor V were conducted by using burro antibovine factor V antibody and the technique of immunoelectrophoresis. The factor V antigen present in both sources is immunochemically identical, as is the electrophoretic mobility of both factor V preparations. These data serve to justify the conclusion that the factor V isolated as a single-chain 330 000-dalton molecule corresponds to the factor V circulating in plasma.

Factor V was discovered by Owren (1947a), who, in 1943, identified a patient with a congenital lack of this coagulation factor. Subsequent work by Owren (1947b), Ware & Seegers (1948), and Murphy & Seegers (1948) identified factor V as one of the essential components required for the rapid conversion of prothrombin to thrombin.

Early workers applied the term "labile factor" in discussing factor V because of the extreme instability of the protein during manipulation and storage. Because of this instability problem and the "procofactor" nature of the factor V (Nesheim et al., 1979a), the protein proved to be difficult to isolate. The first practical preparations of this protein were provided by Esnouf & Jobin (1967) and Barton & Hanahan (1967), and studies of preparations at this level of purity provided a great deal of insight into the function of factor V and the prothrombinase complex. Numerous subsequent reports of factor V purification have appeared in the literature (Colman, 1969; Dombrose & Seegers, 1974; Chulkova & Hernandez, 1975; Day, 1975; Smith & Hanahan, 1976; and Saraswathi et al., 1978). However, most preparations are complicated by the lack of homogeneity that is evident on electrophoretic analysis of the purified material and numerous conflicting assertions with regard to physical properties, subunits, etc. There is little doubt that one of the principal difficulties confronting investigators attempting to isolate factor V is in the susceptibility of the protein to proteolysis during activation (Nesheim & Mann, 1979; Esmon, 1979) and inactivation (Canfield et al., 1978).

Recently, two laboratories (Nesheim et al., 1978, 1979b; Esmon, 1979) have described preparation methods for factor V which provide a protein which is apparently homogeneous by the standard criteria normally applied to the estimation of protein homogeneity, such as a single component when analyzed by gel electrophoresis etc. We have used a variety of physical and chemical methods to establish the relative homogeneity and gross physical properties of the factor V isolated in our laboratory (Nesheim et al., 1979a,b; Tracy et al., 1979; Bloom et al., 1979; Hibbard & Mann, 1980). The protein was studied not only in the native state but also after denaturation and disulfide bond reduction in 6 M guanidinium chloride by using the technique of sedimentation equilibrium analysis in the analytical ultracentrifuge. Under all conditions, a molecular weight consistent with a single-chain protein of 330 000 was obtained. Sedimentation velocity analysis of the same protein preparation under native conditions indicated a sedimentation coefficient ($s^0_{20,w}$) of 9.19 S, which suggests that the single-chain protein is highly asymmetric.

Esmon (1979) has reported that his factor V preparation technique results in the isolation of a protein with an apparent molecular weight consistent with 330 000 when evaluated by detergent gel electrophoresis. Gel filtration analysis of the same preparation, however, suggested a high molecular weight for the protein, with this value approaching 850 000. The observations of Esmon can be rationalized with those of our laboratory if one examines all the physical data provided by both laboratories for the factor V molecule. The gel electrophoretic behavior and specific activities of the protein isolated in both laboratories are nearly identical, suggesting that the factor V isolated by both laboratories represents the same material. The sedimentation data provided by our laboratory suggest that the protein would likely behave anomalously when compared to globular proteins by using gel filtration. Thus, the gross physical data for the preparations reported by both laboratories are internally consistent.

Recently, Bartlett et al. (1980) have reported gel filtration and electrophoresis studies conducted with partially purified factor V which was obtained under conditions of high inhibitor concentration. These investigators concluded that the factor V they isolate is of much greater physical size than the factor V described by our laboratory and that of Esmon. These investigators also concluded that the single-chain factor V reported by our laboratory represents a proteolytic digestion product of plasma factor V. In addition, Saraswathi et al.

† From the Hematology Research Section, Mayo Clinic, Rochester, Minnesota 55901. *Received May 23, 1980.* This research was supported by Grant HL-17430D and by Blood Banking and Hemostasis Grant HL-07069 (M.E.N. and P.B.T.).

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(1978) have reported that the factor V isolated in their laboratory is composed of multiple, noncovalently associated subunits with a combined molecular weight of 1.2 million. In view of these conflicting reports, the present study was initiated in an attempt to ascertain the relationship between the factor V of apparently high molecular weight reported by Bartlett and co-workers, and Saraswathi and co-workers, and the single-chain molecule reported by our laboratory and that of Esmon.

Materials and Methods

Tris base was obtained from either Sigma or Schwarz/Mann. Soybean trypsin inhibitor was from Sigma; 6% agarose was obtained from Bio-Rad. Diisopropyl phosphorofluoridate (DFP)[1] was obtained from Sigma and benzamidine hydrochloride from Aldrich. Dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared as previously described (Nesheim et al., 1979c). Rabbit brain thromboplastin for factor V assays was prepared according to the method of Bowie et al. (1971). [^{125}I]NaI and [^{45}Ca]$CaCl_2$ were obtained from New England Nuclear. "Tagit" reagent [*N*-succinimidyl 3-(4-hydroxyphenyl)propionate] was from Sigma. Bovine blood was obtained either by venipuncture from the Institute Hills Farm of the Mayo Foundation or by postmortem exsanguination at the Rock Dell Meat Shop, Rock Dell, MN. Bovine factor V was isolated and assayed for procoagulant activity as previously described (Nesheim et al., 1979b). Mule antisera to bovine factor V were prepared as previously described (Tracy et al., 1979). Bovine factor V was labeled with ^{125}I by using the method of Bolton & Hunter (1973) as previously described (Tracy et al., 1979). Blue dextran was obtained from Pharmacia while bovine thyroglobulin, urease, catalase, rabbit muscle aldolase, and soybean trypsin inhibitor were obtained from Sigma. Factor Va was prepared by incubation of factor V with thrombin with the assessment of activated cofactor activity by means of the standard factor Va assay. Following development of maximum activity, factor Va was chromatographed on QAE-cellulose with the protein applied to the column in 0.02 M imidazole hydrochloride, 0.05 M sodium chloride, and 5.0 mM $CaCl_2$, pH 6.5, and eluted with a linear gradient of NaCl from 0.10 to 0.30 M.

Gel filtration experiments were performed on a 1.2 × 95 cm column containing 6% agarose (Bio-Rad). The column was prepared in, and equilibrated with, 0.01 M Tris-HCl and 0.10 M NaCl, pH 7.0, and operated at a flow rate of 5.3 mL/h with a hydrostatic head of 60 cm. Fractions were monitored for protein by absorbnance at 280 nm, for ^{125}I-labeled factor V(Va) by γ counting, for [^{45}Ca]$CaCl_2$ by scintillation counting, and for blue dextran absorbance at 610 nm. For experiments involving gel filtration of plasma, bovine blood was collected by venipuncture into an anticoagulant mixture. Following collection, the blood contained the following inhibitors at the final concentration indicated in parenthesis: trisodium citrate (0.285%), benzamidine hydrochloride (1 mM), soybean trypsin inhibitor (0.02%), DAPA (10 μM), DFP (2.0 mM), and PGE_1 (5.0 μM). Serum was prepared by collecting blood into plastic tubes in the absence of anticoagulant and allowing it to clot for 5 h. The clot was then removed and the sample centrifuged to remove residual debris.

Elution volumes (V_e) for substances eluted from the gel filtration column were estimated from the points of maximum concentration in the eluting buffer, and distribution coefficients (K_d) were calculated by using the expression

$$K_d = \frac{V_e - V_{Bd}}{V_i - V_{Bd}} \quad (1)$$

in which V_{Bd} is the elution position of blue dextran and V_i is the elution volume for $^{45}CaCl_2$.

The effective hydrodynamic radii (R_s) for proteins used to calibrate the gel filtration column were computed by using the expression

$$R_s = \frac{M_r(1 - \bar{v}\rho)}{6\pi\eta s N} \quad (2)$$

in which M_r is the molecular weight of the protein, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of water at 20 °C, η is the viscosity (in poise) of water at 20 °C, s is the reduced sedimentation coefficient of the protein in water at 20 °C extrapolated to 0 concentration, and N is Avogadro's number.

The molecular weights, sedimentation coefficients, partial specific volumes, and computed effective hydrodynamic radii for the standard proteins used to calibrate the column are presented in Table I. Gel filtration calibration curves were established by relating the empirical distribution coefficients, K_d, to the effective hydrodynamic radius of the polymers used, by use of the expression developed by Ackers (1967):

$$R_s = A - B\ \mathrm{erfc}^{-1}(K_d) \quad (3)$$

Acker's equation is based upon the assumption that for a given lot of resin a Gaussian distribution of pore sizes exists, and, thus, K_d can be treated as an explicit statistical function. In eq 3, the terms A and B are adjustable parameters, and $\mathrm{erfc}^{-1}(K_d)$ is the error function complement of K_d obtained from a table of statistical constants.[2] This equation has been shown to provide a linear representation for gel filtration data in terms of the Stokes radius over an extremely broad range (Mann & Fish, 1972; Mann et al., 1973).

All ultracentrifugation experiments were performed in 0.1 M NaCl, 0.025 M Tris-HCl, and 5 mM $CaCl_2$, pH 7.4. Sedimentation equilibrium experiments were performed by using the short-column, high-speed technique of Yphantis (1964). Initial protein concentration for these studies was 0.03%. Most sedimentation velocity experiments were carried

Table I: Physical Properties of Proteins Used to Calibrate Gel Filtration Columns

protein	$M_r \times 10^{-5}$	$s^0_{20,w} \times 10^{13}$ (s^{-1})	$\bar{v}$ (cm^3/g)	R_s (Å)	$\mathrm{erfc}^{-1}(K_d)$
thyroglobulin[a]	6.7	19	0.713	89	0.679
urease[b]	4.8	18.6	0.730	61.5	0.441
catalase[c]	2.6	11.6	0.730	53.2	0.382
aldolase[d]	1.6	7.9	0.742	46	0.333
factor V[e]	3.3	9.2	0.712	91.3	0.708

[a] Edelhoch & deChrombrugghe (1966). [b] Sumner et al. (1938). [c] Tanford & Lovrien (1962). [d] Kawahara & Tanford (1966). [e] Nesheim et al. (1979b).

[1] Abbreviations used: DFP, diisopropyl phosphorofluoridate; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; QAE-cellulose, diethyl(2-hydroxypropyl)aminoethylcellulose; PGE_1, prostaglandin E_1.

[2] The inverse error function complement of K_d, $\mathrm{erfc}^{-1}(K_d)$, may be extracted from tabular values of the error function and its inverse found in probability tables of many handbooks. Erfc is related to the error function, erf, by $\mathrm{erfc}(K_d) = \mathrm{erf}(1 - K_d)$ and its inverse by $\mathrm{erfc}^{-1}(K_d) = \mathrm{erf}^{-1}(1 - K_d)$ = the number whose error function is $1 - K_d$. To obtain $\mathrm{erfc}^{-1}(K_d)$ from the tables, (1) determine $1 - K_d$, (2) find the value $1 - K_d$ under the "erf X column", and (3) read the corresponding $\mathrm{erf}^{-1}(1 - K_d)$ from the "X column".

out in double-sector synthetic boundary cells with the protein boundary observed by using Schlieren optics. Sedimentation velocity experiments were also conducted by using the optical scanner and double-sector cells.

The sedimentation coefficient is related to the molecular weight by the expression (Tanford, 1960)

$$s^0_{20,w} = \frac{M_r(1 - \bar{v}\rho)}{Nf} \quad (4)$$

in which f is the frictional coefficient of the component with molecular weight M_r. The frictional coefficient f is a function of the Stokes radius, and vice versa:

$$R_s = \frac{f}{6\pi\eta} \quad (5)$$

In eq 5 the term η refers to the viscosity of water at 20 °C. These two expressions may be combined to obtain a relationship which expresses the molecular weight of the hydrodynamic unit expressed in terms of its reduced sedimentation coefficient and the Stokes radius:

$$M_r = \frac{s^0_{20,w}NR_s6\pi\eta}{1 - \bar{v}\rho} \quad (6)$$

Thus, sedimentation data obtained in the analytical ultracentrifuge and effective hydrodynamic radius data obtained from gel filtration measurements can provide for analysis of the molecular weight of a protein, irrespective of its gross shape. This approach avoids the obvious pitfall of attempting to use sedimentation velocity or gel filtration data directly to estimate the molecular weight of a native protein, since both of these terms depend upon the shape factors which are expressed in the frictional coefficient term.

Immunochemical comparisons of purified factor V, factor Va, and plasma were carried out by using purified burro antibovine factor V antibody. Immunoelectrophoresis was conducted in 1% agar by using the procedure of Grabar & Williams (1953). Electrophoresis was carried out in 0.05 M sodium barbital buffer, pH 8.6, at a final ionic strength of 0.05 M. Following immunodiffusion, the immunoelectrophoretograms were stained with amido black and destained by diffusion.

Results

The gel filtration behavior of factor V isolated by the procedure of Nesheim et al. (1979b) and factor Va obtained after thrombin cleavage and QAE chromatography is presented in Figure 1. The relative activity[3] of factor V (closed circles) or factor Va (open circles) is plotted as a function of fraction number. The elution position of blue dextran is indicated by the term V_0 while the elution position of $^{45}CaCl_2$ is indicated by the term V_1. The relative elution positions of thyroglobulin, urease, catalase, and aldolase are identified in Figure 1. It can be seen that factor V, isolated by the procedure of Nesheim et al. (1979b), elutes slightly ahead of thyroglobulin while QAE-isolated factor Va is eluted between catalase and aldolase. During chromatography on this gel filtration column, factor V was not converted to factor Va to any detectable extent.

The chromatographic data of Figure 1 were plotted according to the procedure of Ackers (1967) by using the experimental data obtained for the proteins used to calibrate the

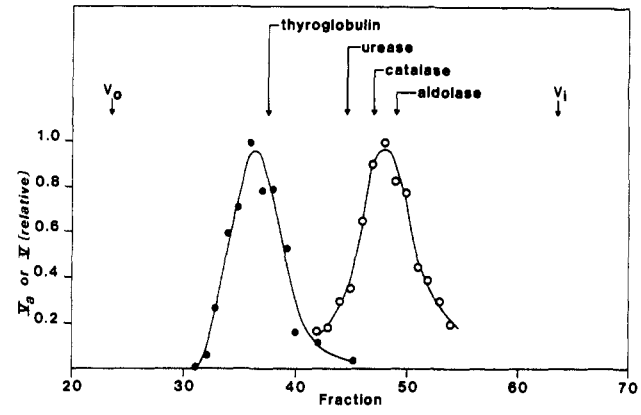

FIGURE 1: Gel filtration chromatography profiles for factor V (closed circles) and factor Va (open circles). The relative activity is plotted vs. fraction number. The elution positions of blue dextran (V_0), [^{45}Ca]$CaCl_2$ (V_i), and the protein markers thyroglobulin, urease, catalase, and aldolase are indicated.

column, aldolase, catalase, urease, and thyroglobulin. The physical data used to calculate the Stokes radius, R_s, for these proteins are presented in Table I, as are the $erfc^{-1}(K_d)$ values determined experimentally. The R_s determined for factor V in these experiments is 93 Å. The value is in good agreement with the value for R_s predicted for the asymmetric factor V molecule from the sedimentation equilibrium molecular weight of 330 000 and the reduced sedimentation coefficient ($s^0_{20,w}$) obtained for this protein (9.19 S). If one combines the $s^0_{20,w}$ obtained for factor V (9.19 S) and the R_s obtained with the gel filtration column (93 Å), a molecular weight of 336 000 is obtained for the factor V hydrodynamic unit by using eq 6. This value is in excellent agreement with that obtained for the isolated factor V native molecular weight determined by sedimentation equilibrium, 330 000.

In contrast to factor V, factor Va isolated on QAE-cellulose is substantially reduced in R_s. The value obtained from the gel filtration column is 50.5 Å. Factor Va, isolated by QAE-cellulose chromatography, was subjected to sedimentation velocity analysis. A value of 7.65 S was obtained at a concentration of 0.1 mg/mL, and the material appeared to be homogeneous during transport. Utilization of the R_s value determined by gel filtration (50.5 Å) and the sedimentation velocity data for factor Va results in an estimated molecular weight of 152 000. Sedimentation equilibrium analysis of the same preparation of factor Va was conducted. The plot of ln C vs. r^2 for this experiment was curved, indicating either that the protein was inhomogeneous with respect to molecular weight or that it was undergoing association–dissociation equilibria. The whole-cell molecular weight average obtained for this preparation was 185 000. Since factor Va is composed of multiple, noncovalently associated subunits, we cannot be certain at the present time whether the discrepancy between the sedimentation equilibrium weight average value (for the heterogeneous molecular weight dispersion) and the value obtained on gel filtration sedimentation velocity is due to association of the protein at the higher concentrations extant in the sedimentation equilibrium experiment or dissociation of the protein subunits during gel filtration analysis, or that both phenomena exist. In any event, it is clear that the molecular weight of the factor Va obtained from chromatography on QAE must be in the range of 152 000–185 000, and, clearly, factor Va is hydrodynamically smaller than factor V in this solvent system (0.1 M NaCl and 0.025 M Tris, pH 7.0).

Purified single-chain ^{125}I-labeled factor V, radioiodinated by using the procedure of Bolton & Hunter (1973), was subjected to gel filtration analysis. In this instance, the ra-

[3] For all assessments of procofactor (factor V) activity, samples were treated with thrombin (1 unit/mL) for 60 s prior to measure, 37 °C.

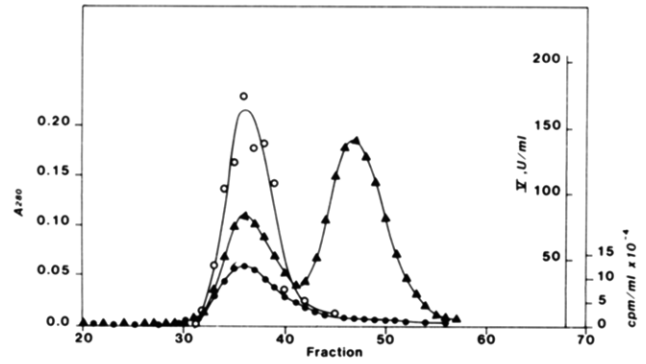

FIGURE 2: Gel filtration chromatographic profile for a mixture of purified factor V and catalase. The absorbance at 280 nm (closed triangles), factor V procoagulant activity (open circles), and ^{125}I-labeled factor V radioactivity (closed circles) are plotted as a function of fraction number.

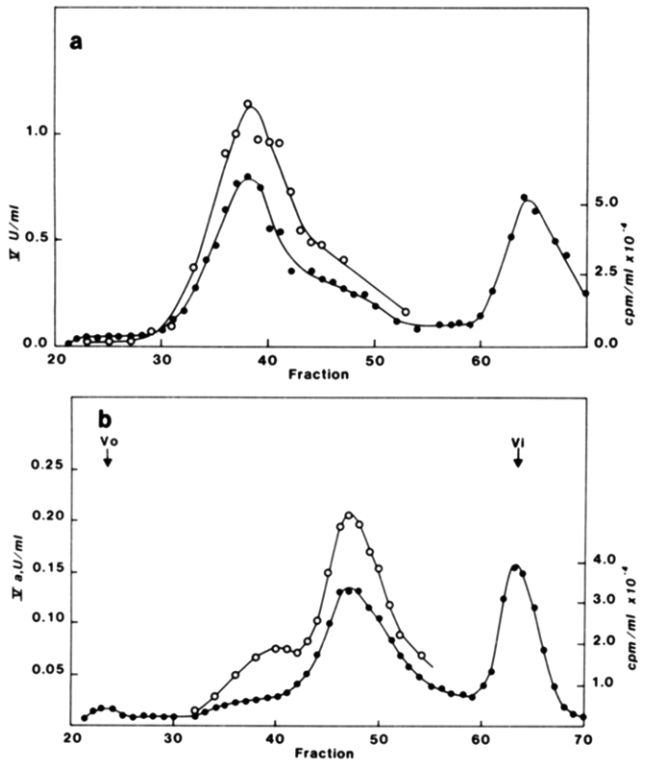

FIGURE 3: (a) Elution profile of a mixture of anticoagulated and inhibited plasma and ^{125}I-labeled isolated factor V. Factor V activity (open circles) and ^{125}I radioactivity (closed circles) are plotted vs. fraction number. Free iodide is present in the sample. (b) Chromatography profile for factor V activity in serum and thrombin-treated ^{125}I-labeled factor V. Factor V procoagulant activity and ^{125}I radioactivity are plotted vs. fraction number. Free iodide is present in the sample.

diolabeled protein was mixed with unlabeled factor V and catalase and applied to the same gel filtration column described in Figure 1. It can be seen (Figure 2) that the gel filtration behavior of factor V, in terms of absorbance at 280 nm (closed triangles) the radioactivity (closed circles), and the factor V activity[3] (open circles), all cochromatograph. Catalase, which has a R_s slightly larger than factor Va, is seen to be completely resolved from the factor V protein on the column.

Fresh bovine plasma was collected by venipuncture into a cocktail containing a variety of anticoagulants and inhibitors, including soybean trypsin inhibitor, DAPA, DFP, and PGE_1. Isolated, radiolabeled factor V was then added and the mixture subjected to chromatography on the same agarose column. The results, analyzed in terms of radioactivity and factor V

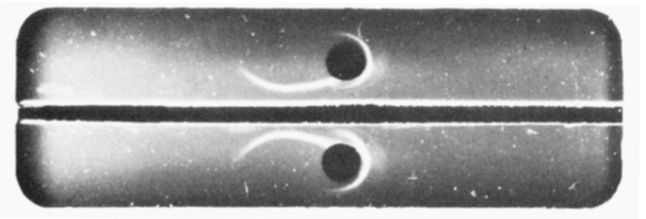

FIGURE 4: Immunoelectrophoretogram comparing bovine factor V mixed with bovine plasma (upper well) and purified factor V (lower well). The antisera used are burro antibovine factor V.

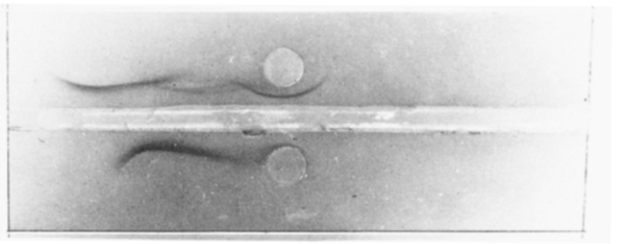

FIGURE 5: Immunoelectrophoretogram comparing thrombin-activated bovine factor V (factor Va)(upper well) with bovine plasma (lower well). The antisera used are burro antibovine factor V.

activity[3] present in the eluted fractions, are presented in Figure 3a. Here it can be clearly seen that the factor V activity present in the inhibited plasma sample coelutes with the factor V preparation which was radioiodinated after isolation and mixed with the plasma sample. Virtually the same R_s is obtained for factor V applied to the column as an unpurified anticoagulated plasma sample and factor V applied as the isolated protein.

In contrast, when serum is cochromatographed with isolated, radioiodinated factor V which has been intentionally activated by the addition of thrombin, the result presented in Figure 3b is obtained. In this case, the resulting chromatograph illustrates some residual unactivated factor V present in the serum sample (open circles) with the majority of the factor Va activity eluting at exactly the same position as the radioactive, isolated factor Va. Thus, both factor V and factor Va obtained after isolation and radioiodination are virtually identical in R_s with their plasma and serum counterparts.

Factor V isolated in the laboratory and factor V present in plasma were compared immunochemically by using burro antisera raised against purified bovine factor V. Figure 4 is a photograph of the immunoelectrophoretogram obtained when purified bovine factor V (lower well) and a mixture of purified bovine factor V and bovine plasma (upper well) were subjected to electrophoresis and subsequent immunodiffusion. A single precipitin arc is obtained in both cases. These data indicate (1) that purified factor V is immunochemically homogeneous when examined with its complementary antibody and (2) that the factor V antibody, raised in a burro against the purified antigen isolated in the laboratory, recognizes unpurified plasma factor V as being identical with the purified factor V.

In order to test the resolving power of the immunoelectrophoretic technique and the ability of the antibody to detect components derived during activation of factor V, an experiment was performed in which activated factor V (upper well) was subjected to immunoelectrophoretic analysis and compared to bovine plasma (lower well). The results are presented in Figure 5: it can be seen that with factor Va at least four arcs are observed, indicating at least four distinguishable components when analyzed by the technique of immunoelectrophoresis. This latter experiment is simply a control, indicating the resolving power of the technique when multiple antigenic

components are present in the system. Thus, the technique of electrophoresis in agar, followed by immunochemical identification, is supportive of the contention that the isolated factor V is identical electrophoretically and immunochemically with the factor V circulating in plasma.

Discussion

The gel filtration behavior of the factor V purified in our laboratory from bovine blood is entirely consistent with the hydrodynamic properties predicted from the sedimentation velocity and sedimentation equilibrium molecular weight data previously published for this protein (Nesheim et al., 1979b). Furthermore, the R_s determined from gel filtration, 93 Å, is nearly equivalent to the Stokes radius of thyroglobulin, a protein of better than twice the molecular weight. All of these data are consistent with our initial conclusion: factor V is a highly asymmetric rodlike single-chain molecule with a molecular weight of 330 000. The gel filtration derived R_s reported by Esmon (1979) for his native factor V preparation is 95 Å, and, thus, not only the sodium dodecyl sulfate gel electrophoretic behavior of the preparations purified by the two laboratories but also the hydrodynamic properties under native conditions are virtually identical.

The gel filtration behavior of the factor V isolated by our laboratory is indistinguishable from the gel filtration behavior of factor V in unfractionated bovine plasma which was carefully prepared by venipuncture in the presence of blood-clotting and platelet inhibitors. This observation indicates that from a hydrodynamic standpoint the factor V molecule we isolate most likely represents the undegraded plasma precursor of the activated factor Va molecule. In addition, immunochemical evaluation of unfractionated plasma factor V, using the technique of immunoelectrophoresis vs. purified burro anti-bovine factor V antibody, supports the contention that the factor V preparation reported by Nesheim et al. (1979b) provides for the isolation of undegraded plasma factor V.

The physical assessment of the molecular size of bovine factor Va is at present less than explicit, owing to the combination of subunit dissociation and molecular association processes occurring during the gel filtration or centripetal fractionation processes used to estimate either R_s by gel filtration or $\bar{M}_w$ by sedimentation equilibrium. However, it is clear that the molecular weight of activated factor V must lie in the range 152 000–185 000. The R_s obtained for factor Va is on the order of 50.5 Å, a value somewhat smaller than that reported by Esmon (1979), 59 Å. It should be noted here that our gel filtration experiments were performed in the absence of added calcium, as the addition of this metal ion would have precluded comparative experiments with unactivated plasma factor V samples. The dissociation constant of the tightly bound calcium in factor V is less than 10^{-8} M, and calcium at this concentration is routinely present in our buffer systems. The gel filtration experiment reported by Esmon was conducted in the presence of a higher calcium concentration, and this may also have influenced the aggregation state of factor Va. At present, it is impossible to say whether the discrepancy between the values reported by the two laboratories represents a difference in the association state, the technique of activation of factor V to factor Va, or some variability in the calibration of the gel filtration columns used to estimate R_s. Immunochemical assessment of factor V after activation by thrombin (i.e., factor Va) indicates a variety of electrophoretically separable, immunochemically recognizable species in factor Va.

Bartlett et al. (1980) have recently reported the gel filtration behavior of a partially purifed factor V preparation and have used this technique to estimate the molecular size of the factor V. On the basis of the gel filtration behavior of their partially purified factor V, these authors have concluded that the factor V they isolated is of much higher molecular weight than that isolated by the procedure of Nesheim et al. (1979b) and Esmon (1979). Further Bartlett et al. concluded that the protein prepared in both of the latter laboratories was highly degraded in view of its (assumed) smaller molecular weight when compared to their preparation. The gel filtration column used by Bartlett et al. was calibrated in terms of the molecular weights of a series of highly globular standard proteins. This approach is clearly subject to the pitfall of using gel filtration behavior to estimate the molecular weight of a protein of unknown shape. These authors also applied an electrophoresis transport system to estimate the molecular weight of factor V. However, this technique is subject to the same pitfall as gel filtration. Comparative techniques such as gel filtration provide valid estimations of molecular weight only when proteins of equivalent frictional ratio are compared. In contrast to the assertion of Bartlett and co-workers, the gel filtration behavior they obtained for their preparation of factor V was not at all inconsistent with the hydrodynamic behavior one would anticipate for the asymmetric 330 000-dalton factor V molecule. In fact, analysis of the data provided by Bartlett et al. in terms of R_s, using their protein standard elution position and the Ackers' (1967) procedure, suggests that the factor V they isolated has an R_s between 69 and 74 Å. Thus, their factor V not only is not larger than that reported by Nesheim et al., and by Esmon, but also may, in fact, be smaller.

Saraswathi et al. (1978) have reported that factor V has a molecular weight in the range of 1 200 000 and is composed of two heavy-chain (M_r 290 000) and two light-chain dimers (M_r 400 000). More recently, Ittyerah et al. (1980) have concluded that the heavy chain (M_r 290 000) represents a protein equivalent to the single-chain, M_r 330 000 factor V of Nesheim et al. (1979b). These investigators further conclude that the light-chain dimer not present in the preparations of Esmon (1979) and Nesheim et al. (1979b) was somehow inadvertently lost during isolation, perhaps because of exposure to solvents of high ionic strength. Ittyerah et al. (1980) have reported that immunoelectrophoretic analysis of plasma and their purified factor V vs. antibody raised to the purified factor V shows two discrete immunoprecipitin bands.

In contrast to these observations, our hydrodynamic data indicate no association of any consequence with any other plasma protein on the part of either the isolated single-chain factor V or the factor V antigen present in plasma. In addition, hydrodynamic behavior on gel filtration of plasma factor V indicates virtually the same R_s as that of the isolated protein. Thus, our data do not support the conclusion that there are any other associated subunit structures in addition to the single chain, M_r 330 000 factor V molecule. Further, since immunoelectrophoretic analysis of factor V antigen in plasma and purified factor V shows these to be identical, it is unlikely that any components represented antigenically or in terms of protein mobility have been deleted during isolation. Rather, it is more likely that the "light-chain protein" represents a contaminant in the preparation of Saraswathi et al. (1978).

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Estimation of Globular Protein Secondary Structure from Circular Dichroism†

Stephen W. Provencher* and Jürgen Glöckner

ABSTRACT: A new method is developed in which a circular dichroism (CD) spectrum is analyzed directly as a linear combination of the CD spectra (from 190 to 240 nm) of 16 proteins whose secondary structures are known from X-ray crystallography. This avoids the dilemma encountered in previous methods of trying to define single reference CD spectra that were supposed to characterize such broad and variable classes as helix, β sheet, β turn, and "remainder". It also permits a more accurate and flexible analysis. The usual instability in using so many parameters is automatically controlled by a simple constrained statistical regularization procedure (similar to ridge regression). Sixteen tests were made by removing 1 spectrum at a time from the set of 16 and analyzing it in terms of the other 15. The product moment correlation coefficients between the computed fractions of helix, β sheet, β turn, and remainder and the fractions from the X-ray data were 0.96, 0.94, 0.31, and 0.49, respectively. Thus, the helix and β-sheet accuracy is very good. (The corresponding values calculated by a previous method with four reference spectra were 0.85, 0.25, −0.31, and 0.46.)

CD[1] is a convenient and widely used method for studying the conformations and conformational changes of globular proteins in solution. The usual procedure for estimating secondary structure composition is to approximate $y(\lambda)$, the mean residue ellipticity of the protein at wavelength λ, simply by a linear superposition of a small set of N_f reference spectra, $r_i(\lambda)$, each of which is supposed to be characteristic of a particular conformational class:

$$y(\lambda) = \sum_{i=1}^{N_f} f_i r_i(\lambda) \quad (1)$$

where f_i is the fraction of residues in class i, and the $r_i(\lambda)$ values are previously determined from CD spectra of model polypeptides (Greenfield et al., 1967) or globular proteins (Saxena & Wetlaufer, 1971; Chen et al., 1972) whose secondary

† From the European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, Federal Republic of Germany. *Received June 3, 1980.*

[1] Abbreviation used: CD, circular dichroism.

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