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Studies on the Structure of Bovine Factor V by Scanning Transmission Electron Microscopy

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We studied purified bovine factor V (mol wt 330,000) by scanning transmission electron microscopy (STEM) of freeze-dried unstained or negatively contrasted preparations. Freeze-dried molecules revealed discrete shapes ranging from roughly spheroidal (100 to 120 nm) to oblong (140 to 200 nm in length × 50 to 100 nm in width). Oblong shapes could often be resolved into two or three distinct domains, ranging from 60 to 100 nm in diameter. A "satellite" nodular structure (30 to 50 nm in diameter) connected to the main molecule by a thin stalk (~10 nm wide) up to 80 nm in length was occasionally seen. Glutaraldehyde-treated preparations yielded the same shapes as were seen in unfixed preparations but revealed better definition of submolecular features and "satellite" nodules. STEM mass analysis confirmed that each of the different shapes represented a monomolecular form of factor V. Negatively stained images revealed objects having the same general shapes as freeze-dried molecules, although greater detail was evident. Some images suggested that molecules consist of five or more discrete parts. Taken together, these observations indicate that factor V molecules are multidomainal, flexible structures that tend to have an irregular oblong shape with an axial ratio between 3:2 and 2:1.

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NATIVE BOVINE[1-6] or human[7-10] factor V is a single-chain molecule having a molecular weight (mol wt) of 330,000.[4,5] Studies of the molecule by sedimentation equilibrium and sedimentation velocity under denaturing and nondenaturing conditions, as well as by gel filtration, indicate substantial asymmetry.[1,2,5,11] The hydrodynamic radius has been calculated to be 9.0 to 9.5 nm, and the molecule behaves in solution like a rod-like structure.[1,2,11] Its mol wt and hydrodynamic properties are similar to those of fibrinogen, although unlike the hexameric disulfide-linked fibrinogen molecule,[12,13] factor V is composed of a single polypeptide chain.

Activated factor V in combination with phospholipid (or platelets) and Ca^{2+}, increases the rate of the factor Xa-catalyzed conversion of prothrombin to thrombin by more than five orders of magnitude.[14,15] Factor Va promotes the assembly of prothrombinase, a multimolecular complex that, in model systems or on platelets in vivo, comprises a Ca^{2+}-dependent 1:1 assembly of factor Xa and factor Va on phospholipid vesicles.[14,16-19] Rate enhancement is accomplished both by facilitation of binding as well as by surface "localization" of the serine protease. This occurs by virtue of the high affinity of factor Va for both phospholipid (or platelets) and factor Xa, plus a 3,000-fold increase in k_{cat} of factor Xa toward prothrombin that is elicited by the presence of the cofactor.[15,17,20]

In an attempt to answer structural and conformational questions concerning factor V, its conversion to factor Va, and formation of the "prothrombinase" complex, we have used scanning transmission electron microscopy (STEM) as a means for visualizing the native factor V molecule. STEM, in particular, provides a powerful tool for the approach not only because of the high image resolution that can be obtained,[21,22] but also because freeze-dried unstained protein molecules or collections of molecules can be visualized readily by this technique and the mass of each object subsequently determined. Images of freeze-dried molecules probably reflect the configurations of molecules in solution[21-24] and, in any case, are not complicated by the usual air drying and negative staining artifacts that accompany more standard electron microscopy specimen-processing techniques.[21] Furthermore, mass analysis of STEM images, coupled with hydrodynamic or electrophoretic data on mol wt, permits unambiguous identification of objects that represent monomolecular, bimolecular, or higher-order complexes or collections of molecules.[21-24]

In this report we present analyses of STEM images of bovine factor V, the results of which should provide a solid basis for further electron microscopy studies of its domains, its activation, and its participation in assembly of the prothrombinase complex.

MATERIALS AND METHODS

Preparation of Bovine Factor V

Factor V was isolated as described by Nesheim et al.[25] The final material (10 mg/mL) was stored at −20 °C in 0.01 mol/L Tris-borate, 1 mmol/L $CaCl_2$, and 50% glycerol solution, pH 6.5. As

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assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis,[26] the material migrated as a single band corresponding to single-chain factor V, and was stable under the conditions of storage for longer than one year.

Scanning Transmission Electron Microscopy

High-resolution STEM was performed at the Brookhaven STEM Biotechnology Resource using a 40-kV probe. The preparation of thin carbon films and the method of specimen application for freeze-drying or negative contrasting were as previously reported.[22] A stock solution of factor V was diluted to a final concentration of 5 to 50 μg/mL with one of the following solutions: 0.15 mol/L NaCl, 0.01 mol/L Tris-HCl buffer, pH 7.4; H_2O; 0.10 mol/L imidazole buffer, pH 7.0. These diluted protein solutions were then applied to a grid and processed for freeze-drying or negative contrasting (ammonium molybdate 2%, or uranyl sulfate 2%).[22] Certain diluted specimens were also incubated for about 30 seconds in 0.10 mol/L imidazole buffer, pH 7.0, containing 0.1% glutaraldehyde, before application to a grid and subsequent processing. Alternatively, glutaraldehyde-containing protein solutions were dialyzed overnight against an imidazole buffer before grid application.

Mass measurements were performed off-line using a "circle" program.[22,27] Tobacco mosaic virus particles that had been added to the specimen were used as an internal mass calibration standard.[21,28]

RESULTS

Freeze-Dried Images of Factor V

Our STEM observations are best considered in context with the known hydrodynamic and physical properties of the molecule (Table 1). Freeze-dried images of factor V deposited at 10 μg/mL revealed discrete particulate forms that were roughly spheroidal to oblong in shape (Fig 1). The diameters of the spheroidal forms were about 10 to 12 nm, whereas the oblong shapes were 14 to 20 nm in length and 8 to 10 nm in width.

Glutaraldehyde-treated molecules (Fig 1b) gave substantially the same appearance as unfixed molecules, except that oblong forms were more frequent and revealed somewhat better definition of submolecular features. Oblong shapes could often be resolved into two approximately spheroidal domains, the smaller of which (5 to 9 nm) usually amounted to ~⅓ the size of the larger (8 to 12 nm) (panels d, e, g, and n). Some objects had an hourglass appearance (panels d and g). In some cases, elongated forms appeared as three distinct contiguous domains (panels i and j). This form and others were better resolved in images of negatively stained preparations (see later). Commonly, a spheroidal "satellite" nodule (3 to 5 nm) was quite distinct from the main molecule (as far away as 8 nm) and evidently connected to it by a narrow stalk (arrows, panels f, h, l, and m).

Mass analysis of unstained freeze-dried images was used to investigate whether the pleomorphic shapes described above represented monomolecular forms of factor V. The observed mass of these forms was evaluated for a set of particle measurements as a function of the radius of integration (Fig 2). The apparent mass increased as the integration radius increased; 96% of the mass in this series of monomolecular particle measurements was included by a radius of 9 nm, and the remainder at an integration radius of 10 nm. This finding is consistent with the observation that the longest dimensions of factor V molecules range up to 20 nm, and that satellite structures, which are remote from the main mass, are commonly observed. At the optimum integration radius of 9 to 10 nm, the mass of factor V molecules corresponded well to the established mol wt of the molecule (Table 1) and indicated that each of these pleomorphic shapes reflected randomly disposed single molecules of factor V. The estimated mass of glutaraldehyde-fixed molecules was somewhat higher than that of unfixed molecules, and is consistent with the fact that intramolecular fixation of proteins by glutaraldehyde results in mass increases that are related to their lysine content.[29,30]

Negatively Stained Preparations

Negatively stained preparations revealed objects that had the same general shape as did freeze-dried molecules, although considerably more submolecular detail was evident. Such details were generally not discernible in the spheroidal particles, suggesting that these forms either represented relatively folded or compact versions of factor V molecules, or that their positioning on the grid surface presented an end-on view, or some combination of the two possibilities. In some cases, as was observed in unstained preparations of molecules, a roughly spherical portion of a molecule

Table 1. Mass Analysis of Bovine Factor V

Preparation or Object Analyzed	Molecular Weight (kd): By Physical and Other Measurements	Molecular Weight (kd): By STEM Mass Analysis	n	SD (kd)	SE (kd)
Factor V	330	320	28	±43	±8
Glutaraldehyde-fixed factor V	376	336	29	±37	±7

Other relevant hydrodynamic properties of factor V include $s_{20,w}$, 9.2S; stokes radius, 9.12 nm; frictional ratio, f/f_{min}, 2.01.[1,4,5] Factor V contains 148 lysine residues/molecule.[1] Assuming the addition of three glutaraldehyde (FW 104) residues per lysine amino group,[29,30] the expected mass of a fully reacted glutaraldehyde-fixed factor V molecule would be increased by 14% to 376,000.

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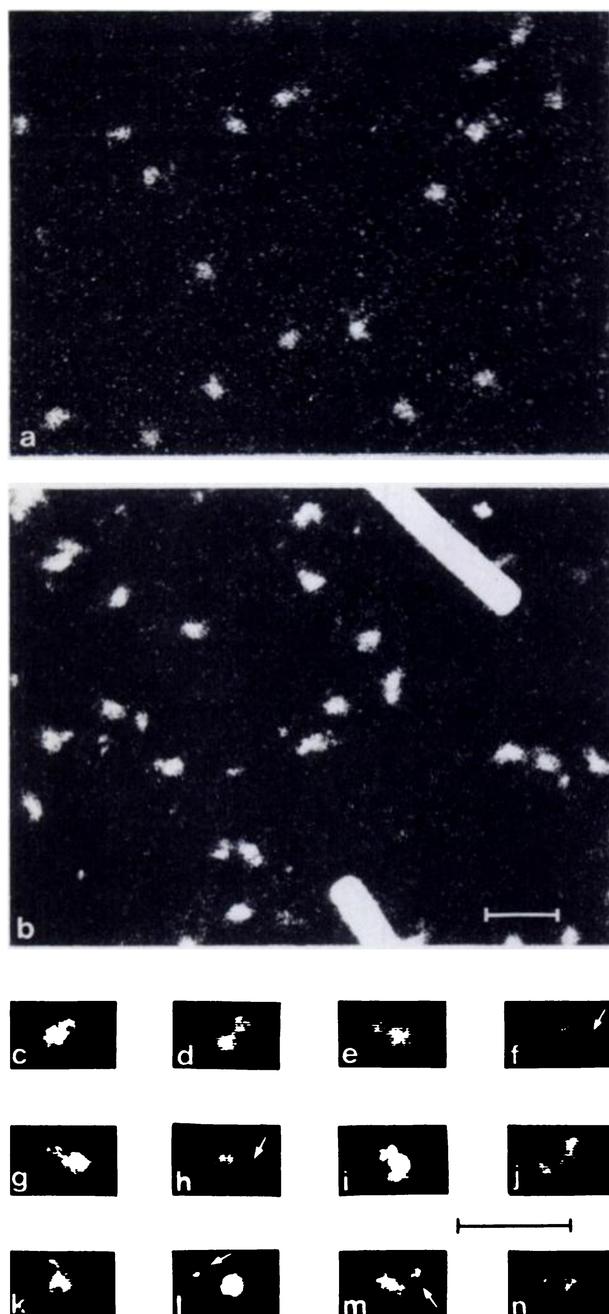

Fig 1. STEM images of freeze-dried factor V. Panel a, full field view of factor V molecules that had been deposited at a protein concentration of 10 μg/mL in a Tris-buffered 0.1 mol/L NaCl solution, pH 7.4, washed two times with buffer, and then washed three times with water before freeze-drying. Panel b, full field view of factor V molecules that had been diluted to a concentration of 10 μg/mL in 0.1 mol/L imidazole buffer, pH 7.0, containing 0.1% glutaraldehyde, deposited on the grid and washed three times with water before freeze-drying. Tobacco mosaic virus, which appear in this field as cylindrical electron-dense shapes ~18 nm in diameter, was added to the specimen with the final water wash. Magnification ×250,000; bar = 40 nm. Below panel b is a gallery of single molecules (each verified by mass analysis) selected from several micrographs of freeze-dried factor V prepared in the absence (c through f) or presence of glutaraldehyde (g through n). Magnification ×375,000; bar = 40 nm. The arrows in panels f, h, l, and m indicate the presence of the "satellite" structure.

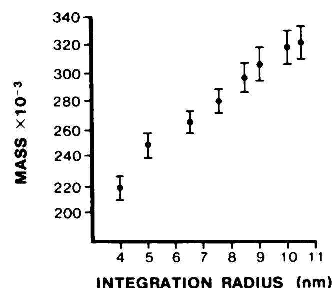

Fig 2. Mol wt values for freeze-dried factor V molecules that had been deposited in 0.1 mol/L imidazole buffer, pH 7.0. The mass is plotted as a function of the radius of integration (nm). Vertical bars indicate the standard error for each determination.

was distinctly separated from the main portion of the molecule, although remaining attached to it by a thin stalk about 1 nm wide (arrow, panel a; panels f and g). Glutaraldehyde-fixed negatively stained factor V molecules presented essentially the same assortment of shapes that were seen in unfixed negatively contrasted preparations.

Many images of oblong shapes indicated that there were more than three distinguishable domains, although they were not always clearly defined. In some images it is easy to identify at least five more-or-less discrete parts (panel d). In such projections one of the outer domains was resolved into two smaller structures in the shape of a barbell, whereas the middle portion of the molecule had a complex shape with at least two recognizable components. It is difficult to discern which portion of the molecule gives rise to the "satellite" structure, although in many projections it appears to originate from an outer barbell-shaped region (panel f).

DISCUSSION

This study presents high-resolution STEM images of the bovine factor V molecule. Mass analysis of freeze-dried preparations provides unambiguous evidence that the various shapes we have described represent monomolecular forms of factor V. Structural pleomorphism, as discussed later, is attributable to factors other than molecular heterogeneity.

Many factor V molecules can be resolved into at least three domains, and often as many as five more-or-less discrete domains can be seen. Among these structures is included resolution of one of the outer domains into a barbell-shaped structure (Fig 3d). Furthermore, at least one portion of the molecule is capable of extending from the main structure as a satellite nodule. It is not clear yet from which region such a structure arises, although it appears to originate from a barbell-shaped region. Factor V molecules appear to be flexible in that the domains can appear in different spatial relationships with respect to one another, some giving an extended appearance (Fig 3b

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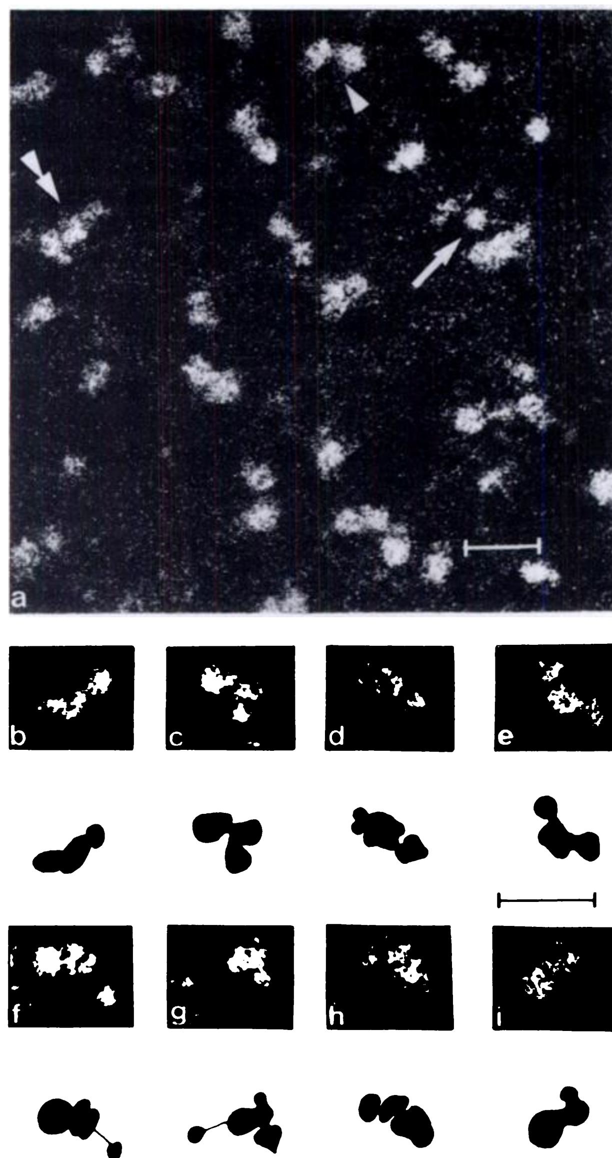

Fig 3. STEM images of negatively stained factor V. The material in the full field view, panel a, had been deposited at a concentration of 20 μg/mL in Tris-buffered, 0.1 mol/L NaCl solution, pH 7.4, and then negatively contrasted with ammonium molybdate. Magnification ×500,000; bar = 20 nm. The arrow in panel a indicates the satellite nodule extending from a factor V molecule on its right. The arrowhead indicates an hourglass form, whereas the double arrowhead indicates a tridomainal form with a nodular projection on one outer domain that could represent an unextended satellite nodule. Beneath panel a is a gallery of single molecules that had been deposited in TRIS buffer, pH 7.4 (b through g), or H_2O (h and i) and then negatively contrasted with uranyl sulfate. Interpretive silhouette drawings of the various shapes appear beneath these panels. Magnification ×625,000; bar = 20 nm.

and e), others somewhat folded (Fig 3c and h). Thus, structural pleomorphism of factor V is attributable to partial folding or unfolding of molecules or to differences in positioning of molecules on the grid surface.

Satellite nodules are observed more commonly in glutaraldehyde-fixed freeze-dried images than in unfixed freeze-dried preparations. One possible explanation is that unfixed structures become more spread on the grid surface than do glutaraldehyde-treated molecules, and thus are less evident to the naked eye. The observed length of many factor V molecules (up to 200 nm) plus the distance between satellite structures and the main molecular mass may explain why an integration radius of more than 9 nm is needed to include the entire mass of a population of factor V molecules (Fig 2).

Mann et al[5] suggested on the basis of hydrodynamic data that factor V is a highly asymmetric molecule, possibly having a rod-like shape. The STEM data obtained in this study indicate that the factor V molecule is asymmetric and, exclusive of satellite nodules, has an axial ratio in the range of 3:2 to 2:1. Although this axial ratio alone does not deviate greatly from a spherical shape, other factors, such as the presence of an extended satellite nodular structure, could contribute significantly to hydrodynamic properties, indicating a high degree of asymmetry.

The three to five distinguishable domains we have visualized may correspond to domains ultimately separated by proteolytic cleavage during activation. Single-chain factor V, when fully activated by thrombin, yields four peptides derived from regions existing outside of disulfide bonds.[31] Two of these, with respective masses of 94 kd and 74 kd, are derived from the NH_2 and COOH terminii of the parent factor V, respectively; together they constitute the two-subunit factor Va molecule. The other two fragments, with masses of 71 kd and 120 kd, respectively, represent activation peptides with unknown function(s). Studies now in progress, concerned with specific identification and characterization of the various domains of factor V, should permit us to correlate its domainal structure and hydrodynamic properties in solution, with its activation to Va and with its participation in assembly of the prothrombinase complex.*

NOTE ADDED IN PROOF

Lampe et al[32] have recently published negatively contrasted images of bovine factor V that were obtained by conventional transmission electron microscopy. Their images showed less detail than ours and suggested a structure comprised of two major globular

*In preliminary STEM analyses of negatively stained factor Va, we have identified a structure resembling that shown in Fig 3 panel d whose shape corresponds closely to the middle portion of the molecule plus the outer "barbell" domain, but that lacks the other outer segment of the molecule. This observation suggests that factor Va is derived from this portion of the factor V molecule and that it undergoes no major structural rearrangements in the transition from factor V to Va.

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domains, having about the same overall dimensions as the molecules we have observed.

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