Apparent Molecular Weight of Purified Human Factor VIII Procoagulant Protein Compared With Purified and Plasma Factor VIII Procoagulant Protein Antigen

By Mark J. Weinstein, Carol A. Fulcher, Leslie E. Chute, and Theodore S. Zimmerman

We have compared apparent molecular weights of purified factor VIII procoagulant protein (VIII:C) and VIII:C antigen (VIII:CAg) by two different NaDodSO₄ gel electrophoretic techniques. In a discontinuous NaDodSO₄-7.5% polyacrylamide system, reduced and unreduced VIII:C, purified from commercial factor VIII concentrates by a monoclonal antibody immunoadsorption technique, showed a major doublet at mol wt 0.79 and 0.8 × 10⁶ and less intense bands extending up to 1.9 × 10⁶. In NaDodSO₄-4% polyacrylamide/0.5% agarose gels (NaDodSO₄-4% PAAGE), purified VIII:C had a major band of mol wt 1.0 × 10⁶, with minor bands of mol wt 0.96, 1.1, 1.4, 1.6, 1.8, 2.2, and 2.4 × 10⁶. In NaDodSO₄-4% PAAGE of ¹²⁵I-anti-VIII:C-Fab-VIII:CAg complexes, the major and minor forms of VIII:CAg in purified VIII:C had the same molecular weight as above when calculated by subtracting the molecular weight of ¹²⁵I-Fab from ¹²⁵I-Fab–VIII:CAg. In both plasma and factor VIII concentrate, a band of mol wt 2.4 × 10⁶ predominated, and minor VIII:CAg forms of mol wt 2.6, 1.8, 1.2 and 1.0 × 10⁶ were also visible. We conclude that the molecular weight of plasma VIII:CAg forms agree with those derived from protein stains of purified VIII:C in the NaDodSO₄-4% PAAGE system, but that consistently lower molecular weight values are obtained for purified VIII:C in the discontinuous system. Both native and either disaggregated or proteolysed VIII:C species are present in the purified VIII:C preparation.

While factor VIII procoagulant protein (VIII:C) plays a critical role in hemostasis, little is known about its physical properties. Its presence in only trace amounts in normal plasma and its susceptibility to proteolysis have hampered its purification and subsequent characterization.

Weinstein et al.¹ have developed an immunologic analytical technique to examine VIII:CAg with a minimum of manipulation. Samples of whole plasma were incubated with ¹²⁵I-labeled anti-human VIII:CAg Fab (¹²⁵I-Fab) and electrophoresed in sodium dodecyl sulfate-3% polyacrylamide/0.5% agarose gels (NaDodSO₄-3% PAAGE). ¹²⁵I-Fab–VIII:CAg complexes were stable under the conditions used, and apparent molecular weights were calculated from migration distances.

In this electrophoretic system, the major intact form of VIII:CAg had an apparent molecular weight of 2.7 × 10⁶; minor species were also present. Thrombin-induced proteolysis of VIII:CAg rapidly produced a single antigenic form, mol wt 1.2 × 10⁶.

In contrast to these molecular weight estimates of plasma VIII:CAg, based on relative migration of antigen–antibody complexes, purified VIII:C protein obtained by Fulcher and Zimmerman² in preparative amounts had substantially different molecular weight values when electrophoresed in a standard NaDodSO₄ gel system. Using discontinuous NaDodSO₄-5% polyacrylamide gel electrophoresis³ of reduced proteins and staining with Coomassie blue, most VIII:C was in the form of a doublet of mol wt 7.9 and 8.0 × 10⁶. Less strongly staining bands were also visible, with molecular weights up to 1.9 × 10⁶. After thrombin treatment, all bands disappeared, and VIII:C was converted to bands with 7.1, 7.2, 5.4 × 10⁶ mol wt and some material of lower molecular weight.

In the present investigation, we have used a NaDodSO₄-4% PAAGE method to examine VIII:CAg in plasma, factor VIII concentrate, and purified VIII:C protein. The higher concentration of acrylamide in these gels, compared to the original procedure,¹ gave better resolution of closely spaced bands. Molecular weight values calculated for the free VIII:CAg by this technique were compared with those derived for the purified protein, visible on the same polyacrylamide/agarose gel. These were also compared with those obtained by NaDodSO₄-polyacrylamide gel electrophoresis of reduced VIII:C in the discontinuous buffer system. VIII:CAg forms remaining after thrombin treatment of purified VIII:C and whole plasma were also analyzed.

MATERIALS AND METHODS

Purification of VIII:C

VIII:C was purified from commercial factor VIII concentrate as described previously,⁴ with the following modifications. Following elution of VIII:C from the monoclonal anti-von Willebrand factor antibody column and concentration by ultrafiltration, the VIII:C...
pool was subjected to immunoabsorption with monoclonal antibodies to fibronectin, fibronectin, and von Willebrand factor. The anti-fibronectin antibody was a gift of Dr. Deane Mosher. The anti-fibronectin antibody clone was provided by Dr. Fred Jensen. The anti-von Willebrand factor antibody was produced as previously described. The monoclonal antibodies were coupled to agarose at a concentration of 2-4 mg/ml. A mixture of equal parts of each antibody–agarose conjugate was prepared and added to the VIII:C pool at a ratio of agarose to pool of 1:5 (v/v). The mixture was slowly rotated for 60 min at room temperature; the agarose was removed by centrifugation and the process repeated.

The VIII:C pool was then subjected to aminohexyl-agarose chromatography as previously described. In some experiments, the starting factor VIII concentrate and subsequent pools of eluted material were treated with 1 M (p-amidinophenyl)methanesulfonyl fluoride, and 1 M benzamidine HCl was included in all buffers.

NaDodSO4-polyacrylamide gel electrophoresis of reduced proteins in the discontinuous buffer system of Laemmli and staining with Coomassie blue R250 was carried out as previously described. Reduced fibronectin, mol wt 200,000; phosphorylase-b, mol wt 95,000; bovine serum albumin, mol wt 68,000; IgG heavy chain, mol wt 50,000; and ovalbumin, mol wt 43,000 were used as molecular weight markers. Gels were dried onto filter paper before photography. Purified human α-thrombin was a gift of Dr. John W. Fenton II.

With the exception of raising the final acrylamide concentration from 3% to 4%, for the procedures for NaDodSO4-PAAGE, 125I-Fab and sample preparation, and autoradiography were the same as those published previously. Plasma, factor VIII concentrate, or purified VIII:C protein were diluted with 0.125 M NaCl/0.028 M sodium barbital, pH 7.3 (VBS), to 0.5–3 U/ml VIII:C activity. Samples were incubated at 37°C for 2 hr with a solution of 125I-Fab/diisopropyl fluorophosphate/hirudin/polyethylene glycol, which allowed the formation of 125I-Fab complexes while minimizing the chance of proteolysis. Following incubation at 37°C for 15 min with an equal volume of 2% NaDodSO4/8 M urea/2 mM EDTA/0.05 M Tris-HCl, pH 8.0, samples were electrophoresed for 20 min at 30 V, 15 min at 70 V, and 2.5 hr at 125 V.

For experiments employing Coomassie blue stain to detect purified VIII:C protein by NaDodSO4-PAAGE, the protein was first dialyzed against VBS containing 10 KIU/ml Trasylol. The purified protein (0.5 mg/ml, approximately 2,300 U/mg), in some cases treated with thrombin, was incubated at 60°C for 15 min with an equal volume of the NaDodSO4, denaturating buffer. Samples were also incubated at 37°C for 15 min in the same buffer with no change in results. Aliquots of 40 μl were applied to the gel.

In the continuous buffer systems, plasma proteins, including albumin, (mol wt 66,300), transferrin (76,500), IgG (150,000), fibrinogen (340,000), and albumin polymers, were used as molecular weight markers. The apparent molecular weight of protein was determined by interpolation on a straight line derived by plotting migration distance versus logarithm of molecular weight. The migration distance was determined by interpolation on a straight line derived by plotting migration distance versus logarithm of molecular weight.

RESULTS

Purified VIII:C protein, analyzed by 125I-Fab–NaDodSO4-4% PAAGE, was compared with VIII:C in whole plasma and commercial factor VIII concentrate (Fig. 1). The major 125I-Fab–VIIIICAg complex in plasma had an electrophoretic mobility corresponding to an apparent mol wt 2.9 x 105. Minor bands were present with molecular weights of 3.1, 2.3, 1.7, and 1.5 x 105. Similarly, 125I-Fab–VIIIICAg complexes in factor VIII concentrate had the same mobilities as those in plasma, but more of the lower mol wt 1.7 and 1.5 x 105 bands were apparent relative to the major 2.9 x 105 complex. Subtracting 0.5 x 105 (the molecular weight of 125I-Fab) from these values gives free antigen molecular weights of 2.4 x 105 for the major species and 2.6, 1.8, 1.2, and 1.0 x 105 for minor species (Table 1). The values in the succeeding experiments were all calculated by subtracting the molecular weight of Fab from the observed molecular weight.

125I-Fab–VIIIICAg complexes, derived from the purified VIII:C solution, showed the same basic pattern as those in plasma and factor VIII concentrate, but the intensity of the bands was different. 125I-

Table 1. Apparent Molecular Weight (x 10^5)

<table>
<thead>
<tr>
<th>Factor VIII Coagulant Protein</th>
<th>NaDodSO4-4% PAAGE</th>
<th>NaDodSO4-7.5% PAAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Thrombin Proteolysis</td>
<td>Untreated</td>
</tr>
<tr>
<td>VIII:C*</td>
<td>VIII:CAg†</td>
<td>VIII:C*</td>
</tr>
<tr>
<td>2.6</td>
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</tr>
<tr>
<td>1.0</td>
<td>1.1</td>
<td>0.96</td>
</tr>
</tbody>
</table>

†VIII:C in plasma. Similar antigenic forms were present in cryoprecipitate and purified VIII:C.

VIII:CAg, factor VIII coagulant antigen.

VIII:C, purified factor VIII protein.

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Fab–VIII:C Ag of mol wt $1.0 \times 10^5$ was the most intense, while those at $1.2, 1.8,$ and $2.4 \times 10^5$ were less prominent. Mixtures (1:1) of hemophilic plasma and purified VIII:C protein did not give different band patterns or band intensities (data not shown).

Electrophoresis of Purified VIII:C Protein

Purified VIII:C protein, at a concentration high enough to be visible by Coomassie blue stain, was analyzed by NaDodSO$_4$-4% PAAGE without $^{125}$I-Fab (Fig. 2). The most prominent band had mol wt $9.9 \times 10^4$, with an adjacent minor band of $9.3 \times 10^4$; Other minor bands were present with mol wt $2.5, 2.2, 1.8, 1.6, 1.4,$ and $1.1 \times 10^5$.

Following reduction, the purified VIII:C was analyzed by NaDodSO$_4$ gel electrophoresis in a 7.5% polyacrylamide gel using the discontinuous buffer system of Laemmli. Under these conditions, a major doublet was seen with mol wt $7.9$ and $8.0 \times 10^4$; a prominent band was evident at $9.2 \times 10^4$; and additional bands were present up to mol wt $1.88 \times 10^5$ (Fig. 3). In the absence of reduction, no significant differences were seen in the pattern of VIII:C bands, and there were only slight changes in their relative mobilities.

Thrombin Proteolysis of VIII:C Ag

Whole plasma was treated with thrombin and the resulting changes in VIII:C Ag were analyzed by NaDodSO$_4$-4% PAAGE (Fig. 4). Three minutes after the addition of thrombin (0.05 U/ml), most $^{125}$I-Fab–VIII:C Ag was in the mol wt $1.0 \times 10^5$ form, and the intensities of the various bands were similar to VIII:C Ag in the purified VIII:C protein preparation.

![Fig. 2. Purified VIII:C protein (0.5 mg/ml), before (−) and after (+) thrombin (10 U/ml, 15 min, 22°C) treatment analyzed by NaDodSO$_4$ PAAGE (10 μg applied to gel) and stained with Coomassie blue.](image)

After 15-min incubation with 0.5 U/ml of thrombin, the mol wt $8.0 \times 10^4$ band was predominant.

Similarly, purified VIII:C protein was incubated with thrombin and analyzed by the immunologic electrophoretic procedure. Within 1 min after thrombin addition (0.1 U/ml), most material was in the mol wt $1.0 \times 10^5$ form. Modest amounts of the $8.0 \times 10^4$ form were also visible. After 15 min with 0.5 U/ml thrombin, most VIII:C Ag had mol wt $8.0 \times 10^4$.

Thrombin Proteolysis of Purified VIII:C Protein

Using high concentrations of purified VIII:C protein, thrombin-derived digestion products were visible on Coomassie blue stained NaDodSO$_4$-4% PAAGE (Fig. 2). After 15-min incubation with 10 U/ml
thrombin, most VIII:C protein had mol wt $9.1 \times 10^4$. Less intense bands were present with mol wt $8.6$, $7.1$, and $5.8 \times 10^4$. Thrombin, at a final concentration of $0.5$ or $1.0$ U/ml, gave similar results. When analysis was performed on thrombin (10 U/ml, 15 min) treated VIII:C using the 7.5% polyacrylamide discontinuous gel system, bands were present at mol wt $4.4$, $5.4$, $7.1$, and $7.2 \times 10^4$ (Fig. 3).

**DISCUSSION**

The availability of substantial amounts of a purified human VIII:C preparation has allowed us to reconcile the approximate molecular weight values for plasma and purified VIII:C calculated using $^{125}$I-Fab PAAGE. The molecular weight values obtained for purified VIII:C electrophoresed in PAAGE and stained with Coomassie blue were approximately the same as those obtained in this system for purified VIII:C complexed with $^{125}$I-Fab, when the molecular weight of the Fab was subtracted from that of the complex. These results are in agreement with a 1:1 molecular stoichiometry for Fab-VIII:CAg binding.

The apparent molecular weights calculated for VIII:CAg forms in the present study are lower than those reported previously. This result is in part caused by the increase in acrylamide concentration from 3% to 4%. Although the theoretical reasons for this are not known, similar observations have been made on von Willebrand factor molecular weight determined in acrylamide-agarose gels of increasing acrylamide concentration. The increased resolving power of the NaDodSO$_4$-4% PAAGE versus the NaDodSO$_4$-3% PAAGE also contributed to lowering the apparent molecular weight of VIII:CAg forms. The predominant VIII:CAg form in whole plasma, for example, appeared as a broad band of mol wt $2.7 \times 10^4$ on NaDodSO$_4$-3% PAAGE. This was resolved into a major and a minor species on the less porous gel, the former having mol wt $2.4 \times 10^4$ and the latter $2.6 \times 10^4$.

The VIII:C molecular weights calculated here using NaDodSO$_4$-4% PAAGE are based on relative migration of unreduced, incompletely denatured Fab-VIII:C complexes and are intended only for the purpose of comparing different samples seen in this electrophoresis system. Although the molecular weights determined here for human VIII:C agree with those calculated by other investigators using techniques such as gel filtration or ultrafiltration, these methods are highly dependent on the aggregation state and tertiary structure of VIII:C. The molecular weights previously reported for purified human VIII:C and porcine VIII:C preparations were calculated from electrophoresis of reduced, completely denatured protein, using discontinuous NaDodSO$_4$ polyacrylamide gel systems. These differences in technique probably account for the different values obtained.

In the $^{125}$I-Fab PAAGE technique, the use of Fab fragments of restricted immunologic specificity limits the visualization to only those VIII:CAg forms that contain the appropriate epitope and that react stably with the Fab under the conditions used. As shown here, the two lowest molecular weight thrombin degradation products stainable by Coomassie blue were not identified by the $^{125}$I-Fab.

A major advantage of the $^{125}$I-Fab technique is its ability to estimate relative molecular weights of impure VIII:CAg. Thus, we were able to compare purified VIII:C with that in the commercial concentrate used as starting material and in fresh plasma. Significantly more low molecular weight material was present in the purified VIII:C than in the starting material. This was true even when 1 mM benzamidine was included in all buffers and when the starting material and all elution pools were treated with ($p$-amidinophenyl)methanesulfonyl fluoride. Though inclusion of these inhibitors does not exclude the possibility of proteolysis, the possibility that disaggregation occurred during the purification process must also be considered. In either case, the band of highest molecular weight in the purified VIII:C appears to be present in plasma and probably represents a native form of VIII:C.

**ACKNOWLEDGMENT**

We thank J. D. Abercrombie for expert manuscript preparation.

**REFERENCES**

Apparent molecular weight of purified human factor VIII procoagulant protein compared with purified and plasma factor VIII procoagulant protein antigen

MJ Weinstein, CA Fulcher, LE Chute and TS Zimmerman