CONCISE REPORT

The Complex Multimeric Composition of Factor VIII/von Willebrand Factor

By Zaverio M. Ruggeri and Theodore S. Zimmerman

We have analyzed the multimeric structure of factor VIII/von Willebrand factor in plasma by sodium dodecyl sulfate electrophoresis using gels of varying porosity and a discontinuous buffer system. Factor VIII/von Willebrand factor bands were identified by reaction with 125I-labeled affinity-purified antibody and subsequent autoradiography. In 1% agarose gels, normal plasma displayed a series of sharply defined oligomers. However, increasing the agarose concentration to 2.0% or utilizing mixtures of 0.8% agarose-1.75% acrylamide revealed two bands of lesser intensity interposed between the major bands. When the acrylamide concentration in the gels was increased to 2.5%, bands with a faster mobility than IgM and fibronectin were now evident. Type IIA von Willebrand’s disease showed not only an absence of the larger multimers but also a relative increase in several of the newly identified bands as compared to type IIB, type I, and normal. These studies suggest that factor VIII/von Willebrand factor in IIA von Willebrand’s disease is structurally different from that in other forms of the disorder. They also indicate that the multimeric composition of factor VIII/von Willebrand factor is more complex than can be explained by simple linear polymerization of a single protomer.

RECENT STUDIES of factor VIII/von Willebrand factor (FVIII/vWF), either purified, or in plasma, have found it to consist of a multimeric series with a protomer variously estimated to have an mol wt of between 0.5 and 1.9 x 10^6 daltons. We have now utilized a discontinuous buffer system in conjunction with agarose and agarose-acrylamide gels of increasing concentrations to better resolve the FVIII/vWF found in plasma. Our studies demonstrate the presence of previously unidentified bands, which suggests that the multimeric composition of FVIII/vWF is more complex than had been thought. These results cannot readily be explained on the basis of a linear polymerization of a single protomer entity organized into a linear series of oligomers. In addition, we have found that the relative concentration of several newly identified bands differs appreciably between types IIA and IIB von Willebrand’s disease (vWd), providing further evidence that distinct molecular abnormalities of FVIII/vWF underlie these two variants of the disease.

MATERIALS AND METHODS

Materials

Seakem Agarose HGT(P) (ultra-pure, high gelling temperature) and Gel Bond Film were purchased from Marine Colloids Division, FMC Corp., Rockland, Md. Acrylamide, N,N'-methylene-bis-acrylamide, sodium dodecyl sulfate (SDS), glycine, and ammonium persulfate (electrophoresis purity reagents) were from Bio-Rad Laboratories, Richmond, Calif. Whatman 3MM chromatography paper was obtained from VWR Scientific, San Francisco, Calif. Urea (ultrapure grade) was from Schwarz-Mann, Orangeburg, N.Y. All other reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

Citrated plasma was prepared and stored as recently described. Samples from the following patients were analyzed: 3 patients with vWd type I; 10 patients with variant vWd type IIA; 6 patients with variant vWd type IIB; and 7 patients with severe, homozygous-like vWd. All these patients have been fully characterized in a previous publication.

SDS Gel Electrophoresis

A modification of the discontinuous buffer system of Laemmli was used. Gels were poured onto a plastic support (Gel Bond Film) of 12.7 x 11.4 cm size and were 1.2 mm thick. The buffer used for preparing running gels had a final concentration of 0.375 M Tris, 0.1% SDS, and was brought to pH 8.8 with concentrated HCl. Agarose gels were prepared at concentrations between 1% and 2% and could be used 2 hr after pouring or stored in a moist chamber overnight. Agarose-acrylamide gels contained 0.8% agarose and acrylamide concentrations between 1.75% and 3.5%, with 5% cross-linking. The acrylamide solution was warmed to 60°C before mixing, with agarose kept at the same temperature. Polymerizing agents were 3-dimethylamino-propionitrile (0.25%) and ammonium persulfate (0.025%), the latter added immediately before pouring the gel. As soon as agarose was solidified, the gels were transferred into a moist chamber, flushed with a constant flow of nitrogen for 1 hr, and then left in the nitrogen atmosphere overnight at room temperature.

The stacking gel contained 0.8% agarose in 0.125 M Tris buffer, 0.1% SDS, adjusted to pH 6.8 with concentrated HCl, and was used to replace a 12.7 x 2.5 cm strip of running gel not more than 1 hr before starting the electrophoresis. Six sample wells (11 mm long...
and 2 mm wide) were cut in the stacking gel at 10 mm from the interface with the running gel. Plasma to be analyzed was diluted 1:20 in 10 mM Tris–1 mM EDTA (disodium salt) buffer, adjusted to pH 8 with HCl, and containing 2% SDS, 8 M urea, and 0.005% bromophenol blue as tracking dye. After incubation at 60°C for 15 min, 20-μL samples were applied to each well and electrophoresis started at a constant current of 10 mA/gel in an LKB-Multiphor 2117 (LKB, Bromma, Sweden) cooled to 15°C. The electrophoresis buffer was composed of 0.05 M Tris, 0.384 M glycine, 0.1% SDS, and was adjusted to pH 8.35 with 50% sodium hydroxide. The gels were connected to the buffer reservoirs by means of Whatman 3MM paper wicks. After the samples had moved out of the wells, these were filled with stacking gel and the current increased to 2.5 mA/gel. Electrophoresis was stopped when the tracking dye reached the anodal end of the gel, usually within 5–6 hr.

After electrophoresis, the gels were fixed, washed, and reacted with 125I-labeled affinity purified anti-FVIII/vWF antibody raised in emus, as recently described. Purified human IgM and fibronectin were obtained as recently described. These reference proteins (0.5–1 μg) were run in parallel with plasma samples and identified by staining with Coomassie Brilliant Blue R.

RESULTS

The use of a discontinuous buffer system allowed better resolution of the FVIII/vWF multimeric structure than our previously described homogeneous system. The absence of larger multimers from type IIB vWD and the absence of both large and intermediate forms from type IIA was readily appreciated in 1% agarose gels (Fig. 1). At this agarose concentration, however, the smallest multimers moved with or very close to the dye front and were not resolved.

When the agarose concentration in the slab gels was increased to 2%, a more complex picture became evident. In 10 different normal plasmas, a series of major bands (1–5 and larger) with intervening bands (single prime and double prime) of lesser intensity were now apparent (Fig. 1). Some unresolved material still migrated with or very close to the dye front. The faster of the major bands in normal plasmas (band 1) had a mobility close to that of the IgM monomer. The other major bands displayed a progressively decreasing mobility that closely paralleled that of corresponding oligomers of chemically crosslinked IgM (Fig. 1). In type IIA vWD, the newly identified intervening bands were more pronounced than in either type IIB, type I (not shown), or normal plasma (Fig. 1) and approached the major bands in intensity.

In order to better resolve the faster migrating bands, mixtures of agarose–acrylamide were used in the gels. All the FVIII/vWF multimers entered gels of 1.75% acrylamide concentration, but resolution of the larger multimers was not as good as in 2% agarose gels. However, resolution of the faster moving bands was improved, and only a small portion of FVIII/vWF-related material moved close to the tracking dye (Fig. 2). In contrast to 2% agarose, band 1 now displayed a slightly faster mobility than the IgM monomer, as did the other major bands (2–5) when compared to the corresponding cross-linked IgM oligomers (Fig. 2). Two bands of faster mobility than band 1 were evident in these gels, and the relative intensity of the fastest was consistently increased in plasmas of IIA vWD patients (Fig. 2). In vWD type I, only the major bands (1–5 and larger) were readily evident, whereas in vWD type IIB, the relative inten-

---

**Fig. 1.** SDS-electrophoresis in 1% (left) and 2% (right) agarose gels of normal plasma (N); plasma from a patient with vWD type IIA (IIA); a patient with vWD type IIB (IIB); and a patient with severe vWD (S). The arrow indicates the interface between stacking and running gel. The cathode was at the top and the anode at the bottom of the gel. Numbers from 1 to 5 indicate the smallest major bands in normal plasma, while the intervening bands are indicated by primes. The position of IgM and its crosslinked oligomers is shown by white marks.
sity of different bands was similar to normal with the exception of the largest multimers, which were decreased or absent (Fig. 2).

A more detailed resolution of the faster moving bands was obtained in gels of 2.5% acrylamide concentration. However, only the first three major bands entered these gels (Fig. 2). At this acrylamide concentration, the IgM monomer mobility was slower than that of both bands 1 and 2. Band 1 now displayed a mobility identical to that of fibronectin (Fig. 2). As with gels of larger porosity, at least two additional bands could be identified between each major multimer, and the intensity of these was relatively increased in type IIA vWD. Moreover, at least 7 additional bands with mobility greater than band 1 were evident (bands a–g; Fig. 2). The intensity of these bands was decreased in type I vWD and similar to normal in type IIB vWD. In type IIA vWD the intensity of several of these faster bands was characteristically increased relative to others (compare band f with g). The pattern observed in frozen normal citrated plasma stored for over 6 mo was identical to that of freshly prepared, diisopropyl fluorophosphate (DFP) treated normal plasma electrophoresed immediately after collection.

When the relative mobilities of each individual FVIII/vWF oligomer, as well as IgM and fibronectin, were analyzed at different gel concentrations (1.75%, 2.0%, 2.5%, and 3.5% acrylamide), the resulting plots gave straight lines. However, the FVIII/vWF oligomers could not be considered as ideal SDS–protein complexes for purposes of molecular weight determination since their individual plots intersected at between 0.5% and 1.25% acrylamide and not at the ideal zero gel concentration as required (Ferguson plot). Therefore, precise molecular weight calculations were not possible.

DISCUSSION

This study demonstrates that the multimeric composition of FVIII/vWF has a greater degree of complexity than previously thought. It is unlikely that our findings are artifacts of the discontinuous buffer system. In experiments not shown here, we performed a similar analysis using a homogeneous Tris–borate buffer system in 0.5% agarose–3% acrylamide SDS gels. Although the larger multimers (above band 3) did not enter the gels, smaller material was resolved in a manner similar to that observed with the discontinuous system. We also addressed the possibility of in vitro degradation by electrophoresing fresh DFP-treated plasma samples within 15 min of venepuncture. These showed no differences from samples frozen for 6 mo or more. In addition, highly purified normal FVIII/vWF gave a pattern similar to that seen in fresh plasma, though the faster moving bands had been relatively depleted during the isolation procedure.

Previous reports have suggested that FVIII/vWF exists as a linear series of oligomers of a protomer, which in turn is composed of a variable number of identical subunits. The protomer has been postulated to be a dimer or tetramer of the basic subunit for human FVIII/vWF, or as a 6-mer or 8-mer for porcine FVIII/vWF. Our results, however, do not
easily accommodate such a model. Rather, the different relative intensities of the major and intervening bands of FVIII/vWF in normal plasma are more consistent with an asymmetrical model of polymerization. Alternatively, these findings might be explained by the existence of different linear series of oligomers originated by distinct protomers present in different concentrations. However, any explanation must address the altered proportion of FVIII/vWF bands in IIA vWd. This selective increase in specific bands suggests that the abnormality in these patients is not limited to absence of the larger multimers, but probably involves alterations in the basic structure of FVIII/vWF.

Evaluating possible explanations for the complex multimeric pattern is hampered by our inability to assign precise molecular weight values to the individual FVIII/vWF bands. It is likely that the nonideal behavior of unreduced FVIII/vWF in these SDS systems is responsible for the varying mobility of the major bands relative to IgM and its polymers observed at different gel concentrations. This nonideal behavior may also explain the differing molecular weight assignments other investigators have given to the FVIII/vWF multimers. In spite of this uncertainty as to the precise molecular weight of the major bands, it is apparent that there are forms of FVIII/vWF in plasma that are smaller than previously suspected. Whether they represent metabolic breakdown products of larger oligomers or, conversely, represent building blocks of these larger forms is presently unknown.

ACKNOWLEDGMENT

We wish to thank James Roberts and Rose Voss for skillful technical assistance and Roberta Novak for preparation of the manuscript.

REFERENCES

The complex multimeric composition of factor VIII/von Willebrand factor

ZM Ruggeri and TS Zimmerman