CONCISE REPORT

Factor VIII-Related Protein Circulates in Normal Human Plasma as High Molecular Weight Multimers

By Leon W. Hoyer and John R. Shainoff

The size of human factor VIII-related protein in plasma has been determined by sodium dodecyl sulfate (SDS) glyoxyl agarose electrophoresis. The protein was immobilized after the electrophoresis by coupling it to the modified agarose, and it was identified by autoradiography using purified rabbit anti-factor VIII-related antigen (VIIIIR:Ag). A series of multimeric forms was identified with M$_r$ of 0.85-12 $\times$ 10$^6$. The distribution of VIIIIR:Ag multimers was the same in heparin and citrate anticoagulated plasmas and in serum, and the pattern was the same after freezing as in plasma kept at 37°C from the time of venipuncture until the electrophoresis was complete. These observations indicate that VIIIIR:Ag circulates in normal plasma as a population of very large multimers and that the size distribution is not an artifact induced by purification methods, freezing, or calcium chelation.

Many recent studies have sought an understanding of factor VIII structure and function. An important unresolved issue has been the size in plasma of factor-VIII-related protein (VIIIIR), the component of the factor VIII complex that is identified by heterologous antibodies to factor VIII (VIIIIR:Ag) and is responsible for ristocetin cofactor and other platelet-related (von Willebrand factor) activities. While most agarose gel filtration and ultracentrifugation studies have indicated that VIIIIR is very large (>10$^6$ daltons), this concept has been questioned by the results of recent gel filtration and analytic ultrafiltration studies carried out directly on plasma. While it is possible that anticoagulation or storage at low temperatures might be responsible for an artifactual aggregation of VIIIIR in vitro, as suggested by the two studies, it is also possible that the differences reflect problems associated with size measurements inferred from agarose gel filtration and ultrafiltration data.

For this reason, studies were carried out with fresh human plasma kept at 37°C while it was separated from cellular elements and analyzed by sodium dodecyl sulfate (SDS) agarose electrophoresis. Under these conditions, plasma VIIIIR has been identified in plasma as a series of multimers of a protein with an apparent $M_r$ of 0.85 $\times$ 10$^6$. The multimeric pattern is the same as that previously demonstrated for purified VIIIIR.$^6$,$^10$

In the course of these studies, a new method was developed for measuring the size of this trace protein. The method employs a newly developed protein-immobilizing gel, glyoxyl agarose,$^{11,12}$ which by virtue of readily controllable reactivity was used initially as an inert support medium for separating proteins at a neutral pH and then as a gel matrix to which the proteins were fixed through covalent attachment. VIIIIR, having been separated according to size by SDS electrophoresis and then immobilized, was washed free of SDS, incubated with labeled anti-VIIIIR:Ag and identified by autoradiography.

MATERIALS AND METHODS

Standard agarose was obtained from Litex Co., Denmark (HSA type, lots 474 and 545) and glyoxyl-modified agarose was prepared from Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.). Glycidol (2,3 epoxypropanol-1) and SDS were obtained from Eastman Kodak Co. (Rochester, N.Y.); sodium cyanoborohydride and dimethylsuberimidate were obtained from Aldrich Chemical Co. (Milwaukee, Wisc.); Triton X-100 was obtained from Sigma Chemical Co. (St. Louis, Mo.). Gel bond polyester film (7-mil thickness) was obtained from Marine Colloids (Rockland, Me.) and sodium heparin, porcine intestinal mucosa, was obtained from Organon, Inc. (West Orange, N.J.). All other chemicals were reagent grade.$^{13}$

$^{13}$I-labeled rabbit anti-VIIIIR:Ag was purified as previously described.$^{13}$ Before use in these experiments, it was incubated at 37°C for 2 hr and at 4°C for 16 hr with an equal volume of plasma from a patient with severe von Willebrand’s disease (VIIIIR:Ag < 0.01 U/ml) and centrifuged at 8730 g for 5 min at room temperature. The proteins used for molecular weight markers included purified human fibrinogen (IMCO, Stockholm, Sweden), human IgM purified from the plasma of two patients with macroglomuler nemia, and IgM crosslinked with dimethyl suberimidate.$^{14}$ The composition of the borate-buffered saline (BS) has been published.$^{15}$

Glyoxyl agarose$^{11}$ was prepared by incubating 200 ml of washed 4% agarose with 100 ml of 1 M NaOH, 0.002 M NaBH$_4$, and 30 ml glycidol at room temperature for 16 hr with gentle agitation. The agarose was then washed with distilled water on a Büchner funnel and suspended in 500 ml 0.06 M sodium metaperiodate adjusted to pH 7. After 60 min incubation at room temperature, the modified

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agarose was washed on a Büchner funnel with distilled water, transferred to a beaker, and suspended in a volume of distilled water equal to 1/2 of the agarose settled volume. The mixture—approximately 2% in agarose—was heated until all of the agarose dissolved and 20–50 ml aliquots were stored at 4°C.

Blood samples, obtained from laboratory personnel by venipuncture in accordance with a protocol approved by the University Human Experimentation Committee, were anticoagulated with citrate (1/50 volume of 0.5 M sodium citrate) or heparin (10 U/ml blood) in polystyrene tubes prewarmed to 37°C. The blood was immediately centrifuged at 37°C (3000 g for 15 min), and an aliquot of plasma—or of a dilution of plasma in BS—was added to 4 volumes of the SDS incubation mixture held at 37°C. This mixture included 0.88 mg/ml iodacetamide, 12.5 mg/ml SDS, 0.01 M sodium phosphate, pH 7.0. A 1/25 volume of 0.5% bromphenol blue was added after 2-hr incubation. Serum, obtained from blood added directly to glass tubes and held at 37°C for 1 hr, and marker proteins dissolved in BS at 2–3 mg/ml, were analyzed in the same manner.

SDS-agarose electrophoresis was carried out in horizontal slab gels containing 1.25% unmodified agarose, 0.25% glyoxyl agarose, 0.1% SDS, and 0.05 M sodium phosphate, pH 7.0. The 10 × 18 × 0.2 cm gels were cast on a polyester backing using a mold assembly with a plastic U-frame spacer. Twenty-microliter samples were added to 0.1 × 1.0 cm slots cut in the agarose with a metal punch. Electrophoresis was carried out on a temperature-controlled surface at 5 V/cm for 60–80 min so that the bromphenol blue marker migrated 4–4.5 cm. The agarose gels were connected to the electrode chambers (each containing 1 liter of 0.1 M sodium phosphate, pH 7.0, with 0.1% SDS) by cellulose Ultra-Wicks (Bio Rad Laboratories, Richmond, Calif.). After electrophoresis and measurement of dye migration, the marker protein lanes were stained with 0.27% Coomassie Blue R-250 (Eastman Kodak) in methanol, and water (1:4:4 by volume) for 30 min. They were destained in acetic acid, methanol, and water (1:4:4 by volume).

The samples examined for VIIIIR:Ag migration were fixed for 2 hr at room temperature by immersion in 400 ml of 0.2 M sodium carbonate, pH 10, containing 0.02 M sodium cyanoborohydride and 1% Triton X-100. The gels were then rinsed and washed for 2 hr at room temperature with 1 liter BS gently agitated with a magnetic stirrer. The gels were subsequently incubated at room temperature for 16–24 hr with 125I-labeled anti-VIIIIR:AG (100,000–500,000 cpm) in BS containing 1 mg/ml normal human IgG (IMCO); the total volume in the plastic dish was 10–30 ml, and the contents were gently rocked during the incubation. The gels were then washed over a 48-hr period with 3 changes of BS (1 liter each), washed with deionized water for 6 hr, pressed, and dried. Autoradiography was carried out with Kodak XR-1 film using two Lanex screens at –70°C for 16–24 hr. Equivalent results were obtained using Kodak No Screen film at 4°C for 3–7 days.

RESULTS

SDS-agarose electrophoresis of fresh human plasma revealed a series of VIIIIR:Ag bands of M, spanning 0.85–12 × 10⁶ daltons (Fig. 1). The most rapidly migrating VIIIIR:Ag had slightly faster mobility than IgM; no smaller forms were detected in any experiment. The multimeric pattern was identical in seven separate experiments in which fresh plasma was anticoagulated with citrate or heparin and kept at 37°C until the electrophoresis was initiated, in 7 experiments in which citrate or heparin anticoagulated plasma was frozen at –70°C for periods of 1 hr to 3 mo prior to analysis, and in 2 experiments in which freshly prepared serum was examined.

In other studies of conditions that might affect the electrophoretic separations, similar patterns were obtained for fresh plasma samples run in an electrophoresis chamber in which the surface temperature was held at 10°C (running tap water), room temperature, or at 37°C. The inclusion of 8 M urea in the incubation buffer also had no effect. Studies in which the glyoxyl agarose or the fixation step (sodium cyanoborohydride at pH 10) were omitted gave much less satisfactory patterns: only a faint outline of the largest polymers could be detected.

The migration of the polymers in each experiment was compared to that of highly purified fibrinogen (340,000), IgM (0.95 × 10⁶), and IgM polymers (1.9–4.8 × 10⁶). The migration of the smallest VIIIIR:Ag band was slightly faster than that of the monomeric form of two different IgM samples obtained from patients with Waldenstrom’s macroglobulinemia. The M, estimated from measurements made in 12 separate experiments, was 0.85 ± 0.03 × 10⁶ (mean ± SEM). As many as eight separate VIIIIR:Ag bands could be detected in the autoradiograms. The differences in M, of successive bands were 0.8–1.2 × 10⁶. In addition, poorly resolved VIIIIR:Ag migrated with an M, of ca. 8 × 10⁶ to 12 × 10⁶. The complete multimeric pattern was identified in plasma diluted as much as 1:16 (ca. 2 ng VIIIIR).

DISCUSSION

Although most chromatographic and ultracentrifuge studies have suggested that plasma VIIIIR:Ag is a large heterogeneous protein, two recent reports have been interpreted as evidence that VIIIIR:Ag circulates in plasma as a molecule that is smaller than 10⁶ daltons. They suggest that the larger forms are the result of in vitro aggregation favored by decalcification and low temperatures. We report here studies of plasma VIIIIR:Ag that demonstrate a pattern of multimers, all greater than 0.85 × 10⁶ daltons, in material kept at 37°C until mixed with an anionic detergent (SDS), an alkylating agent (iodoacetamide), and urea—agents that would tend to prevent VIIIIR:Ag aggregation. Taken together with recent agarose gel filtration studies, our data, and similar results obtained independently by Ruggieri and Zimmerman, suggest that it is very unlikely that the large multimers are aggregates formed in vitro. Although aggregated forms might be dissociated in vitro by the incubation with SDS, there is no basis for an artificial increase in polymer size.

The method used for protein identification in these
studies, autoradiography after incubation of labeled antibody with separated proteins immobilized in glyoxyl agarose, has several major advantages as an analytic technique. Protein resolution is excellent in glyoxyl agarose. has several major advantages as an alternative approach, protein fixation after electrophoresis using mixtures of acids and alcohols, might be as satisfactory, but cannot be relied on to prevent antigen dissolution, especially in the presence of excess antibody. The glyoxyl agarose method avoids these problems and permits precise analysis of proteins separated by their migration in SDS-agarose. It is important to note that this method does not rely on immunoprecipitation to identify the specific proteins.

The absence of detectable protein trailing in Fig. 1 demonstrates that there is little interaction of proteins with glyoxyl agarose at neutral pH values, and standard electrophoretic separations can be carried out in the presence or absence of SDS. Above pH 10, the aldehyde groups of the modified agarose form reversible Schiff bases with amino groups of adjacent proteins and further migration is prevented. This reversible interaction can be converted to a covalent bond by mild reductive amination with sodium cyanoborohydride. Thus, proteins are immobilized in agarose at the point they have reached at the end of the electrophoretic separation. They can then be washed to remove SDS and incubated with identifying reagents. All of the steps are relatively gentle, and antigenic determinants remain readily available after the immobilization. The basic approach has a rather wide potential for application and has been of special value in the analysis of fibrin complexes.

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