JCI The Journal of Clinical Investigation

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J Clin Invest. 1978;62(3):702-709. https://doi.org/10.1172/JCI109178.

Research Article

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Disulfide Bonds and the Quaternary Structure of Factor VIII/von Willebrand Factor

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ABSTRACT Human Factor VIII/von Willebrand factor, purified by calcium citrate-cellulose chromatography and 4% agarose gel filtration was subjected to sodium dodecyl sulfate gel electrophoresis on gels containing 2% acrylamide and 0.5% agarose. We find a series of multimers of which the apparent molecular weight of the higher members was ≅5 million. The higher multimers were isolated by 2% agarose gel filtration. Treatment of the high molecular weight multimers with 2-mercaptoethanol at concentrations of 0.005–0.5% in the presence of 1% dodecyl sulfate resulted in a shift to lower molecular weight multimers. Between mercaptoethanol concentrations of 0.01 and 0.5%, the predominant species was the dimer of the basic subunit. Mercaptoethanol concentrations >0.5% were required to reduce the interchain disulfide bonds of the dimer. An artificial multimeric series was prepared by cross-linking von Willebrand factor subunits with dimethylsuberimidate. Comparison of the multimers produced by reduction with the multimers produced by cross-linking, demonstrated the absence of odd-numbered multimers from the reduced series. Thus, the protomeric unit appears to be the dimer. High molecular weight multimers had both ristocetin cofactor activity and Factor VIII procoagulant activity. Reduction of the protein in the absence of denaturing agents, caused a gradual shift to lower molecular weight species and a concomitant loss of von Willebrand factor activity. In contrast, Factor VIII activity was unchanged by reduction. These studies suggest that the moieties having von Willebrand factor activity and those having Factor VIII activities are covalently linked by disulfide bonds.

Presented in part at the Annual Meeting of the American Society of Hematology, San Diego, Calif., 1977. (*Blood.* **50** [Suppl. 1]: 263.)

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Received for publication 20 December 1977 and in revised form 17 April 1978.

INTRODUCTION

Factor VIII/von Willebrand factor (VIII/VWF)[1] is a protein or protein complex possessing at least two biological functions, procoagulant activity (Factor VIII, antihemophilic factor [AHF]) and the promotion of platelet aggregation (von Willebrand factor, VWF). Several investigators have presented chemical and immunological evidence that these properties may be attributes of the same molecule (1–4) a model that does not necessarily preclude different subunits possessing separate activities, for example, a small procoagulant subunit covalently bound to a larger VWF subunit. In the experiments of Wagner and co-workers (5, 6) and of Weiss and co-workers (7), VWF was separated from AHF activity under mild conditions of increased ionic strength or increased Ca^{++} ion concentration suggesting that the two functional properties reside in separate subunits which are closely associated through noncovalent interactions in the native protein. These observations have been confirmed and extended in several other laboratories (8–10). In similar experiments with bovine Factor VIII, Vehar and Davie (11) found it necessary to activate the protein with thrombin before it could be separated from VWF by gel filtration in $CaCl_2$.

One problem in assigning the two biological activities to separate subunits has been that complete reduction of disulfide bonds in purified VIII/VWF protein leads to only one detectable subunit species (1, 2). However, there are clearly two gene products, because in hemophilia A, an X-linked gene abnormality leads to deficiency in AHF activity with normal VWF, whereas in von Willebrand's disease, an aberrant autosomal gene results in varying deficiencies of both AHF and VWF activities.

The protein or protein subunit responsible for Factor

[1] *Abbreviations used in this paper*: AHF, antihemophilic factor; SDS, sodium dodecyl sulfate; VIII/VWF, Factor VIII/VWF; VWF, von Willebrand factor.

 J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.*, 0021-9738/78/0901-702 $1.00

VIII activity has not been isolated in sufficient quantity to allow characterization though most studies have estimated its molecular weight to be approximately that of the reduced subunit (195–240,000) (6, 11). VWF can be confidently identified with the high molecular weight glycoprotein isolated by gel filtration of cryoprecipitate or plasma.

Molecular weight determinations of purified "native" VIII/VWF have given values of 0.5–5 million (1, 2, 12, 13). The actual molecular weight of the protein in plasma is unknown. Completely reduced purified human or bovine VIII/VWF shows a single subunit band on sodium dodecyl sulfate (SDS) gel electrophoresis with a molecular weight estimated at 195–240,000 (1, 2, 12). Unreduced, the purified protein does not enter 5% polyacrylamide gels. However, on larger-pore gels of lower acrylamide concentration, purified Factor VIII has the appearance of an aggregating multimeric series (14). Both noncovalent interactions (14) and proteolysis (15) have been suggested as possible causes. Austen et al. (16, 17) and Kirby and Mills (18) have demonstrated the sensitivity of both VWF and AHF to reducing agents and to *p*-chloromercuribenzoate, suggesting an important role of sulfhydryl groups and disulfide bonding in their biological activity. However, free sulfhydryl groups have not been detected in the native protein (1, 18).

The present study began after the observation that partial reduction of VWF resulted in two bands on SDS polyacrylamide gel electrophoresis using 5% gels. This suggested that there were at least two classes of disulfide bonds in the protein which differed in ease of reduction.

We present evidence in this paper that VIII/VWF exists, both in purified form and in plasma, as a series of multimers which are held together by disulfide bonds, and that there is ready disulfide interchange between the multimers. The protomer is a dimer of the basic subunit and is held together by disulfide bonds considerably more resistant to reduction or interchange than the bonds between protomers. The multimeric structure may be important for the biological activity of VWF, but a low molecular weight protein species having Factor VIII activity can be produced by minimal reduction of the purified protein with mercaptoethanol.

METHODS

VIII/VWF from normal human plasma was purified by the method of Legaz et al. (1) which uses chromatography of cryoprecipitate on calcium citrate-cellulose followed by gel filtration on 4% agarose (A15M, Bio-Rad Laboratories, Richmond, Calif.). Factor VIII multimers were further separated by gel filtration on 2% agarose. Purified VIII/VWF (2.5 ml) was applied to a 1.5 × 100-cm column of A50M agarose (Bio-Rad Laboratories) in a buffer containing 0.25 M NaCl, 0.5% ϵ-aminocaporic acid, 0.03% sodium azide, and 0.02 M imidazole, pH 6.8. The protein was eluted at room temperature in 1.0-ml fractions at a rate of 9 ml/h. The absorbance at 280 nm of the column effluent was monitored with an Instrumentation Specialties Company, Lincoln, Nebr., absorbance monitor. Protein concentration was determined by the method of Lowry et al. (19) using bovine serum albumin as a standard. The validity of this method for measurement of VIII/VWF was tested by comparison with Kjeldahl nitrogen determinations. The two methods agreed, within 5%, on duplicate samples.

SDS gel electrophoresis was carried out in a large-pore system containing 2% acrylamide and 0.5% agarose (20). The buffer was 0.05 M sodium phosphate, pH 7.2, containing 0.1% SDS. Before application, samples were dissolved in 1% SDS and, in experiments to test the results of reduction, 1:10 vol of the reducing agent was added. After incubation for 30 min at 37°C, the samples were applied to the gels in a 0.025-ml vol. Electrophoresis was carried out at 25°C for ≅2 h. The gels were cut off at the tracking dye front, fixed, and stained in 2.7 mg/ml Coomassie Brilliant Blue A-250 in acetic acid:methanol:water (1:4:4 by vol), and destained in acetic acid:methanol:water (1:4:4, by vol).

Dithiothreitol, 2-mercaptoethanol, iodoacetamide N,N-dimethylsuberimidate 5-5-dithiobis (2-nitrobenzoic) acid and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Polyethylene glycol, was obtained from Union Carbide Corp., New York and benzamidine hydrochloride from the Aldrich Chemical Co., Inc., Milwaukee, Wis.

Factor VIII clotting assays were done by a one-stage method (21, 22) with kaolin and human brain phospholipid (23).

VWF activity was measured by the ristocetin platelet aggregation method with formalin-fixed platelets (24). The VWF activity was estimated by comparison of the maximum extent of aggregation determined in an aggregometer (Chrono-Log Corp., Havertown, Pa.). Standard curves were made by assaying dilutions of normal plasma and of purified VIII/VWF.

VIII/von Willebrand's antigen was assayed by a two-stage solid-phase immunoradiometric assay as described (3).

Antibodies. Anticold insoluble globulin, purified on a cold insoluble globulin immunoadsorbent was the generous gift of Dr. Gary Balian, University of Washington. Anti-human fibrinogen was prepared by immunizing rabbits with purified human fibrinogen (fraction I-4, 99% clottable) and absorbing the antiserum exhaustively with normal human serum.

Cryoprecipitate was produced at the Puget Sound Blood Center as described by Slichter et al. (25).

Partial reduction of VIII/VWF was performed by precipitating the first half of the VWF peak from the Bio-Gel A-50 (Bio-Rad Laboratories) in column with 0.25 vol of 40% polyethylene glycol in 0.25 M NaCl, redissolving the material in a pH 8.0 buffer containing 0.02 M imidazole, 0.25 M NaCl, 0.1 mg/ml SDS, and 1 mg/ml disodium EDTA, and adding 0.1 vol of 2-mercaptoethanol of 10 times the desired final concentration. The final protein concentrations were 0.5–1.0 mg/ml. The VIII/VWF protein preparations used, in the partial reduction experiments, were judged to be free of cold insoluble globulin and fibrinogen by the lack of precipitin lines on immunoelectrophoresis of the protein against monospecific anticold insoluble globulin and antifibrinogen antibodies. VIII/VWF gave a single line on immunoelectrophoresis against rabbit antiserum raised against a crude VIII/VWF preparation.

Dialysis of purified Factor VIII against hypotonic solutions, and subsequent electrophoresis in large-pore polyacrylamide gels was performed as described by Van Mourik et al. (14).

Complete reduction and alkylation were carried out by the method of Legaz et al. (1) except that 65 mM dithiothreitol was used in place of 2-mercaptoethanol.

Time-course of reduction. To study the effect of reduction on biological activity, VIII/VWF (0.1 mg/ml) was dissolved in a pH 6.8 buffer containing 0.02 M imidazole, 0.25 M NaCl, and 0.5% ϵ-aminocaporic acid. 2-mercaptoethanol was added to a final concentration of 50 mM and the mixture incubated at 37°C. A zero-time sample was taken immediately after the addition of mercaptoethanol. Portions (25 μl) were subsequently removed at 5, 10, and 20 min and thereafter at 20 min intervals up to 2 h. The reduction was stopped by adding 25 μl of 50 mM iodoacetamide. After the reaction proceeded for 5 min, 25μl of bovine serum albumin (40 mg/ml) was added and the samples assayed for Factor VIII activity and VWF activity. At the same times that samples were taken for activity assays, 25-μl samples were also removed for electrophoresis. 25 μl of 0.1 M iodoacetamide was added to each sample and the mixture reacted for 3 min at room temperature. 25 μl of 1% SDS was then added. Electrophoresis was carried out in the large-pore gel system.

Cross-linking the VIII/VWF subunit. The association of subunits into multimers was studied by cross-linking with dimethylsuberimidate (26). VWF at a concentration of 1–3 mg/ml in 0.02 M imidazole was desalted on Bio-Gel P-2 into 0.2 M triethanolamine, pH 8.5, containing 0.2 mg/ml SDS. The reaction was carried out at four different concentrations of dimethylsuberimidate. 0.1 vol of dimethylsuberimidate, diluted in this buffer to concentrations of 0.03, 0.3, 3.0, and 30.0 mg/ml was added, and the reaction allowed to proceed for 3 h at room temperature. 0.1 vol of dithiothreitol (10 mg/ml) was then added and the mixture incubated for an additional 30 min at 56°C. 50 μl of the mixture was then layered directly on 2% acrylamide–0.5% agarose gels, as described in the technique for partial reduction. Alternatively, the dithiothreitol-reduced subunits were cross-linked under the same conditions. There was no difference between results obtained by cross-linking the intact protein followed by reduction, and by cross-linking the reduced subunits.

Gel filtration of plasma. Normal human plasma was filtered on Bio-Rad A50 M agarose under the conditions described above for purified VIII/VWF, except that the column buffer used also contained 1 mg/ml bovine serum albumin. Each fraction (1 ml) eluted was assayed for VWF and Factor VIII activity.

Free sulfhydryl groups. For determination of sulfhydryl groups in the native protein, VWF (0.2–0.6 mg/ml) was desalted on Bio-Gel P-2 into 0.08 M sodium phosphate, pH 8, containing 0.5 mg/ml disodium EDTA and 20 mg/ml SDS. Sulfhydryl groups were measured colorimetrically using 5,5'Dithiobis (2-nitrobenzoic) acid (27, 28). For determination of sulfhydryl groups after partial reduction with mercaptoethanol, the partially reduced VWF was precipitated by adding an equal volume of cold 25% TCA. The precipitate was washed four times with 25% TCA, the tubes being flushed with nitrogen each time to exclude oxygen. The precipitate was dissolved in 2 ml 8 M urea–5% SDS and a 1.0-ml aliquot of this solution was mixed with 50 μl of 5,5'dithiobis (2-nitrobenzoic) acid (4 mg/ml). After 15 min the absorbance at 410 nm was measured and adjusted by subtracting the absorbance of reagent and protein blanks. A molar extinction coefficient $E^{410}_{1\,cm}$ = 14,200 for the nitrothiophenolate ion was used to calculate the concentration of sulfhydryl groups (29).

RESULTS

Cross-linking VIII/VWF with dimethylsuberimidate. To provide a homologous series of high molecular weight markers for molecular weight estimation on SDS gels, VWF subunits were cross-linked with dimethylsuberimidate. This produced a series in which the first six bands, starting with the monomer, were well visualized in the large pore gel system used. In calculating molecular weights for the cross-linked multimer, the molecular weight of the monomer was taken to be 240,000 (1, 12). A plot of the logarithm of the multimer molecular weights against their mobility demonstrates that the molecular weights are integral multiples of the monomer molecular weight (Fig. 1).

Unreduced VIII/VWF. The VIII/VWF protein purified by Bio-Gel A-15M filtration did not enter a 5% polyacrylamide gel. When this unreduced preparation was electrophoresed on polyacrylamide agarose gels containing 2% acrylamide, a multimeric series appeared. By comparing the multimers with the artificially cross-linked series, we estimate that the limiting molecular weight aggregates which predominate in unreduced VIII/VWF correspond to 18–30 subunits or molecular weights in the range of 3.6–5.6 million. The higher molecular weight species could be separated from the smaller multimers by rechromatographing the VIII/VWF peak from the 4% agarose on a column of Bio-Gel A-50M. This second gel filtration yielded a broad high molecular weight peak with a shoulder on the trailing edge. The leading edge contained only the higher molecular weight multimers (2×10^6), the trailing edge consisted of lower molecular weight multimers in the series. SDS gel electrophoresis of fractions in the leading part, midportion and trailing part of this protein peak (Fig. 2) illustrates both the heterogeneity of the species present and the tendency for lower molecular weight multimers to elute in later fractions. All species eluting in this peak are much larger than dimers of the subunit, the molecular weight of the fastest band in Fig. 2 being ≅1.4 million. Even

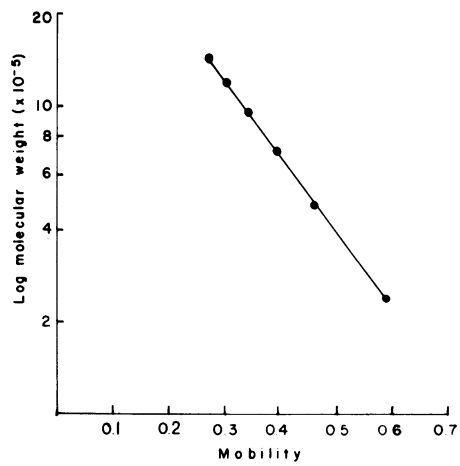

FIGURE 1 Plot of log molecular weight against mobility for multimers of the reduced VIII/VWF subunit cross-linked with dimethylsuberimidate. SDS gel electrophoresis was performed on gels containing 2% polyacrylamide and 0.5% agarose.

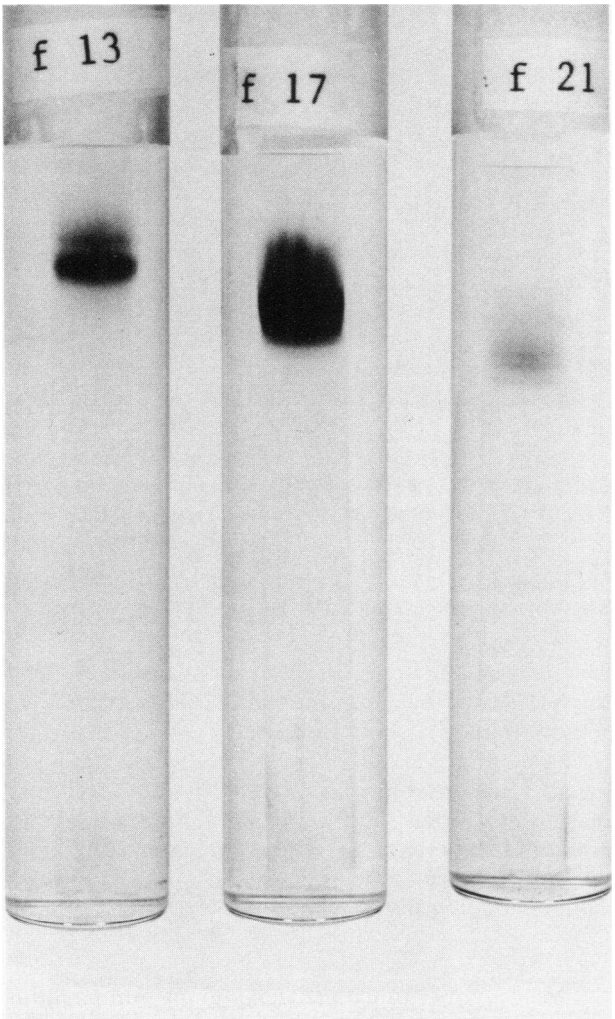

FIGURE 2 SDS gel electrophoresis on 2% polyacrylamide–0.5% agarose gels of unreduced VIII/VWF purified by A-50M agarose gel filtration. The numbers on the tubes are the numbers of the eluate fractions (1 ml) and correspond to the elution diagram shown in Fig. 7. The molecular weight of the fastest-moving band is ≅1.4 million.

when the gels were heavily loaded, as was the case in Fig. 2, fraction 17, no bands corresponding to a low molecular weight protein in the range of 200–250,000 were present.

Reduction of VWF. Partial reduction of the protein from the Bio-Gel A50 purification step with low concentrations of 2-mercaptoethanol, caused a shift to lower molecular weight multimers (Fig. 3). Even at the lowest mercaptoethanol concentration used (0.005%) there was a marked shift away from the 5×10^6 mol wt species to lower molecular weight species. With increasing mercaptoethanol concentration, there was progressive reduction in the size of the multimers. When the concentration was <0.1%, the predominant species had molecular weights in excess of 500,000.

At 2-mercaptoethanol concentrations of 0.5% or greater, the completely reduced VWF subunit of ≅240,000 mol wt appeared, and this was the principle species when the mercaptoethanol concentration was 5%. At mercaptoethanol concentrations between 0.01 and 0.5%, the predominant species had a molecular weight estimated to be 510,000 and was therefore a dimer of the basic subunit. Mercaptoethanol concentrations in excess of 1% were required to achieve significant reduction of interchain disulfide bonds in the dimer within the 30 min incubation period used.

Dialysis of high molecular weight VWF protein from the Bio-Gel A-50 M column for 18 h against buffers of various ionic strength ranging from $\Gamma/2 = 0.015$ (0.01 M phosphate) to $\Gamma/2 = 0.3$ (0.05 M phosphate, 0.09 NaCl) according to the procedure of Van Mourik et al. (14), failed to result in dissociation of the high molecular weight aggregates into lower multimers.

Cross-linking of VWF with dimethylsuberimidate produced a series of multimers with exact correspondence in electrophoretic mobility to the multimers produced by partial reduction of the high molecular weight purified VIII/VWF (Fig. 4). There were also two extra bands in the cross-linked VIII/VWF, corresponding to the trimer and pentamer, neither of which occurred in the partially reduced series. Furthermore, molecular weights determined for the lower three bands produced by reduction with 2-mercaptoethanol (Fig. 4) were 5.1×10^5, 1.1×10^6, and 1.6×10^6. Thus, these bands represent one, two, and three times the dimer molecular weight rather than successive multiples of the monomer.

Free sulfhydryl groups. No free sulfhydryl groups were detected in unreduced, purified VWF. The sensitivity of the determination is such, that as few as one

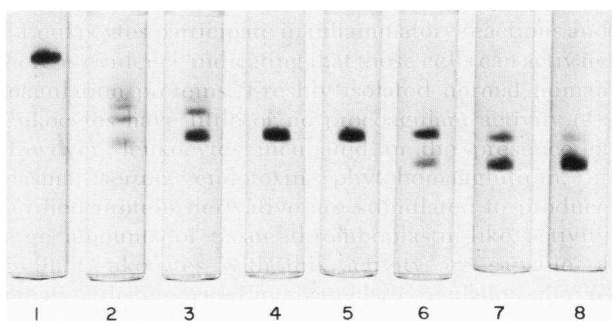

FIGURE 3 Reduction of VIII/VWF with mercaptoethanol. Electrophoresis on 2% polyacrylamide–0.5% agarose SDS gels of VIII/VWF purified on Bio-Gel A-50M agarose. The protein was incubated for 30 min at 37°C with mercaptoethanol at concentrations from 0.005 to 5%. The final mercaptoethanol concentrations in each tube were: 1–0; 2–0.005; 3–0.01; 4–0.05; 5–0.1; 6–0.5; 7–1.0; and 8–5.0%.

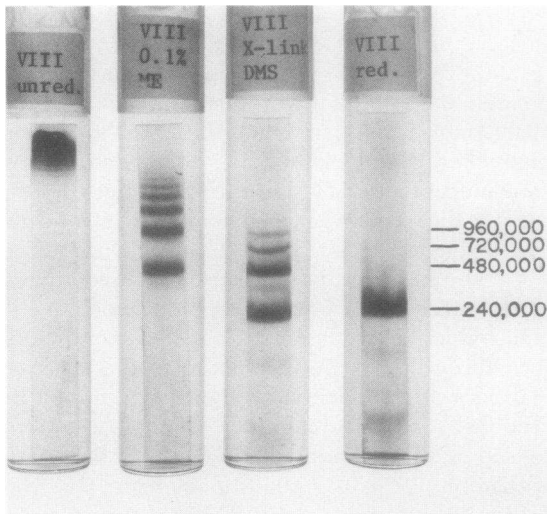

FIGURE 4 SDS electrophoresis on 2% acrylamide–0.5% agarose gels of VIII/VWF. Unred, VIII/VWF from Bio-Gel A-50M column, unreduced; 0.1% ME, VIII/VWF incubated from 30 min at 37°C with mercaptoethanol at a concentration of 0.1%; cross-link DMS, Reduced VIII/VWF subunits cross-linked with dimethylsuberimidate (DMS) (3 mg/ml); Red, VIII/VWF completely reduced with 65 mM dithiothreitol showing 240,000 mol wt subunit. The trimer and pentamer visible in the cross-linked series are missing from the series obtained by reduction with 0.1% mercaptoethanol reduction.

to two sulfhydryl groups per 240,000 mol wt subunits would have been detectable.

Biological activity of VIII/VWF multimers. The purified high molecular weight VIII/VWF protein had both Factor VIII and VWF activities. When this protein is completely reduced and carboxymethylated in the presence of SDS, urea, or guanidine, both activities are lost. We studied the effect of reduction alone on the biological activity by performing a slow reduction of the protein at pH 6.8 with 50 mM mercaptoethanol in the absence of denaturing agents. von Willebrand activity was very sensitive to mild reduction, the activity declining to <5% of the initial activity over 40 min (Fig. 5). In contrast, Factor VIII coagulant activity was stable during the 2-h incubation. The decrease in the size of the multimeric aggregates and the eventual reduction of the protein to the dimer and monomer are shown by SDS gel electrophoresis of the samples removed for the assays (Fig. 6). In the 2-h incubation sample, only the monomeric subunit and the dimer were present. 96% of the original VWF activity had been lost, but the protein retained essentially all of its original Factor VIII activity. The zero-time sample was tested the same as all other samples and contained all reagents, including alkylating agents. Unreduced VIII/VWF lost <5% of its initial Factor VIII activity and VWF activity over a 2-h incubation at 37°C. A control mixture containing all the reagents used, but lacking VIII/VWF, had no procoagulant activity or VWF activity in the assay systems used.

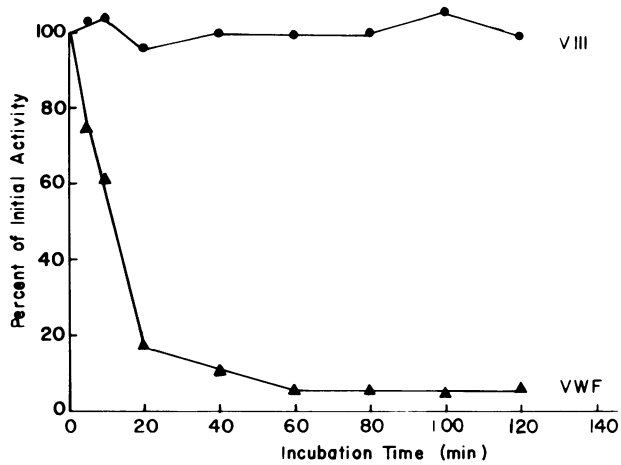

FIGURE 5 Factor VIII and VWF activity of purified VIII/VWF reduced with 50 mM mercaptoethanol at pH 6.8 in the absence of SDS. Symbols used are: (●) Factor VIII coagulant activity; (▲) VWF activity. The initial Factor VIII activity of the protein was 1.7 U/ml, the initial VWF activity was 5.5 U/ml. SDS gels of these samples are shown in Fig. 6.

The distribution of biological activity was examined by gel filtration of purified VIII/VWF and also of normal plasma on an A-50M agarose column. Assays for procoagulant activity and ristocetin aggregation activity were performed on each fraction eluted from the column (Fig. 7). In the case of purified VIII/VWF, the

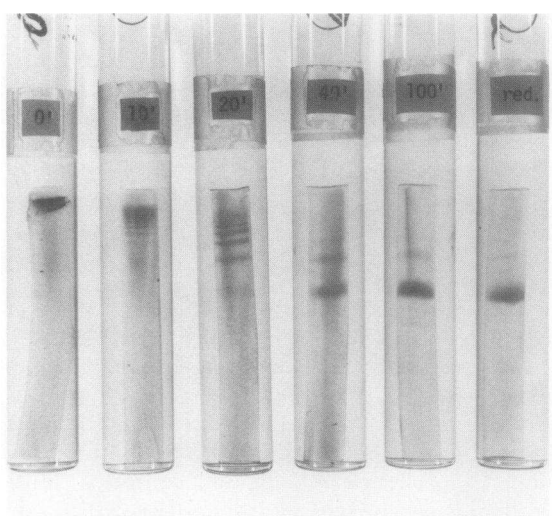

FIGURE 6 Large-pore SDS gel electrophoresis of samples shown in Fig. 5. The incubation time is at the top of each gel. The sample labeled "red" was reduced for 30 min with 65 mM dithiothreitol in 1% SDS, and shows the 240,000 mol wt subunit.

protein concentrations were determined. The Factor VIII and VWF activities were proportionally present in each fraction throughout the protein peak of purified VIII-VWF. On gel filtration of plasma over the same column, the peak of Factor VIII and VWF activities eluted at the same place and with the same distribution as the protein peak of purified VIII/VWF. In the plasma experiments, there was also close correspondence between Factor VIII activity and VWF activity in each fraction across the peak. SDS electrophoresis of various fractions of purified VIII/VWF eluted from the column showed higher molecular-weight multimers in fractions from the leading edge of the peak than in fractions from the trailing edge (Fig. 2).

DISCUSSION

On conventional 5% polyacrylamide SDS gel electrophoresis, unreduced VIII/VWF does not enter the gel. Thus, studies of the homogeneity have been based mostly on electrophoresis of the reduced protein. Electrophoresis in large-pore gels containing 2% acrylamide and 0.5% agarose, shows that unreduced VIII/VWF protein consists of a series of multimers (Fig. 2). The existence of this multimeric series was reported by Van Mourik et al. (14), who attributed it to non-covalent interactions, because they observed dissociation of the high molecular weight aggregates on dialysis against solutions of low ionic strength and pH. With our purified VIII/VWF, we did not observe dissociation on dialysis for 12 h against low ionic strength buffer, but when the high molecular weight fraction was allowed to stand in 1% SDS solution for several days, dissociation did occur, and on electrophoresis the multimeric series was seen. However, in the presence of trace amounts of mercaptoethanol, dissociation of higher aggregates occurs very rapidly to produce a series of multimers within minutes (Fig. 3). The concentrations required for dissociation to the level of the VWF dimer of 480,000 mol wt are on the order of those expected for the catalytic effect of mercaptoethanol in disulfide interchange as demonstrated by Smithies (30).

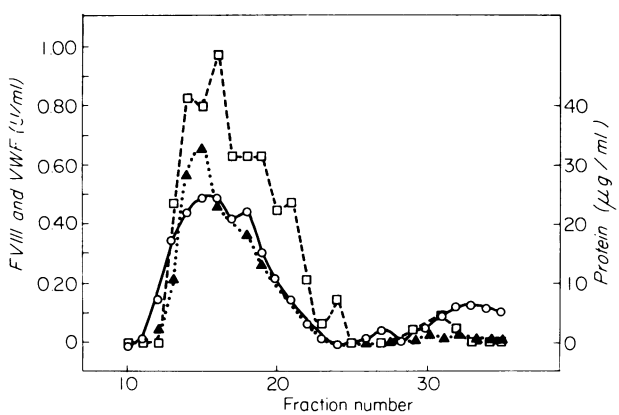

FIGURE 7 Elution of VIII/VWF from Bio-Gel A-50M gel A-50M gel filtration column. (○) Protein; (□) VWF (ristocetin aggregation) activity; (▲) AHF (Factor VIII) activity. SDS gels of the representative fractions through this peak are shown in Fig. 2.

We conclude that the disulfide bonds holding the multimers together are labile, with disulfide interchange occurring slowly—over several days—in the absence of added sulfhydryl reagents, but very rapidly, in minutes, in the presence of traces of mercaptoethanol. The differences between our observations and those of Van Mourik et al. (14) may be because of slight differences in the free sulfhydryl group concentrations in the two preparations.

The protein species formed by random cross-linking of VIII/VWF (26) contain one to five subunits, with the relative quantities of the multimers decreasing with higher molecular weight as expected from the law of mass action (Fig. 4). In addition to allowing estimation of molecular weights, a comparison of the multimers resulting from mercaptoethanol reduction with the cross-linked series shows the absence of bands corresponding to odd-numbered multimers, most clearly the trimer and pentamer. The concentrations of higher artificially cross-linked multimers were too low to allow direct comparisons above the level of the hexamer, but estimation of molecular weights of the reduced series from the band mobilities confirms that they correspond to even multiples of the basic subunit. This indicates that the protomeric unit of the disulfide-bonded aggregate series is the dimer. Furthermore, the disulfide bonds holding together the higher multimers require mercaptoethanol concentrations of <0.01% for disulfide rearrangement, whereas reduction of the interchain disulfide bonds in the dimer requires mercaptoethanol concentration of 0.5–1.0% or 10–20 times higher.

Kaelin (31) reported dissociation of VIII/VWF into a species of 690,000 mol wt by periodate oxidation. This species is of approximately the same size as the VIII/VWF protomer we find after partial reduction. Although it is likely that some of the effect of periodate on VIII/VWF is the result of carbohydrate oxidation, disulfide bonds can also be oxidized by this reagent.

Furlan and Beck (15) have called attention to the sensitivity of VIII/VWF to proteases. It is unlikely that the dissociation of the VIII/VWF polymers, seen with our preparation was the result of proteolysis because high concentrations of proteolytic inhibitors are present during the purification (1).

In gel filtration studies of purified VIII/VWF, Factor VIII activity and VWF activity were each approximately proportional to the protein concentrations in the broad void volume peak (Fig. 7). All the protein

species eluted in this peak had molecular weights, estimated by SDS gel electrophoresis, of >1.4 million. When this experiment was repeated using plasma in place of purified VIII/VWF, the VWF and Factor VIII activities eluted with the same elution volume distribution as the purified protein. Molecular weights could not be directly estimated because of the low VIII/VWF concentration in plasma. These results are consistent with the report by Zimmerman et al. (32) that 80% of VWF in plasma has a sedimentation coefficient >20 *s*.

To study further the relationship of the multimeric structure to the biological activities of VIII/VWF, we reduced the protein at a relatively slow rate (pH 6.8) in the absence of denaturing agents. As expected the disulfide bonds holding the multimers together were the most accessible, but over a 2-hour incubation period all of the interchain disulfide bonds were reduced. Under these conditions there was no loss of coagulant activity, whereas VWF activity was lost before dimers or monomers were detected in the mixture. That this was the result of an alteration in the VWF protein, rather than a nonspecific effect of the reagents upon the ristocetin aggregation assay, is shown by the zero-time sample to which both mercaptoethanol and iodoacetamide had been added. This sample retained full VWF activity.

Previous studies, in many laboratories, have demonstrated the separation of Factor VIII coagulant activity of low molecular weight from high molecular weight VWF (5–7). In most of these reports the molecular weight estimated for the coagulant fraction was in the same range as the subunit molecular weight; ≅200,000. Although the separation of Factor VIII activity from VWF by gel filtration in 0.25 M $CaCl_2$ is not inconsistent with the hypothesis that two different, noncovalently associated proteins make up VIII/VWF complex, our present studies demonstrate that a low molecular weight coagulant species can be separated, by mild reduction, from the high molecular weight purified protein having both Factor VIII and VWF activities.

Thus, our studies provide further evidence that both Factor VIII and VWF activities reside in a covalently bound protein species. The state of aggregation of this protein may be important in determining VWF activity, but the results are consistent with a model in which the 240,000 mol wt subunit possesses Factor VIII clotting activity.

An alternative possibility, suggested by Vehar and Davie (11), is that the protein having Factor VIII activity may be present in such low concentrations that it is not visualized in our present electrophoresis systems. However, data presented by Ratnoff and co-workers (4) and Koutts and co-workers (33) indicate that although there are antigenic differences between VWF and the protein with Factor VIII activity which suggest that they are separate proteins, they are also closely linked both in plasma and in purified VIII/VWF. The present studies indicate that these moieties are linked in the purified protein by disulfide bonds which can be rather easily disrupted under mild conditions.

ACKNOWLEDGMENTS

The authors thank Dr. Eloise R. Giblett and Dr. Earl W. Davie for their helpful discussions and for reviewing drafts of this manuscript.

Supported by National Research and Demonstration Center grant HL-17265 to the Puget Sound Blood Center.

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