Human Factor VIII\textsubscript{a} Subunit Structure

RECONSTITUTION OF FACTOR VIII\textsubscript{a} FROM THE ISOLATED A1/A3-C1-C2 DIMER AND A2 SUBUNIT\textsuperscript{a}

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Heterodimeric human factor VIII was proteolytically activated by catalytic levels of thrombin to yield the (labile) active cofactor factor VIII\textsubscript{a}, possessing an initial specific activity of ~80 units/\mu g. Activation paralleled the generation of fragments A1 and A2 derived from the heavy chain and A3-C1-C2 derived from the light chain. Chromatography of factor VIII\textsubscript{a} on Mono-S buffered at pH 6.0 resulted in separation of the bulk of the A2 fragment from a fraction composed predominantly of A1/A3-C1-C2 dimer plus low levels of A2 fragment. Only the latter fraction contained clotting activity (~20 units/\mu g) which was stable and represented a <10\% yield when compared with the peak activity of unfractionated factor VIII\textsubscript{a}. Further depletion of A2 fragment from Mono-S-purified factor VIII\textsubscript{a}, achieved using an immobilized monoclonal antibody to the A2 domain, yielded a relatively inactive A1/A3-C1-C2 dimer (<0.4 unit/\mu g). Factor VIII\textsubscript{a} (>40 units/\mu g) was reconstituted from the A1/A3-C1-C2 dimer plus the A2 fragment in a reaction that was Me\textsuperscript{2+}-independent and inhibited by moderate ionic strength. Reassociation of A2 required the A1 subunit in that the A2 subunit associated weakly if at all to A3-C1-C2 in the absence of A1. These results indicated that human factor VIII\textsubscript{a} is a trimer represented by the subunits A1/A2/A3-C1-C2 and that the A2 subunit is required for expression of factor VIII\textsubscript{a} activity.

Factor VIII\textsubscript{a}, a protein cofactor for the intrinsic factor Xase enzyme complex, is decreased or defective in individuals with hemophilia A. Factor VIII\textsubscript{a} is synthesized as a single-chain precursor of approximately 300 kDa (1, 2) and is represented by the domain structure A1-A2-B-A3-C1-C2 (3). Factor VIII\textsubscript{a} circulates as a series of Me\textsuperscript{2+}-linked heterodimers (4-6) formed as a result of proteolysis at the B-A3 junction in the precursor plus additional cleavages within the B domain (3). The factor VIII heavy chain is minimally represented by the A1-A2 domains but exhibits significant size heterogeneity resulting from the presence of some or all of the contiguous B domain, whereas the light chain corresponds to the A3-C1-C2 domains derived from the COOH-terminal end of the precursor. Recent electron microscopy studies on porcine (7) and human (8) factor VIII reveal that the A1-A2 and A3-C1-C2 domains of heavy and light chains, respectively, form a globular core structure while the B domain of the heavy chain forms an extended rodlike structure. The intact heterodimeric structure is essential for cofactor function in that the subunits of factor VIII\textsubscript{a} are dissociated by chelating reagents, resulting in loss of clotting activity (4, 9).

Conversion of human factor VIII heterodimers to active cofactor (factor VIII\textsubscript{a}) by thrombin is associated with cleavages in the heavy chain at residue 372 (A1-A2 junction) and residue 740 (A2-B junction) while the light chain is cleaved near its NH\textsubscript{2} terminus at residue 1689 (10). Cleavage of the light chain in itself may contribute to increased specific activity of the cofactor (11) but functions primarily to release factor VIII from its carrier protein, von Willebrand factor (12). Similarly, cleavage of the heavy chain at residue 740 yields a 90-kDa heavy chain form (contiguous A1-A2 subunit) which requires further cleavage (at residue 372) (10) to express factor VIII\textsubscript{a} activity (6). Conversion of Arg\textsuperscript{372} to Ile by site-directed mutagenesis results in a molecule resistant to thrombin cleavage at the modified site, and not activated by the protease (13). The activity associated with factor VIII\textsubscript{a} is markedly labile. Recently, it has been suggested that the spontaneous loss of factor VIII\textsubscript{a} activity results from pH-dependent subunit dissociation (14).

Recent results of Lollar and Parker (15) show that porcine factor VIII\textsubscript{a} is heterotrimeric, represented by the A1, A2, and A3-C1-C2 subunits in equimolar stoichiometry. Following isolation of this material by Mono-S chromatography performed at slightly acidic pH, the recovered factor VIII\textsubscript{a} activity exhibited marked stability. Earlier results from our laboratory obtained from analysis of low concentrations (approximately 0.01 mg/ml) of protein, suggested that human factor VIII\textsubscript{a} consists of an A1/A3-C1-C2 heterodimer with the A2 polypeptide representing an activation fragment (16). This material was unstable at pH 7.2, an effect attributed to a labile conformation rather than dissociation of subunits in the dimer.

In this report we reexamine the subunit structure of human factor VIII\textsubscript{a}, following activation by thrombin using a >10-
fold higher level of protein and employing a chromatographic step shown for the porcine system to yield stable material. While the results obtained show significant dissociation of A2 from the A1/A3-C1-C2 dimer following chromatography, the activity associated with recovered factor VIII, likely results from the trimERIC form of the cofactor. This conclusion is supported by the capacity to reconstitute high specific activity factor VIII, from a relatively inactive A1/A3-C1-C2 dimer plus the A2 subunit.

MATERIALS AND METHODS

Reagents—Human factor VIII concentrate (Koate™) was a generous gift from the Cutter Division of Miles Laboratories. Factor VIII, factor VIII subunits and thrombin-cleaved factor VIII light chain (A3-Cl-C2 subunit) were prepared as described previously (17, 18).

Human α-thrombin (2900 units/mg) was obtained from Enzyme Research Laboratories, Inc. PPACK was purchased from Calbiochem. Reagents used for the production of monoclonal antibodies were as described previously (19). Factor VIII deficient plasma was purchased from George King Biomedical, Inc.

Thrombin Activation of Factor VIII—Factor VIII (90 μg/ml, 4 ml) in 0.01 M Hepes, pH 7.2, 0.2 M NaCl, 0.005 M CaCl₂, and 0.01% (v/v) Tween-20 was reacted with 2 μg (6 units) of human α-thrombin at 22 °C. Assuming a mean molecular mass of 200 kDa for factor VIII (9) and 36.6 kDa for thrombin (20), this represented an enzyme/substrate ratio of 1:30. Reactions were performed in low adsorbing NUNC tubes (Thomas Scientific). Aliquots were removed at the indicated times and assayed for factor VIII activity using a one-stage clotting assay.

Chromatography of Human Factor VIII, Using Mono-S—Factor VIII, at near peak activity was subjected to Mono-S chromatography (Pharmacia LKB Biotechnology Inc.) fast protein liquid chromatography system essentially as described by Lollar and Parker (15). Briefly, at the peak level of factor VIII, activity, PPACK was added to a final concentration of 0.05 μM. The reaction mixture was diluted with an equal volume of 0.5 M histidine, pH 6.0, 0.005 M CaCl₂, and 0.01% Tween-20 and applied to a Mono-S (HR5/5) column equilibrated in 0.1 M histidine, pH 6.0, 0.1 M NaCl, 0.005 M CaCl₂, and 0.01% Tween-20 at a flow rate of 1 ml/min. Sample application was made via a 10-ml super loop, the glass barrel of which had previously been treated with SigmaCoat (Sigma) according to the manufacturer’s instructions. After the sample had been adsorbed, the column was washed with the equilibration buffer until the effluent absorbance was less than 0.002. The column was then developed with a 30 ml linear NaCl gradient (0.1 to 1 M NaCl) in equilibration buffer at a flow rate of 0.5 ml/min and fractions (0.5 ml) were collected in NUNC tubes. Fractions were assayed for factor VIII, by clotting assay and protein concentrations by the method of Bradford (21) using bovine serum albumin as a standard. Column fractions were stored in the refrigerator (4 °C).

Fractionation of Factor VIII.—Reaction of human factor VIII (~0.1 mg/ml) at pH 7.2 with a catalytic level of thrombin resulted in a 40-fold activation of the cofactor (Fig. 1). The factor VIII, activity generated was unstable and decayed with a t½ of approximately 15 min. Factor VIII, at near peak activity (<3 min, post-thrombin addition, see Fig. 1) was treated with PPACK and subjected to Mono-S chromatography developed with a linear NaCl gradient and buffered at pH 6.0 (Fig. 2) essentially as described by Lollar and Parker (15). Two protein peaks were observed with clotting activity associated only with the second. The yield of recovered factor VIII, activity was approximately 6% based upon the peak activity level shown in Fig. 1. However, unlike the unfraccionated material, the Mono-S-
purified factor VIII was significantly more stable, initially decaying to approximately 40% of its original level with a half-life of ~1 day and subsequently retaining this level of activity for at least 1 month when stored at 4 °C. This stabilization of activity following Mono-S chromatography was similar to that observed for porcine factor VIII (15).

Polypeptide composition determined by SDS-PAGE and silver staining revealed that factor VIII(MS) contained two predominant bands of 73 and 51 kDa representing the A3-C1-C2 and A1 fragments, respectively (Fig. 3). Also present in this fraction was a low level of the 43-kDa A2 fragment. Similar composition analysis of the earlier eluting Mono-S peak, which lacked detectable activity, showed the A2 polypeptide, indicating that the bulk of A2 had been separated from the A1/A3-C1-C2 dimer, a result consistent with our earlier observations (16). Based upon the presence of low levels of A2 fragment in factor VIII(MS), it was not clear whether the recovered activity reflected active A1/A3-C1-C2 heterodimer with low levels of contaminating A2 or whether the activity was attributed to low levels of A1/A2/A3-C1-C2 heterotrimer present in the fraction. To evaluate the role of A2, factor VIII(MS) was subjected to chromatography on an immunoaffinity column prepared with a monoclonal antibody, R8B12, directed to within the A2 domain. This antibody has been utilized in our laboratory to effect purification of human factor VIII heterodimers (22). Application of human factor VIII(MS) to this immunoabsorbent resulted in further depletion of the A2 fragment relative to A1/A3-C1-C2 as judged by SDS-PAGE (Fig. 3) and Western blotting (Fig. 4). The resultant material, designated factor VIII(MS/R8), obtained in the unbound column fraction showed significant reduction in both the quantity of A2 and, in parallel, the level of clotting activity. No residual activity was eluted from the column using conditions that elute native factor VIII (22).

Quantitative ELISA using the R8B12 antibody was performed to estimate the concentrations of A2 fragment in factor VIII(MS) and factor VIII(MS/R8) (Table 1). Factor VIII(MS) exhibited an 80% depletion in A2 content when compared with unfractionated factor VIII; a result that paralleled the approximately 4-fold lower specific activity of the former. The amount of A2 fragment present in the R8B12-depleted fraction could not be determined accurately because of its low concentration. From ELISA a maximal level of 0.02 mol A2: mol A1/A3-C1-C2 dimer was determined. Again, the lack of A2 fragment in this fraction paralleled its low specific
activity. These results suggested an integral role for the A2 fragment in factor VIII activity. Attempts to use the R8-affinity column to directly adsorb and elute an A2-containing factor VIII species have not been successful, possibly resulting from perturbation of a weak association of A2 with the A1/A3-C1-C2 dimer. However, little if any factor VIII activity was lost following exposure to a noninteracting antibody (anti-von Willebrand factor propolypeptide) coupled to Affigel, thus ruling out any nonspecific cause for loss of factor VIII activity. This experiment further indicated that the observed stimulatory effect of A2 was not artifactual resulting from a potential active thrombin contamination in the A2 subunit-containing fraction.

The association of A2 subunit with A1/A3-C1-C2 dimer was characterized with regard to the effects of Ca²⁺ concentration and ionic strength (Fig. 6). Surprisingly, the presence of Ca²⁺ inhibited reconstitution of factor VIII activity. This result was in sharp contrast to the requirement for Me²⁺ in forming heterodimeric factor VIII from isolated heavy and light chains (9). Maximal factor VIII activity was generated with <1 mM CaCl₂, a level representing the endogenous Ca²⁺ concentration present in the subunits following their dilution into the reaction mixture. This concentration of Ca²⁺ was ineffective in promoting association of native factor VIII subunits over a similar time course (data not shown). Levels of Ca²⁺ resulting in optimal rates of reassociation of native factor VIII subunits (approximately 50 mM) (9), inhibited the regeneration of factor VIII activity by >90%.
Increasing NaCl concentration also was inhibitory to factor VIII, regeneration although apparently less so than was observed for Ca\(^{2+}\). Again, this result was somewhat different than what had been observed for native factor VIII subunit reconstitution where approximately 0.5 M NaCl was optimal (9). The inhibition caused by Ca\(^{2+}\) cannot be accounted for solely by increased ionic strength of the reaction mixture since 50 mM CaCl\(_2\) (equivalent to 150 mM monovalent salt) resulted in significantly greater inhibition than >1 M NaCl. This result may reflect the chaotropic activity attributed to Ca\(^{2+}\).

The above results suggested that the A2 subunit binds stoichiometrically to the A1/A3-C1-C2 dimer in a Me\(^{2+}\)-independent reaction. In order to determine whether binding of A2 to A3-C1-C2 required the presence of the A1 subunit the following experiment was performed (Fig. 7). The A2 subunit was reacted in the presence or absence of a molar excess of thrombin-activated, human factor VIII light chain (A3-C1-C2 subunit) which had been isolated free from heavy chain-derived fragments. The reactions were run under conditions that promoted association of A2 with A1/A3-C1-C2. During the course of reactions, aliquots were removed and assayed for factor VIII, activity. No activity was detected indicating a requirement for the A1 subunit for functional factor VIII,. However, if A2 bound A3-C1-C2 then one might expect that no factor VIII, activity would be regenerated following subsequent addition of A1/A3-C1-C2.

When A1/A3-C1-C2 dimer was added to either the A2 subunit alone or A2 that had been preincubated with excess A3-C1-C2, equivalent rates and extents of activity were regenerated. This result suggested that the A2 subunit binds weakly if at all to A3-C1-C2 in the absence of A1 subunit. Similar results were observed when native factor VIII light chain was substituted for the A3-C1-C2 subunit in the preincubation mixture or when reactions were supplemented with 10 mM CaCl\(_2\) (data not shown). Control experiments showed that both A5-C1-C2 and native light chain efficiently recombined with intact factor VIII heavy chain (data not shown) indicating the potential of the light chains to yield functional factor VIII. These results suggested that the A1 subunit was required for A2 association in factor VIII,.

**DISCUSSION**

The subunit structure of thrombin-activated, human factor VIII, was investigated following its chromatography on Mono-S alone and in conjunction with an anti-factor VIII heavy chain (A2 domain-specific) monoclonal antibody immunoadsorbent. From these analyses we conclude that human factor VIII, is represented by the A1/A2/A3-C1-C2 heterotrimer and that the A2 subunit is required for expression of factor VIII, activity. These conclusions were based primarily on two observations. First, chromatography of factor VIII, on Mono-S or Mono-S plus the immunoadsorbant yielded an A1/A3-C1-C2 dimer with subunits in equivalent stoichiometry, yet the specific activity of material recovered from the columns paralleled the content of A2 in these fractions. Second, high specific activity factor VIII, (>40 units/\(\mu\)g) could be reconstituted from a relatively inactive (<0.4 unit/\(\mu\)g) A1/A3-C1-C2 dimer plus a stoichiometric amount of purified A2 subunit, the latter possessing no intrinsic activity.

Our results from an earlier study (16) resolved A2 from A1/A3-C1-C2 following fractionation of factor VIII, by either rapid gel filtration or anion-exchange (Mono-Q) chromatography performed at pH 7.2. Based upon coelution of the (labile) activity with the A1/A3-C1-C2 dimer, it was concluded that factor VIII, was dimeric and that the A2 polypeptide represented an activation fragment. No A2 was identified in the active fractions, a result likely reflective of limitations in the amount of factor VIII available for the analysis and lack of immunologic reagents to detect trace levels of A2. We now believe that these earlier conclusions were in error.

Results obtained in the present study are consistent with the studies of Lollar and colleagues using the porcine protein. Thrombin activation of porcine factor VIII yields a labile product, the activity of which was markedly stabilized following Mono-S chromatography (15). Porcine factor VIII, was shown to consist of an A1/A2/A3-C1-C2 heterotrimer with the three polypeptides possessing equal staining intensity following SDS-PAGE. The apparent molecular weight of this material from equilibrium sedimentation analysis, 148,000-161,000, was in good agreement with the summed apparent molecular weights of the subunits, 148,000 determined electrophoretically. Recently, these investigators have shown denaturation of porcine factor VIII, by pH-dependent subunit dissociation (14). Loss of factor VIII, activity at pH 8.0 resulted from dissociation of the A2 subunit since an inactive A1/A3-C1-C2 dimer could be isolated by Mono-S chromatography.

Results from Mono-S chromatography of human factor VIII, qualitatively more closely resembled results obtained for the pH-inactivated porcine factor VIII, rather than native porcine factor VIII, since the bulk of A1/A3-C1-C2 dimer was separated from the A2 subunit. Recovery of human factor VIII, activity was typically <10% when compared with the unfractionated peak activity, whereas Lollar and Parker (15) reported a ~60% yield of porcine factor VIII, activity. Indeed, a direct comparison of the human and porcine proteins suggests a much weaker association of the A2 subunit in human factor VIII,.

In addition, differences in physical properties may exist between the human and porcine A2 subunits. The human A2 subunit was quantitatively adsorbed and eluted on Mono-S and appeared to be quite soluble at nominal concentrations in the elution buffer. On the other hand, porcine A2 subunit, generated following dissociation of factor VIII, at pH 8.0, was recovered in very low yield in the unbound Mono-S fraction and exhibited poor solubility (14).

Me\(^{2+}\) binding is required for factor VIII integrity in that heavy and light chains are dissociated by chelating reagents with resultant loss of clotting activity (4, 9). The native factor VIII subunits efficiently reassociate to form active heterodi-

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2 P. Lollar, personal communication.
mers in the presence of approximately 50 mM Mn⁺⁺ or Ca⁺⁺ (9). Based upon homologies to ceruloplasmin, Vehar et al. (9) observed that consensus type 1 copper-binding residues were present in the A1 and A3 domains but absent in the A2 domain of factor VIII. Thus association of the A2 subunit with other factor VIII, subunits is predicted to be independent of Me⁺⁺. Our results support this hypothesis in that association of the A2 subunit with A1/A3-C1-C2 dimer occurred at <50 mM concentrations of Ca⁺⁺, concentrations which failed to support reassociation of native factor VIII subunits. Furthermore, concentrations of Ca⁺⁺ which resulted in maximal levels of native subunit reassociation inhibited the binding of A2 subunit by >90%. The association of A2 subunit appeared to be primarily electrostatic in that reassociation was inhibited by even moderate concentrations of NaCl. This effect also was in contrast to the reassociation of native subunits which showed aspects of both electrostatic and hydrophobic interactions (9). However, this primarily electrostatic nature of A2 subunit association in factor VIII, was consistent with its observed dissociation at slightly alkaline pH (14).

Binding of the A2 subunit appeared to be dependent upon the presence of A1 in the A1/A3-C1-C2 dimer since A2 associated weakly if at all with isolated A3-C1-C2. The basis for this conclusion was that preincubation of A2 with a molar excess of A3-C1-C2 did not affect the capacity for the A2 subunit to subsequently bind added A1/A3-C1-C2, as measured by regeneration of factor VIII activity. This result suggested that a primary binding interaction of A2 in factor VIII, may be with the A1 subunit rather than with the light chain-derived subunit. Thrombin cleaves the contiguous A1-A2 subunit at residue 372 (3, 10). The residues directly preceding this site (Arg³⁷⁰ to Arg³⁷⁵) contain a high concentration of acidic residues (13 Glu plus Asp) (3). In that the A2 subunit is adsorbed by the cation exchanger, Mono-S, it is interesting to speculate that the stretch of predominantly acidic residues at the end of the A1 subunit might play a role in the retention of A2 subunit in factor VIII, following cleavage at the A1-A2 junction by thrombin.

Although apparently indispensable for activity, the role of the A2 subunit in factor VIII, is unknown. Clearly, A2 may participate directly in the binding of factor IX, and/or its substrate, factor X in the factor Xase complex. However, it must be noted that A2 alone or in the presence of A3-C1-C2 was insufficient to generate factor VIII, activity. Alternatively, A2 may stabilize a labile conformation in the A1/A3-C1-C2 dimer, in that our results and those of others do not exclude the possibility that the dimer is transiently active. The ability to generate the unique reagents A1/A2/A3-C1-C2 and A1/A3-C1-C2 will potentially allow for determination of the function of the A2 subunit in cofactor activity.

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