Factor VIII, a protein cofactor for the intrinsic factor Xase enzyme complex, is decreased or defective in individuals with hemophilia A. Although synthesized as a large single-chain precursor (1, 2) of domain structure Al-A2-B-A3-Cl-C2 (3), factor VIII circulates as a series of Me²⁺-linked heterodimers (4-6) formed as a result of proteolysis at the B-A3 junction plus additional cleavages within B domain (3). The factor VIII heavy chain is minimally represented by the Al-A2 domains but exhibits significant size heterogeneity resulting from the presence of some or all of the contiguous B domain. The factor VIII light chain corresponds to the A3-C1-C2 domains derived from the COOH-terminal end of the precursor. The intact heterodimeric structure is essential for cofactor function since the subunits of factor VIII are dissociated by chelating reagents resulting in loss of clotting activity (4, 7).

Conversion of human factor VIII heterodimers to active cofactor (factor VIIIa) by thrombin is associated with cleavages in the heavy chain at residue 372 (Al-A2 junction) and at residue 740 (A2-B junction) while the light chain is cleaved near its NH₂ terminus at residue 1889 (8). Cleavage of the light chain in itself may contribute to increased specific activity of the cofactor (9), whereas cleavage of the heavy chain at residue 740 yields the contiguous A1-A2 polypeptide that requires further cleavage at residue 372 (8) to express factor VIII activity (6).

Factor VIII is markedly unstable with subsequent loss of activity attributed to a mechanism independent of further thrombin action (10). However, chromatography of thrombin-activated, porcine factor VIII using Mono S under slightly acidic conditions yields stable factor VIII, represented by a heterotrimer of A1, A2, and A3-C1-C2 subunits (11). Recently it was shown that human factor VIII, also is stabilized following similar chromatography and is a trimmer of analogous subunits, but with A2 showing reduced affinity for the A1/A3-C1-C2 dimer (12, 13). The spontaneous decay of factor VIII activity has been attributed to dissociation of the A2 subunit from the A1/A3-C1-C2 dimer (12, 13). However, under conditions of slightly acidic pH and low ionic strength and Ca²⁺, high specific activity factor VIII, can be reconstituted from A2 subunit and dimer (12).

Because of the apparent reversibility of the interaction between A2 and dimer, a study was undertaken to evaluate human factor VIII, stability as related to exogenous A2 subunit, pH, and cofactor concentration. The results obtained suggest that the observed instability of factor VIII is governed by a dissociation constant for the A2-dimer interaction. Furthermore, the capacity for free A1 subunit to inhibit this interaction indicates that the A1 subunit is integral for A2 association within the dimer.

**MATERIALS AND METHODS**

**Reagents**—Human factor VIII concentrate (Koate-HPTM) was generously provided by the Cutter Division of Miles Laboratories. Recombinant human factor VIII (5300 units/mg) was the generous gift of Drs. R. Kaufman and D. Pittman of the Genetics Institute. Factor VIII-deficient plasma was purchased from George King Biomedical, Inc. The murine monoclonal antibody R8B12, specific for the A2 domain of factor VIII, has been described (12). PPACK was purchased from Calbiochem. Human α-thrombin (2900 units/mg) was obtained from Enzyme Research Laboratories, Inc.

*Factor VIII, Factor VIIIa, and Subunits—Factor VIII was purified* ¹

¹The abbreviations used are: factor VIIIa, thrombin-activated factor VIII; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PPACK, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone.

²Factor VIII, subunits are designated relative to the domainal sequence A1-A2-B-A3-C1-C2 (3) and are as follows: A1, residues 1-372; A2, residues 373-740; A3-C1-C2, residues 1690-2332. Noncovalent subunit associations are denoted by (⁄) and covalent associations are denoted by (-), a practice initiated by Lollar and Parker (11).
Interaction between A2 Subunit and A1/A3-C1-C2 Dimer

from Koate-HP as described previously (15). Mono S chromatography of thrombin-activated factor VIII resolved the A2 subunit from a mixture of A1/A2/A3-C1-C2 trimers and A1/A3-C1-C2 dimers (12). Dimers were subsequently purified away from residual A2 subunit by chromatography on the R8B12 immunoabsorbent (12). Alternatively, A2 subunit and A1/A3-C1-C2 dimer were separately isolated following direct application of factor VIII, to the immunosorbent. Dimer eluted in the unbound fraction whereas A2 was eluted from the antibody column with buffer containing ethylene glycol (16). A1 and A3-C1-C2 subunits were isolated following treatment of the dimer (192 nM) with 0.02 M Hepes pH 7.2, 0.15 M NaCl, and 0.01% Tween with 0.04 M EDTA for 2 h at room temperature. This material was diluted with an equal volume of the above buffer and applied to a Mono Q HR5/5 column equilibrated in the above buffer plus 0.01 M EDTA. The A3-C1-C2 subunit eluted in the unbound fraction, whereas A1 subunit was adsorbed and subsequently batch-eluted with buffer containing 0.8 M NaCl. These procedures utilized both the plasma-derived and recombinant factor VIII as starting material with equivalent results. Protein concentrations were determined by the Coomassie dye binding method of Bradford (17) using bovine serum albumin as a standard.

**Thrombin Activation of Factor VIII**—Factor VIII in 0.2 M Tris, pH 7.4, 0.15 M NaCl, 0.005 M CaCl\(_2\), 0.01% (v/v) Tween 20, and bovine serum albumin (1 mg/ml) was reacted with human α-thrombin at 22 °C. Reactions were performed using an enzyme:substrate ratio of 1:25, assuming a mean molecular mass of 220 kDa for factor VIII (18) and 36.6 kDa for thrombin (19). Aliquots were removed at the indicated times and assayed for factor VIII activity using a one-stage clotting assay.

**Determination of K\(_{d}\)** for the A2 Subunit-A1/A3-C1-C2 Dimer Interaction—Values were obtained from stable levels of factor VIII activities at various concentrations using the expression K\(_{d}\) = [A2][A1/A3-C1-C2]/[A1/A2/A3-C1-C2]. A maximum factor VIII specific activity of 84 units/μg (12) was used to indicate when all subunits were in the active trimeric (A1/A2/A3-C1-C2) form. Thus, the ratio of observed specific activity to this maximal value is an indicator of the trimer concentration for a given total factor VIII concentration. It was assumed that [A2] equaled [A1/A3-C1-C2] at all times, and this value was obtained following subtraction of [A1/A2/A3-C1-C2] from total factor VIII.

**Reconstitution of Factor VIII** from A2 Subunit and A1/A3-C1-C2 Dimer—Reactions contained 0.01 M histidine, pH 6.0, 0.025 M NaCl, 0.4 mM CaCl\(_2\), 0.01% (v/v) Tween 20, bovine serum albumin (1 mg/ ml), and A2 subunit and A1/A3-C1-C2 dimer at the indicated concentrations. The above combinations of NaCl and CaCl\(_2\) were from a contribution of endogenous levels of these reagents present in the factor VIII subunit. Reactions were run for 30 min at 22 °C and assayed for clotting activity.

**Electrophoresis—**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli (19). Gradient gels (6-15% polyacrylamide) were cast using a multigel casting chamber (Bio-Rad). Electrophoresis was for 1 h at 180 V. Bands were visualized following staining with silver nitrate (20).

**RESULTS**

**Reversible Binding of A2 Subunit with A1/A3-C1-C2 Dimer**—Thrombin activation of factor VIII results in a marked increase in cofactor activity followed by a thrombin-independent decay of activity. This latter phenomenon likely reflects dissociation of the A2 subunit (12, 14). However, the ability to reconstitute high specific activity factor VIII, from A2 subunit and A1/A3-C1-C2 dimer (12) suggested that dissociation of A2 and hence loss of potentiated activity was reversible. Consistent with this earlier finding was the observed capacity for exogenous A2 subunit to stabilize the labile activity of factor VIII (Fig. 1). For this experiment, factor VIII in the absence or presence of indicated amounts of A2 subunit was reacted with a catalytic level of human α-thrombin. At peak activity, approximately 1-2 min, PPACK was added to inactive thrombin, thereby preventing further proteolysis of factor VIII, and residual clotting activity was determined at the indicated times. In the absence of added A2 subunit we observed the characteristic rapid decay of activity to a more stable, basal level of cofactor activity. This level was found to be dependent upon both reaction pH and cofactor concentration (see below). Reactions containing increased levels of A2 subunit resulted in both a slower rate of factor VIII decay and a higher basal level of clotting activity. At a 28-fold molar excess of free A2 subunit relative to factor VIII, the subsequent decay of factor VIII activity was virtually eliminated over the time course shown. Lower exogenous levels of A2 subunit yielded intermediate extents of basal factor VIII activity. These results were consistent with the hypothesis that spontaneous decay of factor VIII primarily resulted from A2 subunit dissociation. Furthermore, the apparent reversible association of A2 subunit with dimer suggested that factor VIII inactivation was a function of the dissociation constant for this binding interaction.

Results shown in Fig. 2 indicate that the stable, basal level of factor VIII, following peak activity was dependent upon both the initial concentration of the cofactor and the reaction pH. The data presented were obtained following initial activation of factor VIII (112 μg/ml, 510 nM) by thrombin (20 nM) in a reaction mixture buffered at pH 7.4. At peak activity, PPACK was added to inhibit thrombin and the reaction mixture diluted to yield the indicated factor VIII concentration and final pH of either 7.4 (panel A) or 6.0 (panel B). Residual factor VIII activity was determined at the indicated times and used to calculate the specific activity (units/μg factor VIII) for each cofactor concentration. Complete conversion of factor VIII to factor VIII, was supported by gel analysis of the thrombin-cleaved material (results not shown).

The basal activity of factor VIII, was stable over the time course shown (approximately 3 h) as judged by only minor fluctuations in specific activity. However, it was apparent that higher specific activities were observed at high factor VIII, concentrations.

The above data were replotted to illustrate specific activity of factor VIII, as a function of its concentration, and the results obtained at pH 6.0 and 7.4 are shown in Fig. 3. At pH 6.0, factor VIII, specific activity approached a maximum at concentrations greater than 100 nM. The shape of this curve was consistent with a maximum specific activity of 84 units/μg, a value previously determined for the human protein following immediate sampling of the reaction mixture (12). On the other hand, the curve obtained from the pH 7.4 data was essentially linear, and specific activities were reduced
Interaction between A2 Subunit and A1/A3-C1-C2 Dimer

Fig. 2. Effect of factor VIII concentration on specific activity at pH 7.4 (A) and pH 6.0 (B). A, factor VIII (510 nM) was reacted with thrombin (20 nM) at pH 7.4 as described under "Materials and Methods." At peak activity PPACK (0.1 mM) was added to the reaction mixture, and activity was monitored for factor VIII, at 20 nM (△), 445 nM (○), 382 nM (△), 318 nM (△), 255 nM (□), 63 nM (○), 16 nm (●) and 8 nm (○). B, following activation of factor VIII as above, the reaction mixture was adjusted to pH 6.0 and diluted to the indicated concentrations in pH 6.0 buffer: 510 nM (+), 255 nM (□), 63 nM (○), 32 nM (△), 16 nM (●), and 8 nM (○). Data points represent the mean of at least two determinations, and standard deviations were typically <20% of the mean.

compared with values for similar factor VIII concentrations at pH 6. This effect of pH was most pronounced at low factor VIII concentration where as much as a 10-fold greater specific activity was observed at the lower pH.

Since the dissociated A2 subunit and A1/A3-C1-C2 dimer are functionally inactive, residual specific activity of factor VIII can be used to determine the concentration of active A1/A2/A3-C1-C2 trimer and thus measure the affinity of this interaction. K_d values for the A2 subunit-A1/A3-C1-C2 dimer interactions were determined as described under "Materials and Methods." For reactions at pH 6.0 this value was 27.8 ± 5.2 nM (n = 7) whereas reactions at pH 7.4 yielded a 10-fold higher value (258 ± 126 nM, n = 8). This result was consistent with the requirement for slightly acidic pH to maximize factor VIII reconstitution from the A2 subunit and A1/A3-C1-C2 dimer (12).

Since the specific activity for a given factor VIII concentration is dependent upon pH, one would expect an increase in specific activity when the reaction pH is shifted from 7.4 to 6.0. Furthermore, this effect should be most pronounced for low factor VIII concentrations because of the greater disparity in activity at the two pH levels. Results of the pH shift experiments for factor VIII, at two concentrations, 11 and 32 nM, are shown in Fig. 4. For these experiments, factor VIII was activated by thrombin at pH 7.4. Activity was monitored until a stable level was achieved (typically ~60 min). The reaction mixture was then split, and one portion was adjusted to a final pH of 6.0. Shifting the pH of the 11 nM factor VIII reaction resulted in attainment of a new specific activity of ~17 units/μg compared with approximately 4 units/μg in the original reaction. Similarly, the specific activity of the 32 nM factor VIII reaction was increased from ~7 units/μg to ~28 units/μg as the pH was decreased to 6.0. These new values resulting from the pH shift experiment were consistent with the specific activities illustrated in Fig. 3, further indicating that the association of A2 subunit with dimer was fully reversible with the extent of association.

Fig. 3. pH dependence of the effect of factor VIII concentration on specific activity. Mean specific activities for each factor VIII concentration were determined from the data in Fig. 2 and are plotted as a function of concentration for factor VIII at pH 7.4 (●) or pH 6.0 (○).

Fig. 4. Effect of a pH shift on factor VIII specific activity. Factor VIII (11 nM, ■) or 32 nM (○) at pH 7.4 was reacted with thrombin. At peak activity PPACK was added, and clotting activity was monitored at the indicated times. At 60 min, each reaction was split and a portion adjusted to pH 6.0 (open symbols). Data points represent the mean of at least two determinations, and standard deviations were typically <20% of the mean.
governed by an association constant dependent upon the pH of the reaction.

Interaction of the A2 Subunit with the A1 Subunit of the A1/A3-C1-C2 Dimer—The failure of the A3-C1-C2 subunit to inhibit association of A2 with the A1/A3-C1-C2 dimer (12) suggested that the A1 subunit contributed to and/or contained the site in the dimer involved with A2 interaction. To test this hypothesis, we measured the potential for A1 subunit to inhibit association of A2 with the dimer. For this analysis A1 subunit was purified from the A1/A3-C1-C2 dimer following Mono Q chromatography of EDTA-treated dimer. Dissociation of the dimer subunits by the chelating reagent verified that divalent metal ion(s) linked the A1 and A3-Cl-C2 subunit of the dimer subunits by the chelating reagent verified that divalent metal ion(s) linked the A1 and A3-Cl-C2 subunit of the dimer subunits. The A1 subunit was adsorbed to the Mono Q column and subsequently was eluted with buffer containing high salt, whereas the A3-C1-C2 subunit was contained in the unbound column fraction. The purified subunits derived from the A1/A3-C1-C2 dimer are shown in Fig. 5.

The A2 subunit was preincubated in the absence or presence of increasing levels of A1 subunit for 30 min. Following this initial reaction, A1/A3-C1-C2 dimer was added to the mixtures, and following a 30-min incubation, clotting assays were performed to assess the reconstitution of factor VIII. Thus, if A2 binds A1 subunit, one would expect the presence of A1 subunit in the preincubation reactions to inhibit subsequent reassociation of A2 with dimer. As shown in Fig. 6, the presence of A1 indeed inhibited regeneration of factor VIII activity, and this effect was dependent upon the concentration of A1 subunit. A 10-fold molar excess of A1 subunit relative to A2 completely inhibited association of A2 with dimer. This result indicated that the A2 subunit associates with the A1 component of the A1/A3-C1-C2 dimer in factor VIII.

DISCUSSION

Recent studies of the porcine (13, 14) and human (12, 13) proteins suggest that the observed lability of factor VIII results from the dissociation of A2 subunit from the A1/A3-C1-C2 dimer. Dissociation is promoted by slightly alkaline pH (14). In the human protein, this process is reversible, and association of A2 with dimer is promoted by low ionic strength (12) and slightly acidic pH (12, 21). In this report we show that loss of human factor VIII activity, under either stabilizing or destabilizing pH conditions, is primarily a function of the dissociation constant for the A2-dimer interaction.

Several lines of evidence suggest that association of A2 subunit with dimer is fully reversible in the human protein. In an earlier study (12) we showed that high specific activity factor VIII could be reconstituted from isolated A2 subunit and A1/A3-C1-C2 dimer. We now show that exogenous A2 subunit can retard the spontaneous decay of factor VIII activity. This effect, which is proportional to the level of A2 present, likely results from shifting the existing equilibrium for A1/A2/A3-C1-C2 trimer ⇌ A1/A3-C1-C2 dimer + A2 subunit to the left. Since only the heterotrimer form of factor VIII is active (12, 14), the state of association of A2 with dimer is reflected by the specific activity of factor VIII. Specific activity, in turn, is dependent upon the factor VIII concentration for a given reaction pH. At pH 7.4, specific activity is linear with concentration over a broad range (>500 nM). On the other hand, at pH 6.0, specific activity is linear with concentration at low values (<50 nM) with near-maximal specific activity values observed at approximately 200 nM. These values approach the peak specific activity of 84 units/μg observed for factor VIII following initial activation by thrombin (12). Assuming that this latter value represents a maximum attainable specific activity, one can calculate the Kd for the A2-dimer interaction using the ratio of observed specific activity:maximum specific activity as an indicator of active trimer concentration. For pH 6.0, this value is ~28 nM, whereas a value approximately 10-fold greater or ~260 nM was determined for the more physiologic pH. These results are consistent with the enhanced stability of factor VIII at slightly acidic pH values compared with more alkaline conditions (11-14) and suggest that this stability is primarily a function of the dissociation constant for the A2-dimer interaction.

This conclusion is further manifested by experiments where factor VIII, at pH 7.4 is shifted to pH 6.0. These experiments, performed at low factor VIII concentration to maximize the disparity in specific activities at these pH values, indicated that the new specific activity attained following the downward pH shift was similar to that observed for factor VIII, maintained at the lower pH. Lollar and Parker (14) observed a modest increase in porcine factor VIII (1 μM) activity when the reaction mixture was shifted from pH 8.0 to 6.0. This activity increase yielded about 40% of the activity compared with control material maintained at pH 6.0. Because of this failure to achieve the original activity level, these investigators proposed that the pH-induced inactivation of factor VIII included an irreversible step. This effect may result from the
observed insolubility of the porcine A2 subunit (14) and suggests that the porcine and human A2 subunits possess significantly different properties in solution.

The plasma concentration of factor VIII is estimated to be ~1 nM (22). This value is ~200-fold less than the $K_d$ (pH 7.4) for the A2-dimer interaction. Thus, following activation in vivo, essentially all factor VIII, would be expected to be dissociated and inactive. Indeed, this weak affinity interaction under physiologic conditions may provide a regulatory role in limiting factor VIII, activity to a rapid burst rather than a sustained effect. Alternatively, components of the factor Xase-enzyme complex have been shown to stabilize this labile intramolecular interaction. Early work by Lollar et al. (23) demonstrated that in the presence of factor IX, in combination with phospholipid and Ca$^{2+}$ significantly prolonged the dissociation of factor VIII, activity, independent of the active site for factor IX, (24). These results suggested that preincubation of A2 subunit with a molar excess of either substrate did not affect the capacity for A2 to subsequently bind A1/A3-Cl-C2, as judged by reconstitution of factor VIII, activity. Further evidence for this conclusion was that preincubation of A2 subunit with molar excess of factor IX, in a freely reversible reaction governed by a pH-dependent dissociation constant. The markedly weaker affinity of this interaction under physiologic compared with slightly acidic conditions is the primary cause for the observed insolvency of factor VIII, activity. Work is currently in progress to identify structural and sequence determinants involved in this intersubunit interaction.

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