Factor VIII (fVIII) is the plasma protein that is missing or deficient in hemophilia A. In contrast, elevated levels of fVIII are associated with an increased risk of arterial and venous thrombosis. fVIII is activated by thrombin to form a non-covalently linked A1/A2/A3-C1-C2 heterotrimer. At physiological concentrations, fVIIIa decays as a result of A2 subunit dissociation, which may help regulate the balance between hemostasis and thrombosis. A2 subunit dissociation is faster in human fVIIIa than in porcine fVIIIa, which may represent an evolutionary adaptation associated with the development of the upright posture and venous stasis in the lower extremities. To investigate the basis for the different decay kinetics of human and porcine fVIIIa, hybrid fVIII molecules representing all possible combinations of human and porcine A domains were isolated. The kinetics of fVIIIa decay were measured and fit to a model describing a reversible bimolecular reaction in which the dissociation rate constant, $k_d$, and dissociation constant, $K_d$, were the fitted parameters. Substitution of the porcine A1 domain into human fVIIIa produced a dissociation rate constant indistinguishable from porcine fVIIIa. Subsequently, substitution of the second cupredoxin-like A1 subdomain resulted in a dissociation rate constant similar to porcine fVIIIa, whereas substitution of the first cupredoxin-like A1 subdomain resulted in a dissociation rate constant intermediate between human and porcine fVIIIa. We propose that cupredoxin-like A1 subdomains in fVIII contain inter-species differences that are a result of selective pressure on the dissociation rate constant.

fVIIIa functions in blood coagulation as a cofactor for factor IXa during the proteolytic activation of factor X on the phospholipid surface of platelets and monocytes. Cleavage at Arg$^{372}$ activates the factor IXa cofactor function of fVIIIa, as judged by naturally occurring mutations at this site that result in hemophilia A (6, 7) and by site-directed mutagenesis of this site, which produces loss of function (8). Cleavage at Arg$^{40}$ releases the B domain. However, removal of the B domain does not activate fVIII (9, 10). Additionally, cleavage at Arg$^{40}$ does not appear to be necessary for fVIII activation because site-directed mutagenesis of Arg$^{40}$ inhibits cleavage at this site by thrombin but not functional activation (8). Cleavage at Arg$^{1689}$ is responsible for the dissociation of fVIII from vWF (11–13), which is a requisite step during fVIII activation because fVIII bound to vWF cannot bind to phospholipid membranes (14, 15). However, in the absence of vWF, cleavage at Arg$^{1689}$ is not required to produce a fVIIIa molecule with substantial activity (12, 13, 16).

At the plasma concentration of fVIII (~1 nM) at physiological pH, human fVIIIa activity spontaneously decays to undetectable levels with a half-life of 2 min at 23 °C (17–19) as a result of A2 subunit dissociation (4, 5, 20). A2 subunit dissociation is a reversible process, and active, heterotrimeric fVIIIa can be reconstituted using sufficiently high concentrations of purified A2 subunit and A1/A3-C1-C2 dimer (5). The dissociation constant for the binding of the A2 subunit to the A1/A3-C1-C2 dimer is over 2 orders of magnitude greater than the plasma concentration of fVIII (21). Thus, at physiological concentrations the equilibrium lies far toward the dissociated state.

A2 subunit dissociation appears to be an important regulatory feature of the blood coagulation mechanism. Naturally occurring human mutations that increase the rate of A2 subunit dissociation produce hemophilia A (22). Factor V, which is homologous to fVIII, also contains an A1-A2-B-A3-C1-C2 domain sequence but lacks a cleavage site between the A1 and A2 domains. As a result, factor Va is an A1-A2/A3-C1-C2 dimer (23). The dissociation constant for the binding of the subunits of factor Va is significantly below the plasma concentration of factor V (24), indicating that factor Va is stable at physiological concentrations. This suggests that the evolution of fVIII may have included the activation step at Arg$^{372}$ to produce a dissociable A2 subunit to avoid a hypercoagulable state. The rate constant for A2 subunit dissociation of human fVIIIa is ~3-fold greater than that of porcine fVIIIa at 23 °C (21), which may also represent an evolutionary adaptation. In this study, we have investigated the decay kinetics of recombinant hybrid human/porcine fVIIIa molecules. Our results reveal that A1 domain sequence variation is responsible for the difference in the rate of A2 subunit dissociation between human and porcine fVIIIa.

**EXPERIMENTAL PROCEDURES**

**Materials**—Serum-free AIM V medium, oligonucleotides and HEPES were purchased from Invitrogen (Carlsbad, CA). SP-Sepharose, Source Q, mono S, and mono Q chromatography resins were purchased...
from GE Healthcare Life Sciences. Tween 80 was purchased from Pierce. Type III-E egg yolk lyso-phosphatidylcholine and bovine brain lyso-phosphatidylserine (PCPS) (75/25, w/w) vesicles were prepared using an Avanti Polar Lipids Mini-Extruder kit according to instructions supplied by the manufacturer. Recombinant desulfatohirudin was a generous gift from Dr. R. B. Wallis, Ciba-Geigy Pharmaceuticals. Human thrombin was a gift from Dr. SriRam Krishnaswamy, University of Pennsylvania. Human factors IXa and X were purified as described previously (25, 26). Spectrzyme Xa (methoxy carbonyl-D-cyclohexylglycyl-glycyl-arginine-para-nitroanilide acetate) was purchased from American Diagnostica (Stamford, CT).

Construction and Expression of Hybrid fVIII cDNAs—The construction of cDNA constructs encoding B domain-deleted human fVIII (designated HSQ), porcine fVIII (designated POL 1212), and the hybrid human/porcine fVIII molecules designated HP1, HP22, HP30, HP45, HP46, and HP47 (Fig. 1) in the mammalian expression vector ReNeo has been described previously (27, 28). These constructs contain nucleotide sequence between the A2 and A3 domains encoding a linker region of human or porcine origin that includes the Arg-His-Gln-Arg recognition sequence for PACE/furin processing (29, 30). This results in the expression of heterodimeric fVIII as the major secreted species. The hybrid human/porcine A2/A3-C1-C2 constructs HP66, HP67, HP68, HP69, HP70, and HP75 containing the human A2/ap-A3 linker region (Fig. 2) were produced by splicing-by-overlap extension mutagenesis (31) using previously published procedures (32–34). All PCR-generated sequences were confirmed by DNA sequencing. Expression of stably integrated fVIII cDNAs from baby hamster kidney-derived (BHK-M) cells was performed as described previously (27). Geneticin-resistant clones were confirmed by DNA sequencing. Expression of fVIII within serum-free medium. The highest expressing clone of each construct was used for preparative scale purification of recombinant fVIII as described previously (21). With the exception of HP68, fVIII at a nominal concentration of 1, 20, 50, or 100 nM was reacted with 100 nM thrombin at 23 °C for 30 s in 0.15 M NaCl, 0.02 M HEPES, 5 mM CaCl₂, 0.01% Tween 80, pH 7.4, followed by addition of desulfatohirudin (150 nM) to inhibit thrombin. HP68 was activated at nominal concentrations of 1, 5, 10, and 20 nM because of the limited amount of protein. Under these conditions, there is complete cleavage of fVIII within 5 s as judged by SDS-PAGE analysis. At various times, fVIII activation mixtures were diluted to a total fVIII concentration of 0.2 nM into 2 nM factor IXa/20 μM PCPS in 0.15 M NaCl, 0.02 M HEPES, 5 mM CaCl₂, 0.01% Tween 80, pH 7.4, Factor X was then added immediately to a final concentration of 300 nM, and factor Xa formation was measured using the chromogenic substrate Spectrzyme Xa as described previously (36) using a SpectraMax Plus or VersaMax kinetic plate reader (Molecular Devices, Sunnyvale, CA).

For studies of the temperature dependence of the rate of A2 subunit dissociation, fVIII (20 nM) was reacted with 100 nM thrombin at 23 °C for 15 s in 0.15 M NaCl, 0.02 M HEPES, 5 mM CaCl₂, 0.01% Tween 80, pH 7.4. The resulting fVIII was diluted to 1 nM in the same buffer plus 8 nM hirudin equilibrated at 18, 23, 27, 32, or 37 °C at pH 7.4. Adjustment of the buffer pH was necessary because of the variation of the pK_a of HEPES with temperature and was done using a Mettler Toledo InLab 409/120 pH electrode and a Corning 240 pH meter. The pH electrode, which has a potential that varies with temperature according to the Nernst equation, was calibrated at the fVIII decay temperatures using pH 4, 7, and 10 standard National Institute of Standards and Technology traceable buffers with known temperature-dependent pH variation (Fisher Scientific). At various times, activation mixtures were diluted to 0.2 nM fVIII and assayed as described above.

Analysis of the Kinetics of fVIIIa Decay—Decay kinetics of fVIIIa were analyzed using model describing reversible binding of the A2 subunit to the A1/A3-C1-C2 dimer as described previously (21).

$$k'$$

SCHEME 1

The dissociation and association rate constants, k and k', respectively, are related by the dissociation constant, $K_d = k/k'$. 
FIGURE 3. Concentration-dependent decay of human, porcine, and hybrid fVIIIa molecules. Recombinant B domain-deleted fVIII molecules at concentrations of 1 nM (○), 20 nM (■), 50 nM (□), or 100 nM (●) were rapidly activated with thrombin for 30 s at 23 °C at pH 7.4 followed by addition of desulfatohirudin to inactivate thrombin as described under “Experimental Procedures.” At the times indicated, the mixture was diluted into factor Xa/PCPS, the initial velocity of factor X activation was measured, and the fraction of fVIIIa remaining, f, was calculated. Panels: A, human; B, porcine; C, HP1 (HPH); D, HP22 (PPH); E, HP30 (HHP); F, HP45 (HPH); G, HP46 (PHH); and H, HP47 (PHP), where A1-A2-A3 subunit compositions are designated in parentheses. The curves are global nonlinear least-squares fits to the model described by Scheme I with the fitted parameters shown in Table 1 for Experiment 1.
The fraction of fVIIIa as a function of time is given as follows,

\[
f(t) = \frac{a_o - a(t)}{a_o}
\]

(Eq. 1)

where \(a_o\) is the initial concentration of fVIIIa, and \(a(t)\) is the concentration of dissociated product at time \(t\) and is given as follows (37),

\[
a(t) = \frac{a_o x_e (e^{-s_i t} - 1)}{a_o (e^{x_e t} + 1) - x_e}
\]

(Eq. 2)

where

\[
x_e = -K_d + \frac{\sqrt{K_d^2 + 4a_o K_d}}{2}
\]

(Eq. 3)

is the concentration of A1/A3-C1-C2 dimer and A2 subunit at equilibrium and

\[
\lambda(t) = \frac{k(2a_o - x_e)t}{x_e}
\]

(Eq. 4)

Because fVIIIa activity is measured indirectly by measuring factor X activation, \(a_o\) cannot be measured directly but rather was estimated by extrapolation to the ordinate of the line formed from plotting the logarithm of the initial velocity of factor X activation versus time for the 1- and 3-min points. At these early times, reassociation of the A2 subunit and A1/A3-C1-C2 dimer is negligible, as judged by a lack of concentration dependence of extrapolated values. Values of \(f(t)\) as a function of \(a_o\) and \(t\) were fitted by global, weighted nonlinear least-squares regression with \(k\) and \(K_d\) as the fitted parameters using the Marquardt algorithm (38).

The quality of the least-squares regression fits was evaluated by calculating the reduced chi square variable, \(\chi^2_e\), associated with the weighted residuals corresponding to \(m\) decay times and \(n\) initial concentrations, using,

\[
\chi^2_e = \frac{1}{n} \sum_{i=1}^{n} \sum_{j=1}^{m} w_{ij} (f_j(t) - f^*_j(t))^2
\]

(Eq. 5)

where \(f_j\) and \(f^*_j\) are the measured and fitted fractions of fVIIIa remaining, respectively, \(w_{ij}\) is the statistical weight and \(v = mn - 2\) is the number of degrees of freedom for a two-parameter fit. Weights were assigned using the following,

\[
w_{ij} = \frac{1}{s^2_{ij}}
\]

(Eq. 6)

where \(s_{ij}\) represents the standard deviation of \(f_j(t)\). A preliminary set of replicate measurements indicated that \(s_{ij}\) was proportional to \(f(t)\) so that

\[
s_{ij} = \beta f_j(t) + s_o,
\]

(Eq. 7)

where \(\beta\) is the coefficient of variation, and \(s_o\) is the estimated baseline standard deviation when no fVIIIa is present. An estimate of 0.005 was used for \(s_o\) based on 50 replicate measurements in the absence of added fVIII. A value of 0.07 was used for \(\beta\) to produce an average value of \(\chi^2_e\) of approximately one for the data sets. Intra-experimental 95% confidence limits for estimates of \(k\) and \(K_d\) were calculated using Student’s \(t\) distribution with \(v\) degrees of freedom and estimated standard deviations of the fitted parameters that were obtained by multiplying the square root of \(\chi^2_e\) by the diagonal elements of the least-squares error matrix (39).

**RESULTS**

Decay Kinetics of Substituted A Domain Human/Porcine fVIIIa Hybrids—After rapid activation by thrombin, the kinetics of fVIIIa decay are governed by the dissociation rate constant, \(k\), and the dissociation constant, \(K_d\), for the interaction of the A2 subunit with the A1/A3-C1-C2 dimer as shown in Scheme 1 (21). The rate constant for A2 subunit dissociation of human fVIIIa is 3-fold greater than that of porcine fVIIIa at 23 °C (21). We hypothesized that the difference between human and porcine fVIIIa was independent of interactions involving the C1 or C2 domains and constructed recombinant B domain-deleted human, porcine, or hybrid human/porcine fVIII cDNAs encoding all eight possible combinations of human and porcine A domains (Fig. 1). Proteins corresponding to the cDNAs were expressed from a baby hamster kidney-derived cell line and purified. The kinetics of fVIIIa decay were measured at 23 °C using a chromogenic substrate assay of the intrinsic FXase complex under conditions in which fVIIIa was the limiting function component as described under “Experimental Procedures.”

The fVIIIa decay kinetics of the constructs reveal that the activity at 1 nm, which corresponds to the plasma concentration of fVIII, approaches zero after 30 min (Fig. 3). At increasing nominal concentrations of fVIIIa, activity approaches zero-values consistent with reversible binding. The decay of human fVIIIa is faster than porcine fVIIIa at all nominal concentrations (cf. Fig. 3, A and B). Qualitative inspection indicates that some hybrids behave like human fVIIIa, e.g. HP1, which contains a single porcine A2 domain substitution (Fig. 3C), whereas other hybrids behave like porcine fVIIIa, e.g. HP46, which contains a single porcine A1 domain substitution (Fig. 3G). These results indicate that the difference in A2 subunit dissociation between human and porcine fVIIIa is independent of the species composition of the C1 or C2 domain.

The data were fit to a bimolecular equilibrium model represented by Scheme 1 using global, weighted nonlinear least square regression analysis as described under “Experimental Procedures.” The experiment was performed twice for each construct, and the results are summarized in Fig. 4 and Table 1. Reasonable fits to the model were obtained as judged by reduced \(\chi^2\) values that in most cases fell within the 0.95 probability range (0.5–1.7, 22 degrees of freedom). Good reproducibility of the estimates of the dissociation rate constant, \(k\), and dissociation constant, \(K_d\), were obtained as judged by inter-experimental values that were within the 95% confidence limits obtained from intra-experimental regression analysis in most cases. The decay of porcine fVIIIa was ~3-fold slower than human fVIIIa, which is consistent with earlier results using plasma-derived human and porcine fVIIIa (21). All constructs containing the porcine A1 domain, including HP46, which is entirely human except for the porcine A1 domain, exhibited decay rates similar to porcine fVIIIa. In contrast, substitution of only the porcine A2 domain (HP1) resulted in decay rates more similar to human fVIIIa than porcine fVIIIa. Thus, the relatively fast decay of human fVIIIa appears to be due entirely to A1 domain differences between human and porcine fVIIIa.

Although the dissociation constant, \(K_d\), ranged ~4-fold between the constructs, there was not a consistent pattern with respect to A domain composition (cf. Fig. 4, A and B). Additionally, there was only a weak correlation between \(K_d\) and \(x\) (\(r^2 = 0.53\)). If only the dissociation rate constant differed among the constructs, the variables would be related
linearly by $\ln K_a = \ln k - \ln k'$, where $k'$ is the association rate constant (Scheme I). Thus, the results indicate that porcine A domain substitutions produce variable changes in the association rate constant.

Temperature-dependent Decay of Human and Porcine fVIIIa—The Arrhenius equation for the temperature dependence of a rate constant, $k$, is as follows,

$$\ln \frac{k}{k'} = \frac{E_a}{RT} + \ln A \quad \text{(Eq. 8)}$$

where $E_a$ is the activation energy, $A$ is the frequency factor, $R$ is the gas constant, and $T$ is the absolute temperature (40). This equation predicts that there should be a linear relationship between $\ln k$ and $1/T$ with a slope equal to $-E_a/R$ and a common ordinate intercept at $\ln A$ if the difference in rate constant is due solely to a difference in activation energy. Dissociation rate constants for human and porcine fVIIIa were measured at temperatures ranging from 18 °C to 37 °C as described under "Experimental Procedures." Arrhenius plots for human and porcine fVIIIa both were reasonably linear in the 23 °C to 37 °C range (Fig. 5). The decay rates of human fVIIIa at 18 °C and 23 °C points were similar, producing a nonlinear plot below the latter temperature. Regression analysis yielded estimates and 95% confidence limits for $E_a$ of 9.2 ± 4.5 and 13.7 ± 2.0 kcal/mol for human and porcine fVIIIa, respectively. The difference between human and porcine fVIIIa was statistically significant (Student’s $t$ test, $p = 0.02$). Additionally, there was a significant difference in the estimates of $\ln A$ for human and porcine fVIIIa, respectively (Student’s $t$ test, $p = 0.03$). The estimated

![FIGURE 4. Dissociation rate constants and dissociation constants of human, porcine, and hybrid fVIIIa molecules. The mean and range of parameter estimates for $k$ (A) and $K_d$ (B) from the two experiments described in Table 1 are shown in descending order.](image)

![TABLE 1. Kinetic and equilibrium parameter estimates for dissociation of A domain hybrid fVIIIa molecules at 23 °C.](table)

| Construct | A1A2A3$^a$ | Experiment | $k^b$ | $K_d^b$ | $\chi^2c^b$ |
|-----------|------------|------------|-------|--------|-----------|
| Human     | HHH        | 1          | 0.288 ± 0.015 | 149 ± 16 | 0.83      |
|           |            | 2          | 0.284 ± 0.017 | 175 ± 22 | 1.1       |
| Porcine   | PPP        | 1          | 0.110 ± 0.006 | 88 ± 12 | 0.60      |
|           |            | 2          | 0.104 ± 0.004 | 87 ± 10 | 0.38      |
| HP1       | HPH        | 1          | 0.210 ± 0.017 | 133 ± 23 | 1.8       |
|           |            | 2          | 0.220 ± 0.016 | 191 ± 32 | 1.7       |
| HP22      | PPH        | 1          | 0.132 ± 0.008 | 188 ± 35 | 1.2       |
|           |            | 2          | 0.130 ± 0.009 | 240 ± 52 | 1.4       |
| HP30      | HHP        | 1          | 0.349 ± 0.021 | 420 ± 68 | 1.5       |
|           |            | 2          | 0.369 ± 0.039 | 400 ± 112 | 4.6      |
| HP45      | HPP        | 1          | 0.276 ± 0.006 | 185 ± 8 | 0.40      |
|           |            | 2          | 0.276 ± 0.012 | 210 ± 20 | 0.61      |
| HP46      | PHH        | 1          | 0.105 ± 0.004 | 154 ± 20 | 0.43      |
|           |            | 2          | 0.095 ± 0.007 | 137 ± 36 | 1.5       |
| HP47      | PHP        | 1          | 0.126 ± 0.005 | 89 ± 10 | 0.44      |
|           |            | 2          | 0.107 ± 0.007 | 78 ± 15 | 1.00      |

$^a$ A domain composition: H, human; P, porcine.

$^b$ Parameter estimates and 95% confidence limits from nonlinear regression analysis as described under "Experimental Procedures."

$^c$ Reduced $\chi^2$ variable.
The three fVIII A domains each contain two ~20-kDa cupredoxin-like subdomains. Additionally, the A1 domain contains the first porcine cupredoxin-like A1 subdomain (HP70) resulting in a dissociation rate constant intermediate between human and porcine fVIIIa. HP75, which contains the first porcine A1 subdomain and the porcine COOH-terminal A1 acidic region, also displayed an intermediate dissociation rate constant. HP69, which contains both porcine cupredoxin-like A1 subdomains and the human COOH-terminal A1 acidic region, anomalously displayed a dissociation rate constant intermediate between human and porcine fVIIIa. This may indicate an unfavorable interaction between the human acidic region and the first porcine cupredoxin A1 subdomain.

### Discussion

In this study, we found that substitution of the porcine A1 domain into human fVIII is necessary and sufficient to produce an A2 subunit dissociation rate constant that is indistinguishable from porcine fVIIIa (Fig. 4A). Substitution of all possible combinations of the porcine cupredoxin-like A1 subdomains and/or the porcine A1 acidic COOH-terminal region revealed that substitution of the second cupredoxin-like A1 subdomain, producing a construct designated HP68, resulted in an A2 subunit dissociation rate constant that is similar to porcine fVIIIa. Substitution of only the first porcine A1 subdomain (HP70) resulted in a dissociation rate constant intermediate between human and porcine fVIIIa. HP75, which contains the first porcine A1 subdomain and the porcine COOH-terminal A1 acidic region, also displayed an intermediate dissociation rate constant. HP69, which contains both porcine cupredoxin-like A1 subdomains and the human COOH-terminal A1 acidic region, anomalously displayed a dissociation rate constant intermediate between human and porcine fVIIIa. This complex behavior suggests that there is a cooperative interaction between the two cupredoxin-like A1 subdomains that affects the rate of A2 subunit dissociation. Experimental evidence for the interaction of cupredoxin-like A1 subdomains with the A2 subunit of fVIIIa has not been presented, although the acidic COOH-terminal region of the A1 domain has been implicated (41, 42). We did not find differences in fVIIIa decay rates related to the acidic COOH-terminal A1 region.

### Table 2

| Construct | A1 Composition | Experiment | $k^*$ | $K_d$ | $k_d$ |
|-----------|----------------|------------|-------|-------|-------|
| HP66      | hhp            | 1          | 0.278 ± 0.020 | 471 ± 81 | 2.2  |
|           |                | 2          | 0.300 ± 0.036 | 1.9     |
| HP67      | hpp            | 1          | 0.122 ± 0.007 | 460 ± 142 | 1.4  |
|           |                | 2          | 0.115 ± 0.009 | 0.67    |
| HP68      | hph            | 1          | 0.118 ± 0.009 | 127 ± 46 | 1.9  |
|           |                | 2          | 0.123 ± 0.012 | 1.1     |
| HP69      | ppp            | 1          | 0.154 ± 0.009 | 257 ± 45 | 1.2  |
|           |                | 2          | 0.209 ± 0.019 | 1.1     |
| HP70      | pph            | 1          | 0.166 ± 0.008 | 149 ± 17 | 0.63 |
|           |                | 2          | 0.160 ± 0.021 | 2.0     |
| HP75      | pph            | 1          | 0.149 ± 0.007 | 119 ± 15 | 0.68 |
|           |                | 2          | 0.129 ± 0.015 | 1.7     |

* A1 subdomain composition: h, human; p, porcine.
* In experiment 2, decays were analyzed at only 1 nM fVIIIa to obtain the dissociation rate constant.
* Parameter estimates and 95% confidence limits from nonlinear regression analysis as described under “Experimental Procedures.”
* Reduced χ² variable.
dissociation rate constants of human FVIII versus HP66, HP70 versus HP75, and HP67 versus HP68). This may be because COOH-terminal A1 residues involved in binding the A2 subunit are conserved between human and porcine FVIII. Both A1 subdomains make contacts with the A2 domain in a homology model of the FVIII A domains (43) derived from the x-ray structure of ceruloplasmin (44), which is consistent with the results of this study. The activation of FVIII by thrombin includes cleavage at Arg372 between the A1 and A2 domains and presumably leads to an alteration of quaternary structure. Thus, the homology model of FVIII prior to activation may not reveal differences between

**Figure 6. Decay rates of A1 subdomain constructs.** Decays of porcine FVIII (○); HP46, ppp (▼); HP67, hpp (■); HP68, hph (◇); HP70, phh (▲); HP69, php (●); HP66, hpp (▲); HP75, php (▲); and human FVIII (●) were measured at a FVIII starting concentration of 1 nm at 23 °C as described under “Experimental Procedures.” The curves are nonlinear least-squares fits to a single exponential decay with the fitted parameters shown in Table 2, Experiment 2.

**Figure 7. Dissociation rate constants and dissociation constants of A1 subdomain hybrid FVIII molecules.** The mean and range of parameter estimates for $k_A$ and the $K_d$ estimate obtained from a single experiment (B) corresponding to the data in Table 2 are shown in descending order. For comparison, results for human and porcine FVIII are shown using the data in Table 1.
human and porcine fVIIIa that are responsible for differences in decay rates.

While the biochemical phenomenon of A2 subunit dissociation has been known for several years (4), more recently its physiological importance has been recognized by the identification of hemophilia A mutations that produce abnormally fast A2 subunit dissociation (22). These patients originally were identified based on having higher fVIII levels (4). The formation of the fibrin clot clots the one-stage assay. In the two-stage coagulation assay, fVIII is activated by thrombin or factor Xa that are formed endogenously during the ~45–90 s period between addition of calcium and formation of the fibrin clot. The one-stage assay is relatively insensitive to A2 subunit dissociation because of the short duration of the assay. In contrast, in commercial chromogenic assays, fVIII is activated by thrombin in a separate incubation mixture for up to 4 min before the chromogenic substrate is added. Similarly, in the two-stage coagulation assay, fVIII is activated in a separate incubation prior to initiation of fibrin clot formation. Thus, the lower activity in the chromogenic or two-stage coagulation assay is consistent with fVIIIa decay due to A2 subunit dissociation during the separate incubation reactions. Amino acid residues that are involved in mutations that produce the fVIII assay discrepancy (Ala284, Ser289 in the A1 domain, Arg527, Arg531, Asn694, and Arg698 in the A2 domain, and Arg1749, Met1932, Met1937, and His1954 in the A3 domain) are conserved between human and porcine fVIII.

In contrast to bleeding that is caused by fVIII deficiency, elevated levels of fVIII are associated with an increased risk of arterial and venous thrombosis (see Ref. 49 for review). The coagulation mechanism is delicately poised with respect to fVIII function to control hemorrhage and avoid thrombosis. Most of the epidemiological studies that have identified these associations have employed the one-stage fVIII assay. Consequently, elevated fVIII levels presumably reflect an increased concentration of normal fVIII molecules. However, the hypercoagulable state associated with increased fVIII levels suggests that A2 subunit dissociation rates may also reflect a balance between hemostasis and thrombosis. The faster human A2 subunit dissociation rate may be an evolutionary adaptation, perhaps associated with the development of an upright posture and venous stasis in the lower extremities. In this regard it is interesting to note that the half-life of murine fVIIIa at 23°C is greater than 30 min, compared with ~2 and 7 min for human and porcine fVIIIa, respectively (50).

There is a poor correlation between $k$ and $k_2$ among constructs containing combinations of substitutions of the A1, A2, or A3 domains, indicating that porcine substitutions also affect the association rate constant, $k'$. Thus, the differential decay rates of human and porcine fVIIIa cannot be explained by a simple thermodynamic model in which the $k_2$ and $k_3$ values. The temperature dependence of the dissociation rate constant indicates that the activation energy for A2 subunit dissociation is 4 kcal/mol lower for human fVIIIa than porcine fVIIIa (Fig. 5). However, there also was a significant difference in the frequency factors of human and porcine fVIIIa. Thus, a simple thermodynamic model cannot explain the differences in dissociation rates of hybrid human/porcine fVIIIa molecules and human fVIIIa.

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REFERENCES

1. Vehar, G. A., Keyt, B., Eaton, D., Rodriguez, H., O’Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lai, W. L., and Capon, D. J. (1984) Nature 312, 337–342.
2. Eaton, D., Rodriguez, H., and Vehar, G. A. (1986) Biochemistry 25, 505–512.
3. Lollar, P., and Parker, C. G. (1989) Biochemistry 28, 666–674.
4. Lollar, P., and Parker, C. G. (1990) J. Biol. Chem. 265, 1688–1692.
5. Fay, P. J., Haidaris, P. J., and Smudzin, T. M. (1991) J. Biol. Chem. 266, 8957–8962.
6. Shima, M., Ware, J., Youshio, A., Fukui, H., and Fulcher, C. A. (1989) Blood 74, 1612–1617.
7. O’Brien, D. P., Patinson, J. K., and Tuddenham, E. G. D. (1990) Blood 75, 1664–1672.
8. Pittman, D. D., and Kaiser, R. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2429–2433.
9. Eaton, D. L., Wood, W. I., Eaton, D., Hask, P. E., Hollingshead, P., Wion, K., Mather, J., Lai, W. L., Vehar, G. A., and Gorman, C. C. (1986) Biochemistry 25, 8343–8347.
10. Mertens, K., Donath, M. J. S. H., van Leen, R. W., De Keyzer-Nellen, M. J. M., Verbeet, M. P., Klaase Bos, J. M., Leye, A., and van Mourik, J. A. (1993) Br. Haematol. 85, 133–142.
11. Lollar, P., Hill-Eubanks, D. C., and Parker, C. G. (1988) J. Biol. Chem. 263, 10451–10455.
12. Aly, A. M., and Hoyert, L. W. (1992) J. Clin. Invest. 89, 1382–1387.
13. Regan, L. M., and Fay, P. J. (1995) J. Biol. Chem. 270, 8546–8552.
14. Lajmanovich, A., Hudry-Clergeon, G., Freyssinet, J. M., and Marguerie, G. (1981) Biochim. Biophys. Acta 678, 132–136.
15. Andersson, L. O., and Brown, J. E. (1981) J. Biol. Chem. 256, 1729–1733.
16. Hill-Eubanks, D. C., Parker, C. G., and Lollar, P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6508–6512.
17. Hultin, M. B., and Jesty, J. (1981) Blood 57, 476–482.
18. Lollar, P., Knutson, G. J., and Fass, D. N. (1984) Blood 63, 1303–1308.
19. Lollar, P., Knutson, G. J., and Fass, D. N. (1985) Biochemistry 24, 8056–8064.
20. Lollar, P., and Parker, E. T. (1991) J. Biol. Chem. 266, 12481–12486.
21. Lollar, P., Parker, E. T., and Fay, P. J. (1992) J. Biol. Chem. 267, 23652–23657.
22. Pipe, S. W., Eckhorst, A. N., McKinley, S. H., Saenko, E. L., and Kaufman, R. J. (1999) Blood 93, 176–183.
23. Ono, T., and Tsuchiya, K. (1959) J. Biol. Chem. 234, 946–953.
24. Krzysztofowicz, S., Russell, G. D., and Mann, K. G. (1989) J. Biol. Chem. 264, 3160–3168.
25. Lollar, P., and Eubanks, C. T. (1996) J. Biol. Chem. 271, 13882–13887.
26. Barrow, R. T., Parker, E. T., Krzysztofowicz, S., and Lollar, P. (1994) J. Biol. Chem. 269, 26796–26800.
27. Seidah, N. G., and Chretien, M. (1997) Curr. Opin. Biotechnol. 8, 602–607.
28. Lollar, P., Larsson, K., Spira, J., Sydow-Backman, M., Almstedt, A., Gray, E. T., and Sandberg, H. (1995) Eur. J. Biochem. 232, 19–27.
29. Klotz, M. R., Ho, S. N., Pullen, J. K., Hunt, D. H., Cai, Z., and Pease, L. R. (1993) J. Biol. Chem. 268, 1303–1308.
30. Seidah, N. G., and Chretien, M. (1997) Curr. Opin. Biotechnol. 8, 602–607.
31. Lind, P., Larsson, K., Spira, J., Sydow-Backman, M., Almstedt, A., Gray, E. T., and Sandberg, H. (1995) Eur. J. Biochem. 232, 19–27.
32. Klotz, M. R., Ho, S. N., Pullen, J. K., Hunt, D. H., Cai, Z., and Pease, L. R. (1993) J. Biol. Chem. 268, 1303–1308.
33. Seidah, N. G., and Chretien, M. (1997) Curr. Opin. Biotechnol. 8, 602–607.
32. Healey, J. F., Lubin, I. M., Nakai, H., Saenko, E. L., Hoyer, L. W., Scandella, D., and Lollar, P. (1995) *J. Biol. Chem.* 270, 14505–14509
33. Healey, J. F., Barrow, R. T., Tamim, H. M., Lubin, I. M., Shima, M., Scandella, D., and Lollar, P. (1998) *Blood* 92, 3701–3709
34. Barrow, R. T., Healey, J. F., Gailani, D., Scandella, D., and Lollar, P. (2000) *Blood* 95, 557–561
35. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci.* 4, 2411–2423
36. Duffy, E. J., and Lollar, P. (1992) *J. Biol. Chem.* 267, 7821–7827
37. Moore, J. W., and Pearson, R. G. (1981) *Kinetics and Mechanism*, pp. 284–333, John Wiley & Sons, New York
38. Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp. 204–246, McGraw-Hill, New York
39. Shoemaker, D. P., Garland, C. W., and Nibler, J. W. (1989) *Experiments in Physical Chemistry*, pp. 801–827, McGraw-Hill, New York
40. Moore, W. J. (1972) pp. 324–420, *Physical Chemistry*, Prentice-Hall, Englewood Cliffs
41. Fay, P. J., Haidaris, P. J., and Huggins, C. F. (1993) *J. Biol. Chem.* 268, 17861–17866
42. O’Brien, L. M., Huggins, C. F., and Fay, P. J. (1997) *Blood* 90, 3943–3950
43. Pemberton, S., Lindley, P., Zaitsev, V., Card, G., Tuddenham, E. G. D., and Kemball-Cook, G. (1997) *Blood* 89, 2413–2421
44. Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A., and Lindley, P. (1996) *J. Biol. Inorg. Chem.* 1, 15–23
45. Parquet-Gernez, A., Mazurier, C., and Goudemand, M. (1988) *Thromb. Haemostasis* 59, 202–206
46. Rudzki, Z., Duncan, E. M., Casey, G. J., Neumann, M., Favaloro, E. J., and Lloyd, J. V. (1996) *Br. J. Haematol.* 94, 400–406
47. Keeling, D. M., Sukhu, K., Kemball-Cook, G., Waseem, N., Bagnall, R., and Lloyd, J. V. (1999) *Br. J. Haematol.* 105, 1123–1126
48. Hakeos, W. H., Miao, H., Sirachainan, N., Kemball-Cook, G., Saenko, E. L., Kaufman, R. J., and Pipe, S. W. (2002) *Thromb. Haemostasis* 88, 781–787
49. Kamphuisen, P. W., Eikenboom, J. C., and Bertina, R. M. (2001) *Arterioscler. Thromb. Vasc. Biol.* 21, 731–738
50. Doering, C. B., Parker, E. T., Healey, J. F., Craddock, H. N., Barrow, R. T., and Lollar, P. (2002) *Thromb. Haemostasis* 88, 450–458
51. Evans, E. (2001) *Annu. Rev. Biophys. Biomol. Struct.* 30, 105–128