

## Topology and Order of Formation of Interchain Disulfide Bonds in von Willebrand Factor

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Interchain disulfide bonds between the subunits in von Willebrand factor (vWf) dimers and in vWf multimers have been studied using some unique features of the cultured human umbilical vein endothelial cell system. Ammonium chloride inhibition of multimerization of vWf allowed selective examination of vWf dimeric molecules, and monoclonal antibody against the vWf propolypeptide was used to separate pro-vWf dimers from mature dimers. After cleavage of dimers and multimers with *Staphylococcus aureus* V-8 protease, the location of interchain disulfide bonds in amino (N)-terminal or carboxyl (C)-terminal frag-

ments was determined by gel electrophoresis under reduced and nonreduced conditions. The first interchain disulfide bonds formed during dimerization are in the C-terminal region of the subunits, whereas interdimer disulfide bonds are located in the N-terminal portion. These data confirm recent electron microscopic projections of disulfide bond locations and provide support to the hypothetical role of the propolypeptide in the multimerization process.

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**T**HE LARGEST von Willebrand factor (vWf) multimers are the most active in in vitro platelet aggregation and binding assays, and their absence in vivo results in type IIA von Willebrand disease (vWd).<sup>1</sup> Therefore, the formation of the interchain disulfide bonds is a crucial aspect of vWf biosynthesis. Studies using cultured endothelial cells indicate that dimers of pro-vWf subunits are formed in the endoplasmic reticulum, and only after these are transported to the Golgi apparatus does interdimer disulfide bond formation begin.<sup>2</sup> This latter process is pH sensitive because it is inhibited in the presence of a weak base such as ammonium chloride.<sup>3</sup> Simultaneous with multimerization, the vWf propolypeptide (von Willebrand antigen II),<sup>4</sup> located at the N-terminal end of the vWf subunit,<sup>5-7</sup> is proteolytically removed,<sup>8,9</sup> and vWf is secreted into the culture medium or stored in the Weibel-Palade bodies.<sup>10</sup>

Many multimeric sizes of vWf are found in plasma.<sup>1</sup> The smallest circulating species is a dimer<sup>11,12</sup> that could correspond to the dimeric molecule formed in the endoplasmic reticulum. Limited proteolysis of plasma vWf with *Staphylococcus aureus* V-8 protease produces a single cleavage in the vWf subunit, yielding two large homodimers,<sup>13,14</sup> indicating that multimerization occurs in a head-to-head and tail-to-tail fashion. Electron microscopic observations confirm this type of arrangement and in addition reveal the vWf multimers to be elongated molecules with the interchain disulfide bonds located near the N-terminals and C-terminals of the subunits.<sup>12,15</sup> In this study, we examine which of these interchain disulfide bonds is formed first (ie, in the endoplasmic reticulum) during dimerization of vWf and compare our result to a preliminary electron microscopic observation which assigns the interchain link of the smallest circulating species as being between the C-terminal regions of the vWf subunits.<sup>16</sup>

### MATERIALS AND METHODS

**Cells and culture conditions.** Endothelial cells were obtained from human umbilical vein by mild proteolytic digestion as described previously<sup>10,17</sup>; only primary cultures were used. Cells were cultured in McCoy's 5A medium (Flow Laboratories, Inc, McLean, VA) containing 20% fetal bovine serum (FBS). Ammonium chloride (Fischer Scientific, Rochester, NY) was dissolved in culture medium. For metabolic labeling, cells were grown for 3 days in the presence of [<sup>35</sup>S]methionine (25  $\mu$ Ci/mL, 1148 Ci/mmol) (New England Nuclear, Billerica, MA).

**Antibodies.** Anti-vWf antiserum was purchased from Calbiochem-Behring (San Diego). Antiserum against human pro-vWf was prepared by immunization of New Zealand albino rabbits with purified von Willebrand antigen II (vW AgII) and vWf. Antiserum was rendered monospecific for pro-vWf by filtration through Sepharose CL-4B coupled with plasma from a patient with severe vWd. This polyclonal antiserum recognizes both the vWf propolypeptide (vW AgII) and the mature vWf multimers. Monoclonal antibodies against the 100-kd propolypeptide of vWf<sup>8</sup> were prepared as follows: Spleen cells from female BALB/c mice hyperimmunized with the propolypeptide were fused to the myeloma cell line P3X63Ag8.653<sup>18</sup> at a ratio of 3:1, using standard techniques with HAT selection.<sup>19</sup> Supernatants from each resulting clone were tested by enzyme-linked immunosorbent assay (ELISA) against the propolypeptide, mature vWf, and fibronectin. Antibody-producing clones with preferential activity against the 100-kd propolypeptide of vWf were expanded and recloned until clonal homogeneity was achieved (two to three subclonings). These were then frozen and stored. The specificity of antibody produced by such clones was confirmed by Western blotting.<sup>20</sup>

**Purification of vWf.** vWf was immunopurified as described previously.<sup>2</sup> For purification of molecules containing the propolypeptide, 300  $\mu$ L of supernatant containing monoclonal antibody was used for each sample.

**S aureus V-8 cleavage.** Purified vWf in electrophoresis sample buffer containing 2% sodium dodecyl sulfate (SDS)<sup>21</sup> was diluted twofold in water containing *S aureus* V-8 protease so that the final concentration of the enzyme was 10  $\mu$ g/mL. After 10-minute digestion at room temperature, the reaction was stopped by adding 1/10 vol of 20% SDS and boiling. Samples were immediately prepared for electrophoresis with or without dithiothreitol.

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**Electrophoresis gels.** Agarose horizontal slab gels were prepared using a solution of 2% agarose, 0.1% SDS in 0.05 mol/L of phosphate buffer at pH 7.0, and running buffer was 0.1 mol/L of phosphate buffer, pH 7.0, with 0.1% SDS. SDS polyacrylamide gels were prepared as described by Laemmli.<sup>21</sup> For two-dimensional gel electrophoresis, protein was first run nonreduced on a 5% polyacrylamide gel. The strip was cut out and incubated with constant shaking for 20 minutes at room temperature in running buffer<sup>21</sup> that contained 50 mmol/L of dithiothreitol. The strip was then transferred to the top of an identical 5% SDS polyacrylamide gel and overlaid with 0.5% agarose and 0.1 mol/L of dithiothreitol in running buffer before electrophoresis. Western blotting analysis was performed as previously described.<sup>22</sup> The primary antibody solution consisted of a mixture of three different anti-vWf propolypeptide monoclonal antibody supernatants each diluted 1:25.

## RESULTS

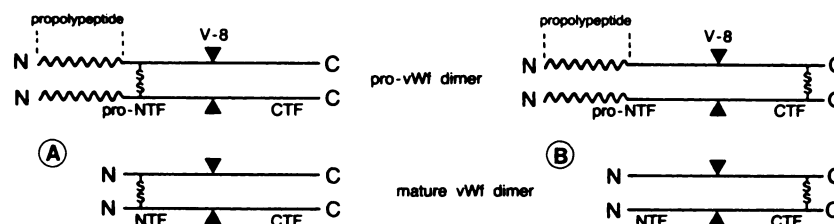
Ammonium chloride inhibits interdimer disulfide bond formation and partially blocks cleavage of prosequence.<sup>3</sup> Therefore, in its presence, endothelial cells in culture secrete dimeric molecules, some of which contain pro-vWf subunits (Fig 1). *S aureus* V-8 protease cleaves the vWf or pro-vWf subunit in two fragments (refs. 13 and 14 and our preliminary results). As diagrammed schematically in Fig 1, the C-terminal fragments released from the two types of subunit would have to be identical, whereas the N-terminal fragment from the pro-vWf subunit should be larger, because it contains in addition the 100-kd prosequence. To localize the interchain disulfide bond(s) in the dimers to the N-terminal or C-terminal portion of the molecule (options A or B, Fig 1), we generated these V-8 protease fragments and determined which were dimeric in the absence of reduction.

Human umbilical vein endothelial cells were metabolically labeled in the presence of 25 mmol/L of ammonium chloride. Culture medium was collected, and molecules containing the pro-vWf sequence were purified by the use of monoclonal antibody prepared against the propolypeptide. vWf molecules containing mature subunits were then isolated from the supernatant with a polyclonal anti-vWf antiserum. The purified protein was examined nonreduced on a 2% agarose gel (Fig 2A), and both reduced and nonreduced protein was examined on a 5% polyacrylamide gel (Fig 2B). The sample purified with the antipropolypeptide monoclonal antibody contained mostly dimers of pro-vWf subunits and free propolypeptide.<sup>4</sup> The protein purified with the polyclonal antiserum was composed predominantly of dimers of mature

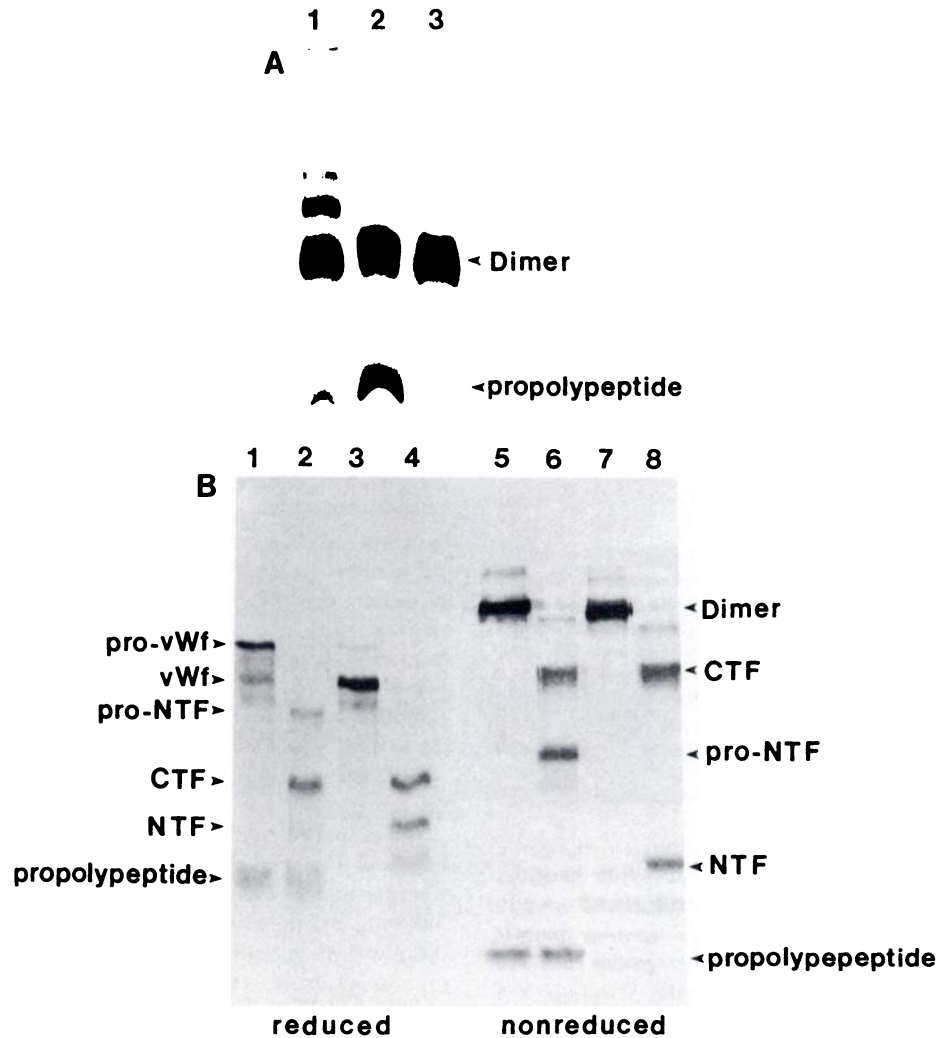
subunits and did not contain the free propolypeptide (Fig 2). Two major bands were produced by V-8 protease cleavage of the mature vWf and pro-vWf dimers. One band was shared between the two samples (Fig 2B, lanes 2 and 4 reduced, lanes 6 and 8 nonreduced) and therefore represents the C-terminal fragment (CTF). As expected, the other N-terminal cleavage fragment was larger when obtained from the pro-vWf dimer (pro-NTF) than from mature vWf dimers (NTF) (Fig 2b). The identification of the fragments was further confirmed by Western blotting as follows: a mixture of pro-vWf and vWf dimers purified from culture medium of ammonium chloride-treated cells by polyclonal antiserum against pro-vWf were digested by V-8 protease. The starting material and the resulting fragments were electrophoresed on a reduced 6% polyacrylamide gel. Western blots of this gel with a mixture of antipropolypeptide monoclonal antibodies (Fig 3) identified the pro-vWf subunit, the propolypeptide, and the pro-NTF, and demonstrated that the large pro-NTF indeed contains the N-terminal portion of the molecule.

Both pro-NTF and NTF migrated faster on gels nonreduced, indicative of the presence of intrachain disulfide bonds (Fig 2B). On the other hand, the C-terminal fragment migrated significantly more slowly nonreduced, suggesting that it was dimeric. To show this more convincingly, the *S aureus* V-8 proteolytic fragments were examined by two-dimensional gel electrophoresis (Fig 4). In the first dimension, the samples were electrophoresed nonreduced on a 5% polyacrylamide gel; then the lanes were cut out, incubated in the presence of a reducing agent, and placed on top of a second 5% polyacrylamide gel (as shown in Fig 4) and electrophoresed. Only CTF migrated well below the diagonal of the gel, confirming that it was dimeric prior to reduction. It appears therefore that the dimers that are the building blocks of vWf polymers<sup>2,23</sup> are held together by disulfide bond(s) localized in the C-terminal region of the polypeptide chain (option B, Fig 1).

The major products of *S aureus* V-8 cleavage of plasma vWf are two large distinct dimeric fragments,<sup>13,14</sup> indicating that both ends of the vWf subunit form interchain disulfide bonds. Because the first link between the subunits of dimers is in the C-terminal region, the interdimer disulfides formed later in the processing of vWF<sup>2</sup> should involve the other (N-terminal) end of the subunit. If this is so, the amount of the monomeric N-terminal fragment should decrease with an increase in size of vWf multimers submitted to *S aureus*



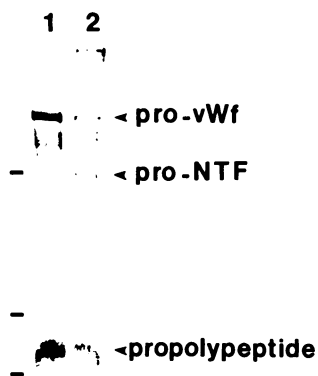
**Fig 1.** Schematic representation of the location of disulfide bonds in the pro-vWf dimer and mature vWf dimer. The arrowheads indicate the approximate cleavage site for *Staphylococcus aureus* V-8 protease. Following cleavage, V-8 protease produces N-terminal fragments (pro-NTF and NTF) and C-terminal fragments (CTF). If the interchain disulfide bond(s) of the dimers is located at the N-terminal part of the molecule (option A), the pro-NTF and NTF will be dimeric, whereas a location of the disulfide bond(s) at the C-terminal end (option B) will produce dimeric CTF.



**Fig 2.** *Staphylococcus aureus* V-8 cleavage of pro-vWf and vWf dimers. (A) The wide range in size of vWf multimers secreted constitutively by metabolically labeled endothelial cell cultures is indicated in lane 1. In the presence of 25 mmol/L of ammonium chloride, only vWf dimers are produced, and these can be separated immunologically (described in text) into pro-vWf dimers (lane 2) and mature vWf dimers (lane 3). Purification of the pro-vWf dimer with specific monoclonal antibody against the prosequence also precipitates free propolypeptide. Autoradiograph of a 2% agarose gel is shown. (B) Autoradiograph of a 5% polyacrylamide gel, the left portion of which (lanes 1 through 4) was obtained with reduced protein. Pro-vWf dimers were examined before V-8 protease cleavage (lanes 1 and 5) and after cleavage (lanes 2 and 6). Mature vWf dimers are similarly shown before protease cleavage (lanes 3 and 7) and after cleavage (lanes 4 and 8). The 100-kd propolypeptide is not cleaved by the V-8 protease. Comparison of migration of the fragments with mol wt markers gives the following mol wts: pro-NTF 200 kd, CTF 145 kd, and NTF 125 kd. Because vWf and pro-vWf migrate anomalously in polyacrylamide gels,<sup>4</sup> these estimated mol wts are likely incorrect. The minor band which migrates more rapidly than the pro-NTF and NTF fragments is a secondary cleavage product of these fragments. The small amount of mature vWf subunit in the pro-vWf preparation (lane 1) indicates that a minor fraction of the dimers synthesized in the presence of ammonium chloride are heterodimeric, that is, they contain both types of subunits. The small amount of pro-vWf subunit present in the vWf preparation (lane 3) reflects incomplete prior removal of all pro-vWf by the antipropolypeptide monoclonal antibody.

V-8 digestion. vWf purified from endothelial cell's culture medium (using anti-vWf antiserum) is predominantly composed of small vWf multimers<sup>22</sup> (Fig 2a), whereas the protein stored in the endothelial cells is highly multimerized.<sup>24</sup> In the endothelial cell's endoplasmic reticulum, there is in addition a large pool of pro-vWf dimers that we can separate from the mature vWf with antipropolypeptide monoclonal antibodies. Metabolically labeled endothelial cell lysate was therefore subjected to a two-step purification procedure. The first step removed the prosequence containing molecules that were

discarded, and the second isolated the remaining high-mol-wt (HMW) vWf multimers, using a polyclonal anti-vWf antiserum. These large cellular multimers were subjected to *S aureus* V-8 digestion in parallel with the predominantly small multimers purified from the culture medium. The starting material and the resulting fragments were analyzed nonreduced on 6% acrylamide gels (Fig 5). Significantly less monomeric N-terminal fragment was generated from the cellular sample (Fig 5, lane 4) than from the secreted vWf (Fig 5, lane 2), indicating that this portion of the subunit



**Fig 3.** Western blot of *Staphylococcus aureus* V-8 cleavage products with antipropolypeptide monoclonal antibodies. Culture medium from metabolically labeled cells in the presence of 25 mmol/L of ammonium chloride was purified with polyclonal anti-pro-vWf antiserum and electrophoresed reduced on a 6% polyacrylamide gel before (lane 1) and after (lane 2) digestion with V-8 protease. The Western blot of this gel was done with a mixture of three monoclonal antibodies directed against the propolypeptide. Only the pro-NTF, not the CTF and NTF, reacted positively with the antibodies. Indications at left show migration of markers: myosin, 200 kd;  $\beta$ -galactosidase, 130 kd; phosphorylase b, 92.5 kd.

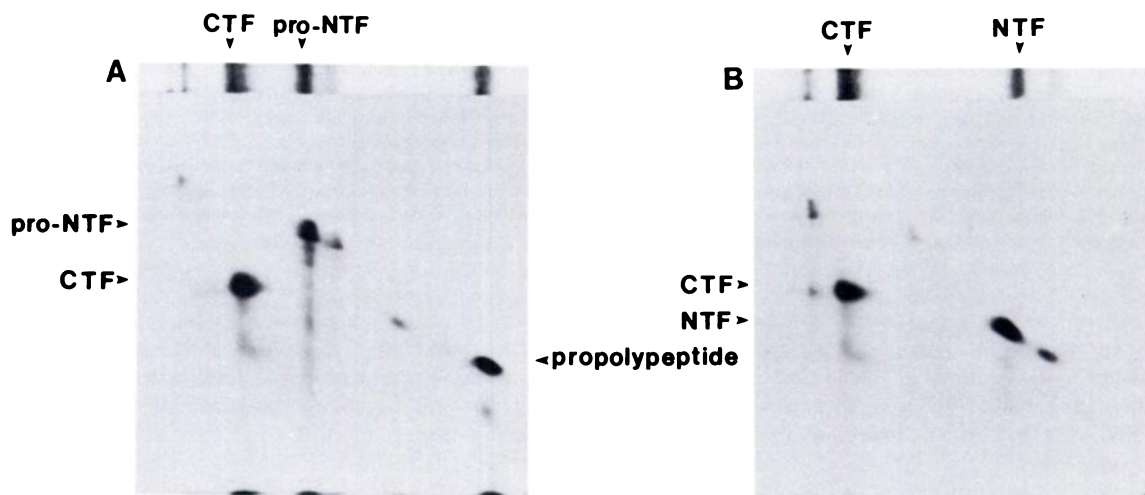
became highly involved in interchain disulfide bonding. Two-dimensional gel electrophoresis of the digested samples (not shown) revealed that the slowly migrating doublet contains both the dimeric C-terminal fragment and the dimeric N-terminal fragment. Because the *S aureus* V-8 cleaves the vWf subunit close to its center, the dimers of the resulting fragments appear similar in size in our gel system. The doublet can be better resolved on low-percentage acrylamide gels in which the CTF dimer migrates slower than the

NTF dimer (not shown). The very small amount of monomeric N-terminal fragment (seen only after long autoradiographic exposures) produced from HMW vWf multimers confirms that this is the part of the subunit involved in the polymerization of the vWf dimers.

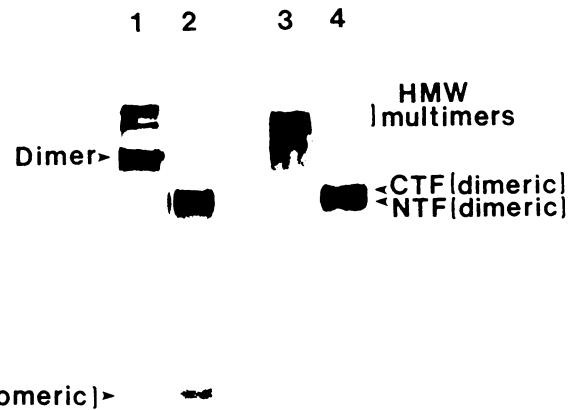
#### DISCUSSION

Although most of the amino acid sequence of the mature vWf subunit is known from molecular cloning studies<sup>5-7</sup> and protein sequencing,<sup>25</sup> the exact number and location of the interchain disulfide bonds in the vWf multimers remains to be elucidated. To localize the interchain disulfide bonds in vWf multimers, the structure of the protein was first simplified to dimers. This was accomplished by culturing human umbilical vein endothelial cells in the presence of a weak base (ammonium chloride) that inhibits the intracellular multimerization of dimers.<sup>3</sup> The resulting dimeric molecules were separated into two groups, namely, those containing pro-vWf subunits and those built of mature subunits. For this purpose, we used monoclonal antibodies prepared against the vWf propolypeptide. The two sets of dimers were submitted to cleavage with *S aureus* V-8 protease, and the resulting fragments were compared reduced and nonreduced and by two-dimensional gel electrophoresis. A shared C-terminal fragment was shown to be dimeric (Figs 2 and 4). Therefore, the first interchain disulfide bonds formed in the biosynthesis of vWf during dimerization are in this C-terminal region of the subunit.

We cannot reconcile why our results showed a C-terminal fragment that was larger than the N-terminal fragment whereas other researchers have reported opposite results following V-8 digestion of mature vWf.<sup>13</sup> It is possible that the presence of SDS in our samples uncovered a different primary cleavage site for the protease, also localized in the center of the subunit, or that the fragments have different electrophoretic properties in our gel system. The sum of the apparent mol wts of the two fragments, ie, 145 plus 125 kd, corresponds to the size of vWf subunit (270 kd) determined



**Fig 4.** Two-dimensional gel electrophoresis of *Staphylococcus aureus* V-8 cleavage products (autoradiographs of 5% polyacrylamide gels). Samples were analyzed nonreduced in the first dimension as shown (left to right) and reduced in the second (top to bottom). Fragments were obtained from (A) pro-vWf dimers and (B) mature vWf dimers.



**Fig 5.** *Staphylococcus aureus* V-8 cleavage of multimeric vWf (autoradiograph of nonreduced 6% polyacrylamide gels). Lanes 1 and 2 show results obtained with constitutively secreted vWf obtained from long-term labeled human endothelial cell medium and lanes 3 and 4 show results with high-mol-wt (HMW) cellular vWf multimers. Lanes 1 and 3 represent the starting material, and lanes 2 and 4 show the cleavage products after V-8 protease digestion. Both CTF and NTF fragments obtained from the HMW multimers are dimeric, whereas the NTF obtained from culture medium vWf is mostly monomeric.

by amino acid sequence and carbohydrate content studies of the protein.<sup>25</sup> Both the N-terminal<sup>7</sup> and C-terminal<sup>5-7</sup> regions of the vWf polypeptide chain are rich in cysteines, but the center portion of the mature subunit contains a stretch of ~600 amino acids that is poor in cysteines,<sup>26</sup> similar to the disulfide distribution of fibronectin.<sup>27</sup> In addition, we showed that vWf dimers are similar to fibronectin dimers,<sup>28,29</sup> in that both are held together at the C-terminal portion of the subunits (Fig 1, option B). In human cells, the formation of the vWf dimers is dependent on N-linked glycosylation of the subunits, since tunicamycin, which inhibits glycosylation, also blocks processing of vWf beyond the monomeric form.<sup>3</sup>

A recent abstract<sup>16</sup> compared the electron microscopic appearance of N-terminal and C-terminal fragments of plasma vWf with that of the smallest circulating form of vWf and suggested that this dimeric molecule is held together in the C-terminal region of the subunits. It appears therefore that these small circulating species of vWf are not degradation products of large multimers but instead represent the dimeric molecules synthesized in the endoplasmic reticulum and secreted by endothelial cells<sup>2,23</sup> and megakaryocytes<sup>30</sup> in culture.

Comparison of the nonreduced fragments obtained from dimeric and multimeric molecules (Figs 2 and 5) reveals that the interdimer disulfide bonds involve the N-terminal region of the subunit. This may occur by direct oxidation of the free sulfhydryl groups present on the pro vWf-dimers,<sup>2</sup> which are absent from mature vWf multimers.<sup>31,32</sup> The multimerization process is likely to begin in the Trans Golgi apparatus<sup>3</sup> and possibly continues during the packaging of the protein into the Weibel-Palade bodies. Loscalzo and colleagues showed that at high concentrations vWf can aggregate by noncovalent forces and proposed that such forces might be important during intracellular assembly of vWf.<sup>33</sup> We hypothesized that the large propolypeptide of vWf may assist in the proper alignment of the pro-vWf dimers for the interdimer disulfide bond formation.<sup>2</sup> The observation that these links are formed in proximity to the prosequence, ie, in the N-terminal region of the subunit, is further support for this hypothesis.

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