Identification of Disulfide-Bridged Substructures within Human von Willebrand Factor†

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ABSTRACT: In the course of identifying substructural domains within the homooligomeric protein von Willebrand factor [270 kilodaltons (kDa) per polypeptide chain], seven large fragments of 8–90 kDa have been generated by limited proteolysis. A monomeric fragment that binds coagulation factor VIIIc is identified as residues 1–272. A fragment that binds platelet glycoprotein Ib is identified as a homodimer containing two pairs of identical chains, i.e., residues 273–511 and 674–728. Disulfide bonds have been identified by several methods, including direct observation of the phenylthiohydantoin of cystine during Edman degradation of isolated peptides. Among half-cystine residues in the amino-terminal 1365-residue region, 52 have been paired. They place structural constraints on folding possibilities within three structural domains. Additional clusters of disulfide bonds are evident. It has been shown that at least 35 disulfides must form intrachain bridges, specifically the cystines among residues 1–272 and 906–1492. Intersubunit disulfide bonds are partially localized in an interior region (residues 283–695) and a carboxyl-terminal region (residues 1908–2050). Each of these regions appears to be linked to a corresponding region of a neighboring subunit in the network of interconnected chains. The difficulties of pairing all 169 half-cystines (per chain) and of distinguishing intrachain from interchain disulfides are evaluated.

Human von Willebrand factor (vWF)[1] is a large, multivalent plasma glycoprotein that mediates the adhesion of platelets to the subendothelium as well as the aggregation of platelets at sites of vascular injury (Weiss et al., 1978). vWF participates in the initial reactions of hemostasis by interacting with specific receptors on the platelet membrane and with components of the vessel wall. The binding of platelets appears to involve glycoprotein Ib (Jenkins et al., 1976) and, in the presence of thrombin or ADP, the glycoprotein IIb/IIIa complex (Ruggeri et al., 1982). The principal target for vWF in the subendothelial connective tissue is probably collagen (Morton et al., 1983), although additional receptors may exist (Wagner et al., 1984). vWF also forms a noncovalent complex with coagulation factor VIII, thus stabilizing it and prolonging its half-life in circulation (Tuddenham et al., 1982).

vWF is synthesized by endothelial cells (Jaffe et al., 1973; Jaffe & Hoyer, 1974) and by megakaryocytes (Nachman et al., 1977) in a large precursor form and secreted into plasma following several processing events, including glycosylation, sulfation, disulfide bond formation, and proteolytic cleavages (Wagner & Marder, 1983, 1984; Browning et al., 1983; Lynch et al., 1983; Ling et al., 1984). The protein circulates in plasma as a series of high molecular weight disulfide-linked multimers that are assembled from identical 270-kDa subunits (Ruggeri & Zimmerman, 1980; Hoyer, 1981; Chopek et al., 1986).

Recently, protein sequence analysis in our laboratory (Titani et al., 1986) and cDNA nucleotide sequencing (Sadler et al., 1985; Shelton-Inloes et al., 1986; Lynch et al., 1985; Ginsburg et al., 1985; Verweij et al., 1986; Bonthron et al., 1986) have established the complete amino acid sequence of human vWF. The single-chain subunit contains 2050 residues and shows an unusually high content of Cys residues (8.2%). All 169 Cys residues are engaged in intrachain or interchain disulfide bond formation (Legaz et al., 1973). Half-cystinyl residues are clustered and particularly abundant in the amino- and carboxyl-terminal regions of the protein, where nearly one-third of these residues are in Cys-X-Cys or in Cys-Cys sequences, indicating an extensive network of cross-links.

Girma et al. (1986a) have reported that limited proteolysis of native vWF by *Staphylococcus aureus* V8 protease proceeds primarily by cleavage of a single peptide bond in each subunit, resulting in the disassembly of the multimeric structures and the generation of two major fragments III and II. Both fragments are disulfide-linked homodimers containing the amino-terminal (residues 1–1365) or the carboxyl-terminal (residues 1366–2050) portions, respectively, of two vWF subunits. Upon prolonged digestion, fragment III is further degraded to a homodimeric fragment III′ (residues 1–910) and two identical monomeric fragments I (residues 911–1365). All fragments can be separated without denaturation or cleavage of disulfide bridges (Girma et al., 1986a; Titani et al., 1986; Fretto et al., 1986). vWF has also been partially characterized in terms of the products of limited proteolysis by various other proteases, notably trypsin (Sixma et al., 1984; Houdijk et al., 1986) and plasmin (Andersen et al., 1980; Hamilton et al., 1985). These fragments were characterized largely by im-

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[1] Abbreviations: CNBr, cyanogen bromide; Cys, half-cystine; DTT, dithiothreitol; FAB, fast atom bombardment; PTH, phenylthiohydantoin; RP-HPLC and SE-HPLC, reversed-phase and size-exclusion high-performance liquid chromatography, respectively; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TFA, trifluoroacetic acid; vWF, human von Willebrand factor; V8, *Staphylococcus aureus* protease V8; kDa, kilodalton(s); TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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munological techniques that permitted mapping of different functional domains, showing affinity for platelets, collagen, and heparin (Fujimura et al., 1986, 1987; Girma et al., 1986b; Pareti et al., 1986; Houdijk et al., 1986). However, these functional regions have not been located in specific substructural domains of the native vWF molecule.

In this study, we applied techniques of limited proteolysis and sequence analysis in order to explore the substructural organization of this multivalent protein. In addition, we present a partial assignment of disulfide bonds within the amino-terminal V8 fragment III, which has been reported to contain functional domains binding platelet glycoprotein Ib, collagen, heparin, and factor VIIIc.

Materials and Methods

Human vWF was isolated from a commercial factor VIII concentrate (a generous gift of Dr. H. Kingdon, Hyland Therapeutics, Division of Travenol Laboratories) as previously described by Titani et al. (1986).

Fragments I, II, and III were prepared by limited proteolysis of the native protein with *Staphylococcus aureus* V8 protease (Miles). The exact procedure and the separation pattern on a Mono Q column have been reported previously (Titani et al., 1986). These fragments were stored at 4 °C in the presence of 5 mM iodoacetic acid and 0.01% sodium azide. For further enzymatic or chemical digestions, the fragments were precipitated by the addition of trichloroacetic acid.

Limited tryptic proteolysis of fragments I, II, and III was performed under the following conditions: Fragment III (1 mg/mL) was dissolved in 0.1 M NH_4HCO_3, pH 8.0, and incubated with TPCK–trypsin (Millipore) at an enzyme:substrate ratio of 1:100 (w/w) for 2 h at 37 °C. The digest was acidified, lyophilized, and purified by SE-HPLC on TSK columns (LKB) and by RP-HPLC. Digestion of fragment II with subtilisin was carried out in 0.1 M NH_4HCO_3, pH 8.0, at a molar ratio of 1:100 for 40 h at 37 °C. Fragment I was treated with trypsin at an enzyme:substrate ratio of 1:25 (w/w) for 2 h at 37 °C.

Methods employed for reduction, S-carboxymethylation, and digestion with CNBr followed procedures previously described (Titani et al., 1984). Aspartyl bonds were selectively cleaved in 2% formic acid under vacuum for 5 h at 110 °C (Inglis, 1983).

Subdigestions with thermolysin (Calbiochem) were performed in 0.1 M 3-(*N*-morpholino)propanesulfonic acid, pH 6.5, and 2 mM $CaCl_2$ containing 1 mM iodoacetic acid to minimize disulfide interchange. The cleavage conditions were optimized for each fragment as follows: fragment I-T5 for 6 h at 40 °C; fragments III-T4-D1 and III-T4-D2 for 6 and 3 h, respectively, at 60 °C, all at an enzyme:substrate ratio of 1:25 (w/w). Fragments III-T2 and III-T4 were cleaved with thermolysin for 20 h at 60 °C, using an enzyme:substrate ratio of 1:12 (w/w).

Mixtures of peptides were usually separated first by SE-HPLC on tandem TSK G3000 SW columns [cf. Titani et al. (1986)], in 6 M guanidine hydrochloride containing either 10 mM phosphate, pH 6.0 (for reduced and alkylated fragments), or 1% phosphoric acid, pH 3.2 (for disulfide-linked peptides). Further purification was achieved by RP-HPLC on Ultrapore RPSC (C3) or SynChropak RP-8 (C8) columns. Thermolytic fragments were first resolved on a SynChropak RP-P (C18) column and then chromatographed on Hypersil ODS (100 × 2.1 mm, Hewlett Packard) or Aquapore RP-18 (30 × 2.1 mm, Brownlee). In each case, an acetonitrile gradient was used in dilute aqueous trifluoroacetic acid. Cys-containing peptides were detected according to the procedure of Sueyoshi et al. (1985), using an aliquot (100–200 pmol) of each RP-HPLC fraction. The reagent was purchased from Wako Pure Chemicals, Osaka. The fluorescence intensities were measured in a Perkin-Elmer MPF-44A fluorescence spectrophotometer with excitation at 385 nm and emission at 520 nm.

SDS–PAGE was performed as described by Laemmli and Favre (1973). Ferguson plots were prepared from the results of electrophoresis at different acrylamide concentrations following the procedure of Kawasaki and Ashwell (1976).

Amino acid compositions were determined in 20-h hydrolysates by RP-HPLC with a Waters Picotag system (Bidlingmeyer et al., 1984). Edman degradations of proteolytic fragments were performed in a Beckman 890C spinning-cup sequencer. Phenylthiohydantoins were identified on a Zorbax PTH column (Glajch et al., 1985). Disulfide-linked peptides were analyzed with an Applied Biosystems 470A sequencer using a program adapted from Hunkapiller et al. (1983) and equipped with a Model 120A PTH analyzer. PTH–cystine was identified after its release in the corresponding cycle as previously reported by Lu et al. (1986).

FAB mass spectrometry was performed in a Kratos MS-50, double-sector instrument, using a glycerol/thioglycerol/HCl matrix as described by Naylor et al. (1986).

Results

Legaz et al. (1973) reported that the human vWF/factor VIIIc complex contained no free sulfhydryl groups. This observation is critical to the present investigation and was confirmed by attempting to label denatured vWF with iodo-[^{14}C]acetic acid by the procedures of Garcia-Pardo et al. (1985). The amount of radioactivity incorporated corresponded to less than 0.1 thiol group per 270-kDa subunit, verifying that all 169 Cys residues are involved in disulfide linkages.

Limited Proteolysis of vWF by S. aureus V8 Protease. The products obtained by V8 digestion of vWF have previously been characterized by Girma et al. (1986a). Two major fragments, III and II, represent residues 1–1365 and 1366–2050, respectively. A minor product, fragment I, is derived from fragment III. The calculated molecular weight of dimeric fragment II (182K) is at variance with that estimated on agarose gels where it moved more slowly than the larger fragment III. In order to clarify the multimeric forms of these fragments, we reexamined the molecular weights of their nonreduced forms using "Ferguson plots" of SDS–PAGE data (Kawasaki & Ashwell, 1976). Fragments II and III exhibited molecular weight values of 225K and 315K, respectively, indicating that both are disulfide-linked dimers (Table I).

Limited Proteolysis of V8 Fragments III and I by Trypsin. Tryptic cleavage of native fragment III with enzyme concentrations as high as 10% (w/w) produced two large segments, III-T2 and III-T4, that were readly separated by SE-HPLC as shown in Figure 1A. Edman degradations and SDS–PAGE experiments established their location within the sequence of vWF.

Analysis of native fragment III-T2 revealed two major sequences in equal quantities, which were interpreted as Lys-Val-Pro-Leu-Asp and Ala-Phe-Val-Leu-Ser. These N-termini correspond to residues 273 and 674 in the vWF sequence (see Figures 2 and 3). Minor tryptic cleavage sites (in total ~20% of major) were also observed at Lys-273, Lys-289, Arg-373, and Lys-448. All of the corresponding peptides were isolated by SE-HPLC and RP-HPLC of reduced and S-carboxymethylated fragment III-T2. Compositional and sequence analyses revealed that these peptides all originated from

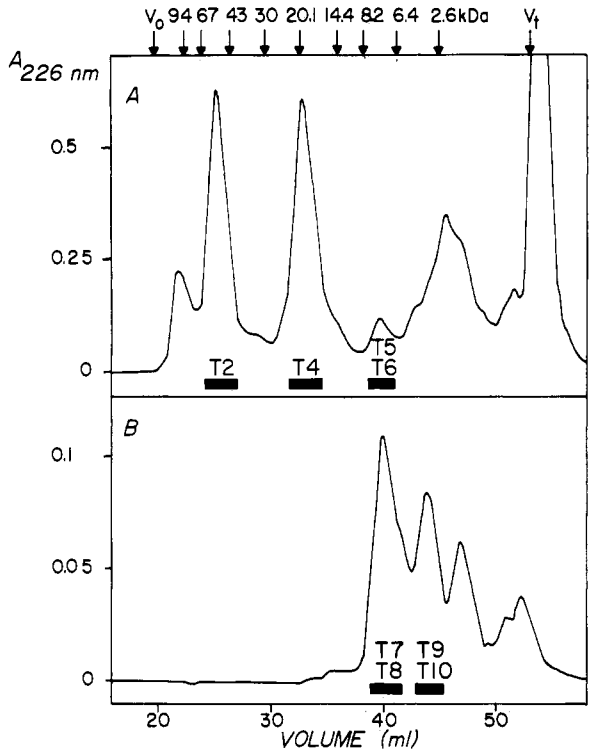

FIGURE 1: Separations by size in 6 M guanidine hydrochloride/1% phosphoric acid, pH 3.2, at a flow rate of 1 mL/min on two columns of TSK G3000 SW (7.5 × 600 mm) connected in series. Fractions were pooled as shown by horizontal bars. V_0, V_t, and the elution positions of standard polypeptides are indicated at the top by vertical arrows. (A) Primary separation of a tryptic digest of unreduced fragment III (10 nmol). Fractions T2 and T4 were purified further on an Ultrapore RPSC column. Peptides T5 and T6 were subsequently separated on a SynChropak RP-8 column. In the text, these peptides are identified with the prefix III-. (B) Primary separation of the tryptic digest of a core fraction obtained by CNBr cleavage of fragment I (12 nmol). Tryptic peptides in pooled fractions were subsequently purified on Ultrapore RPSC and SynChropak RP-8 columns, respectively. In the text, they are identified with the prefix I-M-.

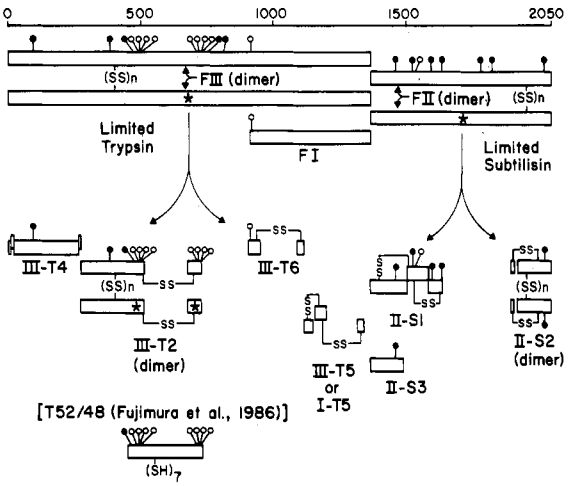

FIGURE 2: Diagrammatic summary of the dimensions and the source within human vWF of 10 major fragments generated by limited digestion. The number line at the top refers to the 2050 residues of vWF. Fragments III, II, and I were generated by Girma et al. (1986a) using *S. aureus* V8 protease. The prefix in the name of each other fragment indicates its source (III or II); the suffix indicates whether it is a product of tryptic (T) or subtilisin (S) cleavage. Fragments are monomeric unless specifically denoted dimers. N-Linked and O-linked carbohydrate chains are indicated by (●) and (○), respectively, but only on one subunit of dimers (stars denote chains *not* decorated with carbohydrate symbols). The three short chains and one long chain of III-T4 are linked to each other by intrasubunit disulfides. The fragment of Fujimura et al. was isolated after disulfide reduction.

residues 273–511 and 674–728 of vWF. On the basis of these results, we identified fragment III-T2 as a two-chain molecule consisting of a 239-residue heavy chain and a 55-residue light chain. The molecular weight of this segment as calculated from the sequence, including the 10 carbohydrate chains, is approximately 44K. SDS–PAGE under nonreducing conditions revealed a main band at 90 kDa, whereas after reduction all bands had molecular weight values smaller than 38K (Figure 4). Thus, in its native form, fragment III-T2 represents a disulfide-linked homodimer of four chains. It was not possible to determine the molecular weight by gel filtration because disulfide-linked fragments routinely exhibited anomalous mobility in guanidine hydrochloride on TSK columns (see Figure 1A).

Edman degradation of unreduced fragment III-T4 yielded four sequences in approximately equal amounts that were assigned as follows: Ser-Leu-Ser-X-Arg, Leu-Val-X-Pro-Ala, Ala-Glu-Gly-Leu-Glu, and Val-Ser-Ser-Gln-X (where X indicates that no PTH was detected). The starting points of these sequences correspond to residues 1, 11, 20, and 264, respectively, of human vWF (Figure 3). Subdigestion with cyanogen bromide and subsequent reduction and S-carboxymethylation generated eight fragments that were identified by amino acid and sequence analyses. The data obtained showed that they were all derived from the amino-terminal 272 residues of vWF. SDS–PAGE of unreduced fragment III-T4 revealed molecular weights of 34K and 31K after reduction (Figure 4). This suggests that only the three short disulfide-linked peptides (residues 1–10, 11–19, and 264–272) are removed from the amino- and carboxyl-terminal regions upon reduction. The molecular weight determined by gel electrophoresis is in agreement with the value calculated from the sequence, including carbohydrate (Table I).

Trypsinolysis of fragment III generated two other small disulfide-linked segments, III-T5 and III-T6, largely from the region corresponding to V8 fragment I (Figure 2). A fragment identical with III-T5 can also be isolated after tryptic cleavage of fragment I. Sequence analysis revealed that fragment T5 from either source consisted of three chains, namely, residues 1115–1150, 1154–1193, and 1318–1336, connected by disulfide bridges (Figure 3). Fragment III-T6 comprised two chains (residues 906–948 and 1091–1114), one overlapping fragment I and the other within it. The amino terminus of fragment I is Gly-911, whereas that of one chain of III-T6 is Cys-906 in the sequence Cys-Cys-Ser-Gly-Glu-Gly (residues 906–911). The two Cys in this sequence cannot be disulfide bonded to any part of fragment I; otherwise, this fragment would have remained joined to a segment of fragment III. Therefore, as discussed later, Cys-906 and Cys-907 must be disulfide bonded to each other or, through two intersubunit bonds, to an identical peptide in a neighboring subunit. The remaining two Cys in III-T6 (residues 923 and 1109) must form the disulfide bond linking its two chains to each other.

Limited Proteolysis of V8 Fragment II by Subtilisin. Digestion by subtilisin occurred mainly in the middle of fragment II, releasing core segments from the amino- and carboxyl-terminal regions (Figure 2). They were isolated by SE-HPLC (Figure 5) and further purified by RP-HPLC. Edman degradations revealed that fragment II-S1 consisted of three disulfide-linked chains with amino termini of Glu-1366, Trp-1508, and Leu-1585, respectively (Figure 6). Following reduction and alkylation, the three chains were separated by HPLC and subdigested with cyanogen bromide or trypsin. Compositional and/or sequence analyses verified that the resulting peptides were all derived from the amino-

Table I: Large Fragments of von Willebrand Factor (Unreduced)

Proteolytic Agent/ Fragment	Observed[a] Mr (kDa) Unreduced	Calculated[b] Mr (kDa) (for monomer)	Monomer/ Dimer	Residue No. in vWF	No. of Residues[c]	No. of Cys[c,d]	No. of Oligosaccharide Chains N-linked	O-linked
S. aureus V8								
III	315	170	D	1-1365	1365	74	5	9
I	50	50	M	911-1365	455	18	--	1
II	225	91	D	1366-2050	685	95	7	1
Trypsin								
III-T4	34	32	M	1-10 11-19 20-263 264-272	272	24	1	--
III-T2	90	44	D	273-511 674-728	294	30	2	8
III-T6	N.D.	8	M	906-948 1091-1114	67	4	--	1
III-T5[e]	N.D.	10	M	1115-1150 1154-1193 1318-1336	95	12	--	--
Subtilisin								
II-S1	46	(40)[f]	M	1366-(1507)[f] 1508-(1584)[f] 1585-1639	(274)[f]	42	4	1
II-S3	25	16	M	1366-1492	127	18	1	--
II-S2	43	18	D	1900-1912 (1926)[g]-2050	138	17	1	--

[a] SDS-PAGE. [b] Predicted from sequence and corrected for approximate molecular weight of N-linked and O-linked oligosaccharides (2.2 and 0.9 kDa, respectively). [c] Per monomer. [d] Half-cystine. [e] This peptide was also obtained by tryptic digestion of fragment I (I-T5). [f] The carboxyl terminus is not clearly established. [g] Approximately 50% of this chain had residue 1929 at the amino terminus.

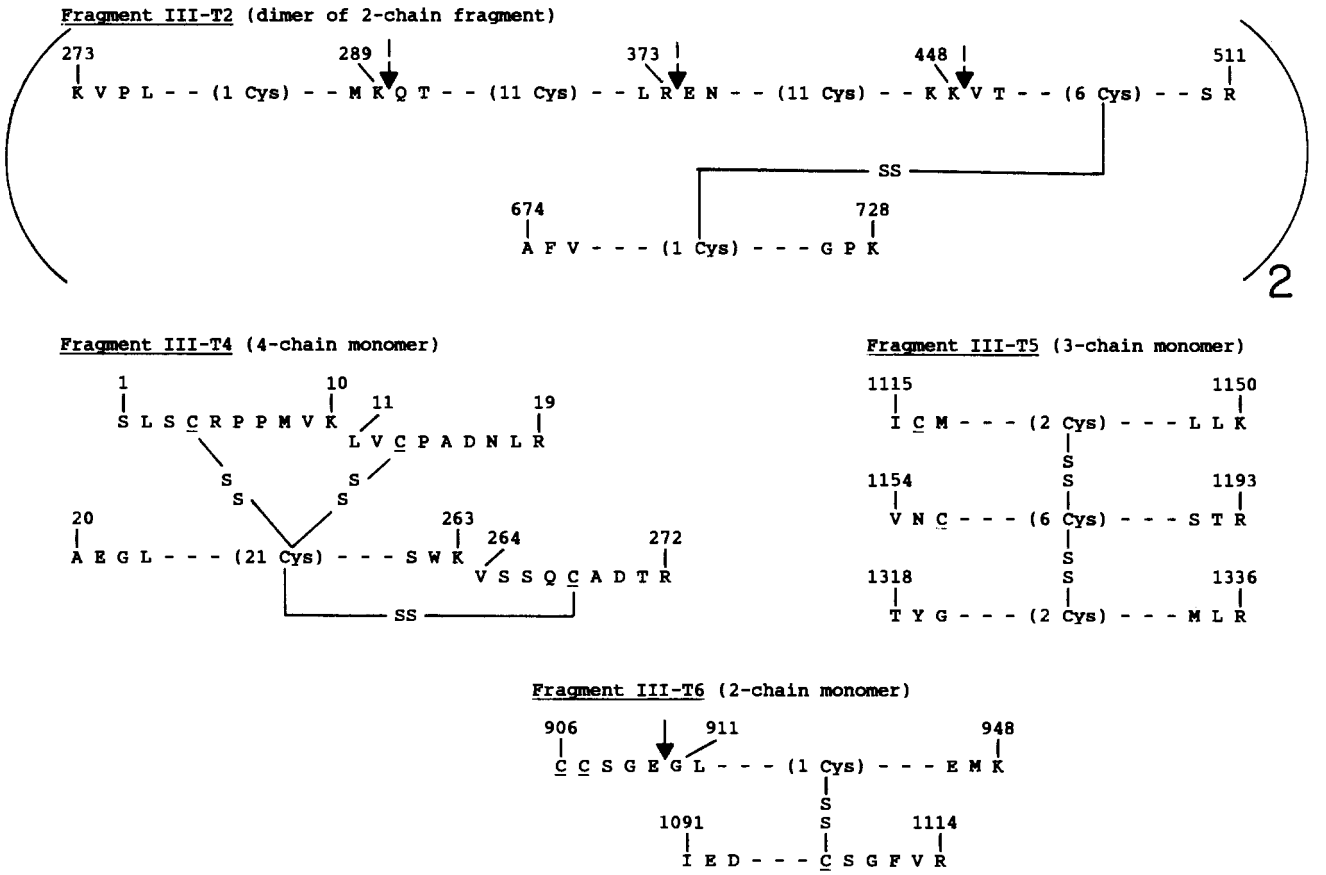

FIGURE 3: Polypeptide chains within the substructural fragments III-T2 through III-T6. Residue numbers and sequences are from Titani et al. (1986). Cys residues are designated either by an underlined C in the sequence or in groups within an unwritten sequence. Positions of oligosaccharide chains are not illustrated here but are shown in Figure 2. Arrows in fragment III-T2 indicate minor tryptic cleavage sites; arrows in fragment III-T6 denote the peptide bond cleaved by V8 protease.

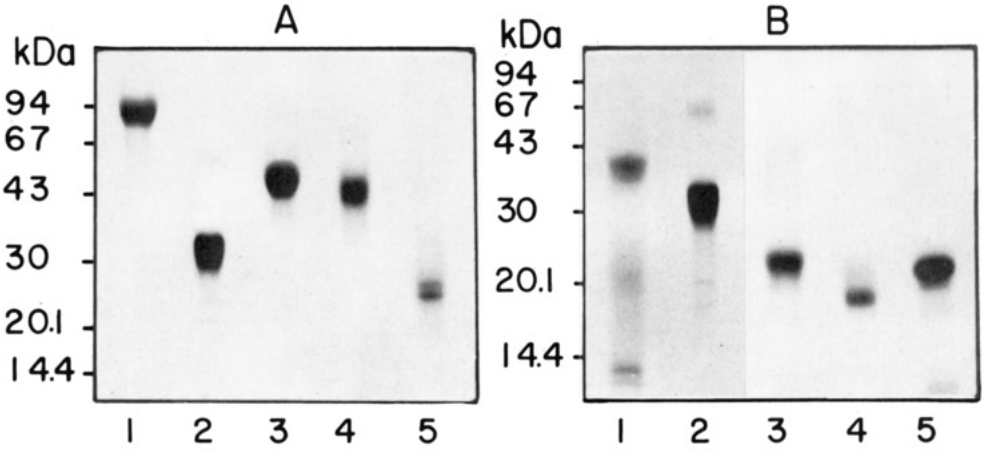

FIGURE 4: SDS–PAGE of major proteolytic fragments of vWF. (A) Unreduced 8–16% gradient gel; (B) reduced 13% gel. Each gel was stained with Coomassie Blue. Markers (left) indicate the positions of molecular weight standards. The fragments are shown in the following order: lane 1, III-T2; lane 2, III-T4; lane 3, II-S1; lane 4, II-S2; lane 5, II-S3.

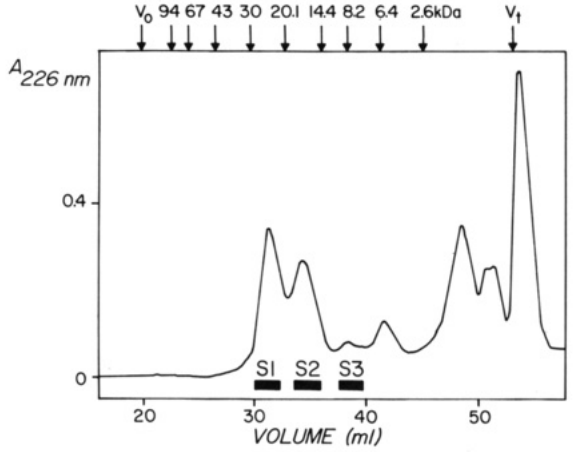

FIGURE 5: Primary separation of the subtilisin digest of unreduced fragment II (10 nmol) on the same HPLC sizing system as in Figure 1. Fractions S1 and S2 were subsequently desalted and subfractionated on an Ultrapore RPSC column; fraction S3 was similarly purified on a SynChropak RP-8 column.

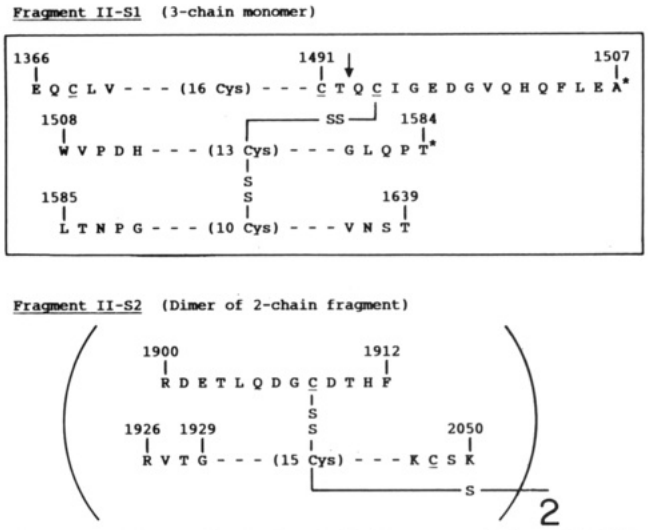

FIGURE 6: Polypeptide chains within fragments II-S1 and II-S2. Residue numbers and sequences are from Titani et al. (1986). Cys residues are designated either by an underlined C in the sequence or in groups within an unwritten sequence. Positions of oligosaccharide chains are not illustrated here but are indicated in Figure 2. Asterisks indicate chain ends that have not been rigorously identified. Fragment II-S3 is not specifically illustrated but was shown to be a single-chain monomer of residues 1366–1492 (ending at the arrow in the upper diagram). This corresponds to a shorter form of the upper chain in II-S1 and provides the evidence that Cys-1494 in II-S1 is in disulfide linkage to one of the other two chains.

terminal part of fragment II. Sequence analysis of the tryptic peptide produced by cleavage at Lys-1622 led to the assignment of Thr-1639 as the carboxyl-terminal residue of fragment II-S1. These data suggest that fragment II-S1 covers residues 1366–1639 of vWF but the carboxyl termini of two of the three chains were not rigorously established. The molecular weight calculated from this sequence, including the five carbohydrate chains (Table I), does agree with that obtained by SDS–PAGE under nonreducing conditions (Figure 4).

The fraction labeled S3 in Figure 5 contained a fragment that was also derived from the amino-terminal portion of fragment II. Sequence analysis revealed a single amino terminus at Glu-1366. Subdigestion of the native fragment II-S3 with cyanogen bromide produced two additional sequences, originating from cleavage at Met-1438 and Met-1480. The resulting three peptides were isolated following reduction and S-carboxymethylation. Sequence analysis of the only peptide lacking homoserine identified Thr-1492 as the carboxyl terminus of fragment II-S3. Comparison of II-S3 with the longest of the three chains in II-S1 (residues 1366–1507) reveals one less Cys residue in II-S3 than in the corresponding chain of II-S1 (Figure 6). That residue, Cys-1494, must form a disulfide bond to one of the other two chains in II-S1; otherwise, II-S3 would have had a second disulfide-linked chain.

A fragment containing the carboxyl-terminal region of vWF was purified from fraction S2 (Figure 5) by RP-HPLC. Three amino termini were observed, corresponding to Arg-1900, Arg-1926, and Gly-1929. Treatment of fragment II-S2 with CNBr and subsequent sequence analysis showed additional sequences, due to cleavage at four internal Met residues. After reduction of the disulfide bonds, all resulting peptides were separated on a SynChropak RP-8 column and characterized by amino acid composition. They corresponded to residues 1900–1912, 1926–1946, 1929–1946, and the four CNBr peptides closest to the carboxyl terminus [M39 through M42 of Titani et al. (1986)]. The peptide with Arg-1900 at its amino terminus was sequenced to its carboxyl terminus. Thus, fragment II-S2 was unambiguously identified as a two-chain segment consisting of residues 1900–1912 and either 1926–2050 or 1929–2050 of vWF. The molecular weight of this fragment, as calculated from the sequence including carbohydrate (18K), is in reasonable agreement with the value obtained by SDS–PAGE under reducing conditions. Under nonreducing conditions, however, the molecular weight is

approximately 43K (Figure 4), thus revealing that in its native form fragment II-S2 is a homodimer containing two pairs of chains.

Retained Binding Functions. vWF has been reported to bind coagulation factor VIIIc, collagen, and heparin as well as glycoproteins Ib and IIb/IIIa. Although not the subject of the present study, evidence of retained binding functions is being sought among the various fragments of limited proteolysis, both in this laboratory and elsewhere.

For example, fragment III has been reported to bind platelet glycoprotein Ib (Girma et al., 1986a,b), to bind collagen, to prevent platelet adhesion to collagen (Sakariassen et al., 1986), and, most recently, to bind coagulation factor VIIIc (Foster et al., 1987). As these studies are extended to smaller fragments, binding domains will become more closely defined. Foster et al. (1987) have shown that fragment III-T4 (25 μg/mL) completely inhibits the binding of factor VIIIc to vWF, whereas III-T2 (100 μg/mL) has no effect. In contrast, fragment III-T2 (100 μM) inhibits the binding of vWF to glycoprotein Ib in the presence of ristocetin (Y. Fujimura and T. S. Zimmerman, personal communication). Analogous measurements are the subject of separate detailed investigations.

Discrimination between Intra- and Intersubunit Disulfide Linkages. Since vWF contains both intra- and intersubunit disulfide bonds, definition of domains within the subunits and of contacts between subunits must discriminate between these two types of bonds. In general, this discrimination is not simple. For example, although intrasubunit bonds may appear to be indicated when an even number of Cys residues is found in an isolated peptide displaying a single amino acid sequence, even in this case it is necessary to demonstrate that the molecular weight of the peptide precludes the possibility of two copies of that chain linked by disulfides contributed from sibling subunits. Such evidence supports the monomeric nature of fragment I (Girma et al., 1986a) and of fragment II-S3 (Table I), both of which must contain only intrasubunit disulfide bonds.

The difficulty of discrimination is compounded if an isolated fragment contains more than one peptide chain, even when it is established that a single copy of each chain accounts for the combined molecular weight as for fragment III-T4 and II-S1 (Table I), because each constituent chain does not necessarily arise from the same parent subunit. In the specific case of III-T4, Hamilton et al. (1985) have isolated from a plasmic digest of vWF an amino-terminal fragment of about 298 residues that includes all four chains of III-T4 in a single chain. One can therefore conclude that all of the disulfide bonds in fragment III-T4 are of the intrasubunit variety. In the case of fragment II-S1, its three chains could be from different subunits; however, all of the Cys located between residues 1368 and 1491 form intrasubunit disulfides in II-S3 and must do so also in II-S1. Cys-1494 must form an interchain disulfide, but it is not clear whether it is linked to a chain derived from the same subunit or from a neighboring subunit.

Fragments of proven dimeric character clearly have some intersubunit bonds. If there is an odd number of Cys per monomer, one intersubunit bond could suffice (e.g., in fragment II-S2); if there is an even number of Cys per monomer, there must be at least two such bonds (e.g., in III-T2). If a peptide displaying a single sequence, and containing one Cys, is isolated from a dimeric fragment, this would identify a symmetrical intersubunit disulfide bond linking identical chains. If a peptide of single sequence containing two Cys is isolated from a dimeric fragment, it is necessary to show (e.g., by mass spectrometry) whether the molecular weight corresponds to one chain with an intrachain bond or to two identical chains sharing two intersubunit bonds. These considerations place limits on the extent to which data concerning individual disulfide placements in dimeric fragments III-T2 and II-S2 can be interpreted. No general procedure is known to distinguish their intersubunit from their intrasubunit disulfides. In what follows, the simpler monomeric fragments are considered first.

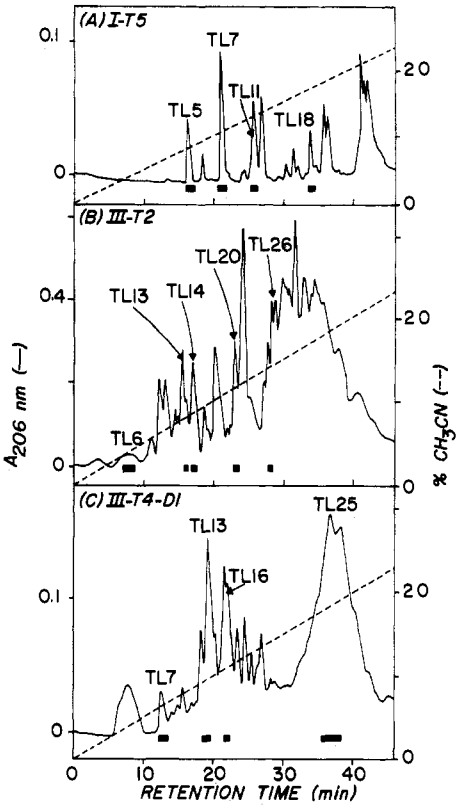

FIGURE 7: RP-HPLC separations of thermolytic peptides derived from (A) fragment I-T5 (4 nmol), (B) fragment III-T2 (6 nmol), and (C) fragment III-T4-D1 (8 nmol). All separations were performed on a SynChropak RP-P (C18) column (4.1 × 250 mm) with a TFA/acetonitrile system. Cys-containing fractions were detected according to the procedure of Sueyoshi et al. (1985) and rechromatographed to purity on a Hypersil ODS (2.1 × 100 mm) or an Aquapore RP-18 column (2.1 × 30 mm).

Assignment of Individual Disulfide Bridges in V8 Fragment I. Of the 18 Cys in this monomeric segment, 1 disulfide bond has already been placed between Cys-923 and Cys-1109 in fragment III-T6. This was confirmed, and two more disulfides were placed in unreduced fragment I by cleaving first with CNBr and then with trypsin. Cleavage with CNBr generated a core fragment comprising five chains disulfide linked to each other and containing all nine intrachain bonds. Digestion with trypsin and separation by SE-HPLC gave rise to peptides I-M-T8, I-M-T9, and I-M-T10 (Figure 1B), each of which had a single disulfide bridge (Table II). The remaining 12 Cys of fragment I were present in fragment I-M-T7. A similar core fragment, I-T5, identical with III-T5 (Figure 1A), was also isolated after direct tryptic treatment of fragment I. Subdigestion of I-T5 with thermolysin led to four more peptides (Figure 7A), three of which (e.g., I-T5-TL5) contained unique disulfides (Table II).

Evidence of the identity of the various small disulfide-linked peptides was of several types. In all cases, amino acid compositions and Edman degradation data permitted their location in the sequence of vWF, even when two or three chains were cross-linked. Since the cystine residues were not reduced,

Table II: Degradation Data and Identification of Disulfides in Fragment I of vWF

Peptide	Structural Data[a]	Disulfide Bond Cys-Cys
I-M-T8[b]	GLQIPTLspapdxsqpldvi LXSGFVR	923-1109
I-M-T9	EQDLEVILHNGAxspgar QGXm	1230-1238
I-M-T10	LTGSXSYVLFqnk TLVQEWTVQRpgqtxqpile	1209-1360
I-T5-TL5	VCT LCG	1187-1322
I-T5-TL7	IXMDEDgnekrpgd VTCQPDgqt	1116-1141
I-T5-TL11	LRPSCPNSQSP IXDENGAND	1164-1325
I-T5-TL18	TLPDQXHT VKVEETCGCRW VNXDRG	1136-(1177 or 1179)-1156

[a] Sequences in capital letters indicate the results of Edman degradation of unreduced peptides; those in lower case letters denote amino acid compositions. X denotes a Cys residue expected from the known sequence but not observed; C denotes the di-PTH of cystine. [b] Only seven cycles of the Edman degradation are illustrated. The upper chain contained 37 residues.

Edman degradations in the Applied Biosystems sequencer did not yield a PTH from a half-cystine residue *until* its other half was also released. In Figure 8, a simple case is illustrated where both halves of the cystine residue are reached simultaneously in the degradation and a major product is seen in the tyrosine position, in addition to a minor product (marked with an asterisk) corresponding to the DTT adduct of dehydroalanine. The ratio of these 2 signals changed from approximately 10:1 to 1:1, when PTH–cystine was released after 10 degradation cycles. The yield of PTH–cystine was generally 40% in the early cycles of an analysis (as in Figure 8), but it decreased to approximately 8% by 10 cycles. Both observations are probably the result of slow breakdown of cystine at the high pH of the coupling reaction in the Edman degradation. It should be noted that these sequence analyses were performed in the Applied Biosystems Model 470A sequencer where reducing agents do not enter the reaction cartridge. DTT is introduced later (in TFA) during conversion to the PTH, but reduction would not be expected under these acidic conditions. The product is analyzed immediately after conversion in the on-line HPLC system, again in acid. Controls indicated that the major product (Figure 8) is the di-PTH of cystine.

The fourth peptide from the thermolytic digest, I-T5-TL18, contained four Cys residues among three peptide chains (Table II). Each of the Cys residues (1177 and 1179) in the chain bearing two must form a disulfide bond with one other chain, but it is not clear whether, for example, Cys-1136 is paired with Cys-1177 or with Cys-1179.

The ninth disulfide bond in fragment I should have been found in the tetrapeptide Thr-Cys-Pro-Cys (residues 1182–1185) as a result of the anomalous thermolytic cleavage of a Trp–Thr bond observed in I-T5-TL18 at residue 1181. However, this peptide was not recovered from the digest, and the pairing of Cys-1183 and Cys-1185 is deduced by difference after the assignment of all other Cys in fragment I to disulfide bonds (Figure 9).

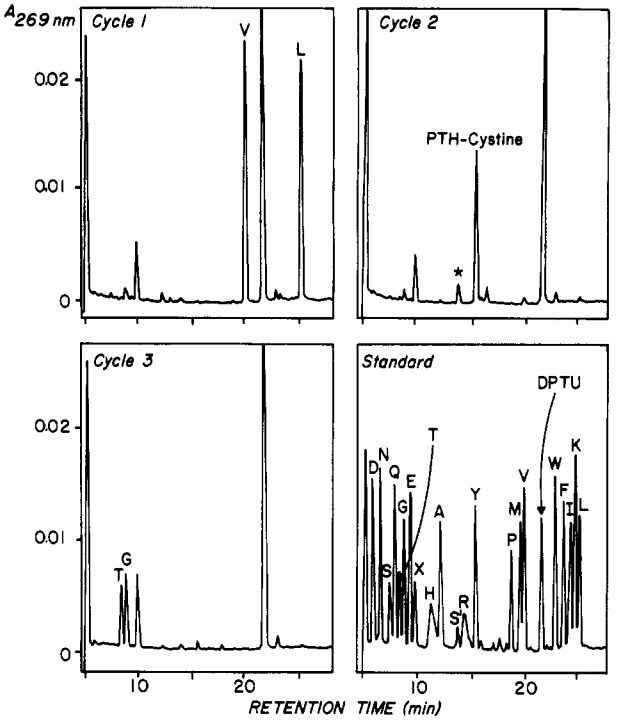

FIGURE 8: Identification of the di-PTH of cystine in the second cycle of Edman degradation of peptide I-T5-TL5 (Table II). The two chains Val-Cys-Thr and Leu-Cys-Gly degrade simultaneously, releasing the PTH of both Val and of Leu in cycle 1. Each chain releases half-cystine in the second cycle. The height of the PTH–cystine peak in cycle 2 is 37% of the sum of PTH–Val and PTH–Leu in cycle 1.

Assignment of Individual Disulfide Bridges in V8 Fragment III. In addition to the region represented by fragment I (residues 911–1365) and fragment III-T6 that overlapped it, dimeric fragment III was the source of fragments III-T4 and III-T2 (Figure 2). Together, these 4 disulfide-containing fragments comprise 1026 residues (of 1365 per monomer of fraction III) that include all 74 Cys residues of fragment III (Table I).

Of the four fragments, only III-T2 was found to be dimeric (Table I). Fragments III-T5 (within fragment I) and III-T4 (the amino-terminal segment) must contain only intrachain bonds. Fragment III-T6 contains two disulfides, one of which is already placed in the overlapping fragment I. The other, involving Cys-906 and Cys-907, could form either two interchain bonds or one intrachain bond. Although intrachain disulfide bonds between adjacent Cys residues are both rare and strained [e.g., see Kao and Karlin (1986)], one appears to exist in this case. Evidence in support of this contention was derived by FAB mass spectrometry of a derivatized V8 digest of fragment III-T6. The fragment (2 nmol) was treated with 1 μg of V8 protease in 1% NH_4HCO_3 for 2 h and then dried and esterified in ethanolic 1 M HCl (Naylor et al., 1986). A positive MH^+ ion of mass 552 was seen, corresponding to the diethyl ester of Cys-Cys-Ser-Gly-Glu with an intramolecular disulfide bond, but none at 1103, the predicted mass of the peptide with interchain bonds.

Fragment III-T4 is monomeric and must contain 12 intrachain disulfide bonds. Seven of these were placed in the following manner. The fragment was first cleaved at Asp residues with dilute formic acid and separated by size on TSK columns (not shown). A major fraction of apparent molecular weight 7K was further resolved by RP-HPLC on a Syn-Chropak RP-8 column. Cleavage at Asp-101 had produced an amino-terminal (D1) and a carboxy-terminal (D2) segment, containing eight and four disulfide bonds, respectively. Sub-

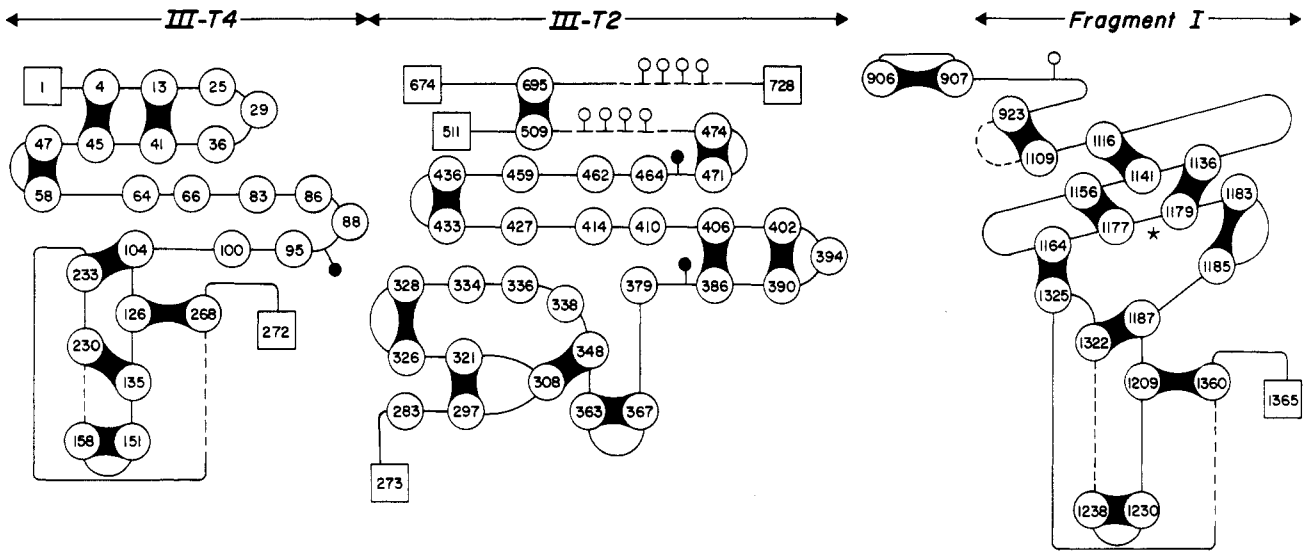

FIGURE 9: Disulfide pairing topology of substructural domains derived from fragment III (cf. Figure 2). Circled numbers denote Cys residue numbers in the vWF sequence. Twenty-six disulfide bridges are indicated by thick columns joining pairs of Cys residues. Unidentified disulfide bridges join all other Cys within the various fragments defined in Figure 2 and Table I. The asterisk indicates that it is not clear whether Cys-1177 (or Cys-1179) is paired with Cys-1136 or Cys-1156. The peptide chain is indicated by a solid line if 25 residues or less and a dashed line if more than 25 residues are present between neighboring Cys. Numbers in squares denote chain ends. Open circles and closed circles indicate O-linked and N-linked carbohydrate chains, respectively. Only the middle fragment (III-T2) is dimeric, but a single monomer is displayed. It is possible that some of its illustrated intrachain disulfides are in fact intersubunit disulfides.

Table III: Degradation Data and Identification of Disulfides in Fragment III of vWF

Peptide	Structural Data[a]	Disulfide Bond Cys-Cys
III-T4-D1-TL7	LVCPA | GX	13-41
III-T4-D1-TL13	LXPPGM | VRHENRC	47-58
III-T4-D1-TL16	SLSCRPPM | VSGC	4-45
III-T4-D2-TL14	LVGNKGCSHPS | VKXKKR	151-158
III-T4-D2-TL23	ATCST | LXGNF	104-233
III-T4-D2-TL33	LFPGECQY | VSSQXA	126-268
III-T4-TL22	LVQDYCGSNPGT | VXG	135-230
III-T6-E[c]	∩ (CCSGE)[d]	906-907
III-T6[e]	∩ XCSGEGLQIPTLSPAPDXSQ | IEDLPTMVTLGNSFLHKLCS	906-907 923-1109
III-T2-D6a	SSCRILTS | VXIY	297-321
III-T2-D6b[b]	FYXSR | eivsylc	509-695
III-T2-TL6	AXQ | LACP	390-402
III-T2-TL13a	YNSCAP | VQX	386-406
III-T2-TL13b	⌐¬ LXPQSCEKRn	363-367
III-T2-TL13c	VFQDCNK | VXA	308-348
III-T2-TL14	⌐¬ IYDIXSCES	326-328
III-T2-TL20	⌐¬ LTXEACQEpgg	471-474
III-T2-TL26	⌐¬ VDPEDXPVCE	433-436

[a] Sequences in capital letters indicate the results of Edman degradation of unreduced peptides; those in lower case letters denote amino acid compositions. X denotes a Cys residue expected from the known sequence but not observed; C denotes the di-PTH of cystine. [b] Glu-689 was blocked. [c] Separated by FAB mass spectrometry as the diethyl derivative after treatment of III-T6 with V8 protease. [d] Determined as mass 552. [e] Only 20 cycles of Edman degradation are illustrated. The upper chain is 43 residues long, the lower one 24 residues.

fragmentation of D1 with thermolysin yielded (Figure 7C) three peptides, TL7, TL13, and TL16, each containing a single disulfide bridge (Table III). The remaining 10 Cys residues of fragment D1 were present in peptide TL-25 (Figure 7C); they form a five-disulfide cluster which has not been resolved (Figure 9). In a similar manner, three disulfide bridges within fragment D2 were assigned (Table III) after thermolytic subdigestion (not shown). The remaining disulfide, connecting

Cys-135 and Cys-230 (Table III), was obtained by direct thermolytic digestion of fragment III-T4 (not shown).

Fragment III-T2 contained 30 Cys and presented the unique problem of its disulfide-linked, dimeric character. Nine of its disulfides are identified in what follows, but in each case, it is not clear whether the observed linkage is intrachain or interchain.

Fragment III-T2 was cleaved at Asp residues, and a 1-kDa fraction was separated as for III-T4 and then followed by RP-HPLC on a SynChropak RP-P column. Peptides were found to link Cys-297 to Cys-321 and Cys-509 to Cys-695 (Table III). The assignment of the latter bridge was complicated by an unexplained α-amino block at Glu-689, thus leaving only one visible sequence during Edman degradation. The amino acid composition of the reduced and carboxymethylated segment containing residues 689–695 clearly verified this disulfide linkage. Seven additional peptides, each containing a single disulfide, were obtained (Figure 7B) after prolonged treatment of III-T2 with thermolysin. Four of these peptides (TL13b, TL14, TL20, and TL26) each consisted of a single chain with two Cys residues (Table III), thus indicating disulfide bond formation between neighboring Cys residues.

Twelve Cys remain to be paired within III-T2, including three clusters of Cys separated by one to three residues (Figure 9). These appear to form networks of disulfides that were quite refractory to the resolution techniques applied.

Discussion

The extremely large size and the multivalent character of the von Willebrand factor molecule offer a challenge in attempts to locate specific domains responsible for its various binding functions. The multichain nature of the protein, the large number of disulfide bonds, and the high oligosaccharide content all complicate the problem of separating fragments of the protein that can be tested for isolated functions. In 1986, Girma et al. characterized fragments I, II, and III, the products of limited proteolysis of vWF by *S. aureus* V8 protease. Electron microscopic examination of these fragments by Fretto et al. (1986) identified the dimeric, rodlike nature ($\sim 2 \times 32$ nm) of the carboxyl-terminal fragment II (residues 1366–2050) and the dimeric, globular character ($\sim 6.5 \times 35$ nm) of the amino-terminal fragment III (residues 1–1365). Only the small fragment I (derived from the carboxyl-terminal end of fragment III), which also appeared to have a globular structure, was found to be monomeric by ultracentrifugal analysis, in agreement with our findings summarized in Table I.

With unreduced fragments, estimates of molecular weight from mobilities on denaturing TSK sizing columns (e.g., Figure 1A) were observed to be consistently smaller (55% ± 10%) than estimates based on Ferguson plots of SDS–PAGE data. Only the latter gave values in accord with calculations based on amino acid sequences, as indicated in Table I. It is not clear why the constraints of disulfide bonds should interfere more with diffusion-based sizing columns than with friction-based SDS–PAGE, but we relied on the latter for estimates of molecular weight.

In the present work, additional fragments of vWF were generated by limited proteolysis with either trypsin or subtilisin. Each fragment was characterized with regard to its location in the primary structure and its dimeric/monomeric character. In addition, 26 disulfide bonds have been placed, accounting for 70% of the Cys in the amino-terminal 65% of the molecule (Figure 9). It should be noted that all of the separated fragments (summarized in Figure 2) were obtained without the need for reduction of disulfide bonds. Thus, each of the monomeric fragments acts as an independent substructural domain that is not otherwise covalently attached to its parent chain. In several cases, these substructural fragments retain binding function. Since the fragments generated in the present study were obtained from parent fragments that had been precipitated with trichloroacetic acid, it appears that those with binding capacity must also retain the ability to renature after acid denaturation. In that sense, these fragments may be considered as independent functional domains derived from the single gene encoding vWF.

Several other fragments of vWF have been generated recently in other laboratories. Fujimura et al. (1986) have shown that a fragment of 48–52 kDa (after reduction) binds the platelet glycoprotein Ib. This fragment corresponded to residues 449–728, clearly overlapping our fragment III-T2 (Figure 2), which also binds platelet glycoprotein Ib. The only region common to both fragments comprises residues 449–511 and 674–728, which must therefore include the binding site for glycoprotein Ib. Recently, it has been reported that the reduced 52/48-kDa tryptic fragment also interacts with collagen (Pareti et al., 1986) and with heparin (Fujimura et al., 1987). That fragment was isolated from a TSK sizing column fraction containing larger fragments (of 76 and 120 kDa in unreduced SDS–PAGE), but it is not known whether their parent fragment was a dimer in the unreduced state or whether two dissimilar chains are united by an intrasubunit disulfide bond. It is also not clear why tryptic digestion gave such different fragments in the two laboratories, but it should be noted that the experiments used different starting material (native protein vs. trichloroacetic acid denatured fragment III) and quite different vWF:trypsin ratios.

A monomeric fragment denoted P34 was generated by digestion of the native multichain protein with plasmin (Hamilton et al., 1985). Its amino terminus was identical with that of the native protein, and it was estimated to contain 298 residues (Fretto et al., 1986), thus completely overlapping our fragment III-T4. Since the latter binds factor VIIIc, it is likely that P34 will also, although Hamilton et al. (1985) did not test this possibility.

Other fragments of vWF have been generated by Sixma et al. (1984), Girma et al. (1986b), Houdijk et al. (1986), and Sakariassen et al. (1986) and characterized largely by immunological techniques, by retention of function, and by SDS–PAGE. Since the precise location of these fragments within the monomeric chain has not been established, it is not possible to compare those fragments directly with those described herein.

In addition to demonstrating that limited proteolytic fragments could be separated from each other with retention of binding function, we have identified disulfide cross-links within fragments derived from the amino-terminal 1365 residues (fragment III). Of the 74 Cys residues in that segment, 52 have been placed in disulfide bonds. They impose restrictions on the folding alternatives, as illustrated in Figure 9. The potential for complexity within the disulfide network among 169 Cys residues per monomer and the unidentified intersubunit disulfide linkages made this task a formidable one. However, it is clear from the ease with which the molecule can be dissected into substructural fragments that most of the disulfide bonds tend to link local (<170 residues) rather than more distant parts of the same polypeptide chain. In retrospect, this might have been predicted from the rodlike structure of the carboxyl-terminal segment of the molecule. It is interesting that the 1365 residues in the globular part of the

molecule (fragment III) could be readily divided into three large domains, III-T4, III-T2, and fragment I, each of which had multiple intrachain disulfide bonds and only one of which (III-T2) must have interchain linkages to sibling chains.

The patterns of disulfide linkages displayed in Figure 9 were compared with other large disulfide-cross-linked plasma proteins for common structural motifs as, for example, in fibronectin and factor XII (Skorstengaard et al., 1984; McMullen & Fujikawa, 1985). As yet, we have not observed structures corresponding to the growth factor and kringle domains of factor XII or the type I and type II structures of fibronectin, suggesting that vWF possesses a novel domain organization.

It has been proposed that the evolutionary history of vWF included several gene duplication events and divergent evolution of partially repeating structures of five different types (Sadler et al., 1985; Shelton-Inloes et al., 1986). At the present stage of our analysis of the disulfide network, it appears that the proposed repeats in structure do not correspond to the substructural domains that are excised by limited proteolysis. Nonetheless, three disulfide bonds in homologous domains D1 and D2 (residues 104–296 and 1187–1374) are paired between corresponding residues (Figure 9), lending support to the proposal of the divergence of duplicated regions. Moreover, each of these two homologous domains contains an internal protease-sensitive locus in a corresponding position (residues 272–273 and 1365–1366). These are the sites of separation of fragments III-T4 from III-T2 and of V8 fragments III (and I) from II, respectively. A similar comparison of repeating regions A1, A2, and A3 is not possible because the six Cys residues among the three regions do not align with each other. These six Cys residues, however, show an unusual disulfide bonding pattern, i.e., Cys-509 to Cys-695 and Cys-923 to Cys-1109, each closing a large loop of precisely 185 amino acids. In contrast, adjacent residues Cys-906 and Cys-907, which are between these loops, form an intrachain disulfide bond. Such an intrachain bond is very unusual [e.g., see Kao and Karlin (1986)], and it is not clear whether it has any structural or functional significance.

Attempts to place disulfide bridges are also hindered by the presence of numerous clusters of Cys residues. For example, there are 8 Cys-Cys, 22 Cys-X-Cys, and 16 Cys-X-Y-Cys sequences, most of which are resistant to proteolytic separation into unique peptides containing single disulfide bonds. Moreover, intersubunit disulfide bonds cannot be readily distinguished from intrachain disulfide bonds, yet this distinction is important in defining the relationship of sibling chains to each other. It is fortunate that several proteolytic segments of vWF have proven to lack intersubunit bonds. However, two large dimeric segments (III-T2 and II-S2) have an undetermined number of intersubunit bonds. It should be noted that electron microscopy has indicated that a region near the carboxyl terminus of fragment II does form an interchain linkage (Fretto et al., 1986). This is consistent with our observation that fragment II-S2 is dimeric. It remains to be determined which of the Cys in each half of the dimer contributes to the cross-links.

In the course of these experiments, we encountered little difficulty with disulfide exchange. In fact, this was observed only in one case, i.e., in a thermolytic digest that generated small disulfide-linked peptides. This was subsequently avoided by decreasing the temperature, increasing the concentration of thermolysin, and, most importantly, adding iodoacetate to scavenge thiolate ions. Disulfide exchange did not appear to be important at the higher pH of the tryptic and subtilisin digests, probably because it was hindered by the relatively rigid three-dimensional structure of these large products of limited proteolysis.

We are continuing attempts to place the remaining disulfides within fragment III-T4 and, by mass spectrometric procedures, to define more completely the disulfide bonds in the dimeric fragment III-T2. An even more challenging analysis remains in fragment II, where 95 Cys are expected to form a complex disulfide network consistent both with its rodlike dimeric structure and with the independence of its substructural segments II-S1 and II-S2. As with III-T2, the C-terminal dimeric segment presents the additional complication of distinguishing between inter- and intrasubunit disulfide bonds. As this information accumulates, it should become possible to delineate the boundaries of the functional domains, the constraints restricting their folding patterns, and the specific covalent linkages between the several polypeptide chains of these multivalent molecules.

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