Determination of the Disulfide Bridges in Factor Va Heavy Chain†

Jiachun Xue,‡ Michael Kalafatis, Jay R. Silveira, Chun Kung,§ and Kenneth G. Mann*

Department of Biochemistry, Given Building, Health Sciences Complex, University of Vermont, College of Medicine, Burlington, Vermont 05405-0068

Received January 20, 1994; Revised Manuscript Received June 29, 1994®

ABSTRACT: The M_r = 94 000 heavy chain of bovine factor Va contains 10 cysteine residues which are distributed in the 2 A domains which make up this portion of the factor V molecule. The A1 domain contains four cysteines while the A2 domain contains six cysteines. The locations of disulfide bridges and free cysteines in bovine factor Va heavy chain were analyzed using iodo[^{14}C]acetamide-labeled factor Va heavy chain digested with trypsin, plasmin, V-8 protease, and cyanogen bromide. Following HPLC separation of the resulting peptides, free cysteines were identified by the incorporation of radioactivity while disulfide-containing peptides were detected using an SBD-F fluorometric assay after reduction. All cysteine–containing peptides were analyzed by amino acid sequence analysis. The four cysteines in the A1 domain are associated with two disulfide bonds, Cys_{139}–Cys_{165} and Cys_{220}–Cys_{301}. One disulfide bond was explicitly identified in the A2 domain; Cys_{471}–Cys_{497}, and a free cysteine was found in the A2 domain at Cys_{538}. Significant difficulties were encountered in preparing identifiable or soluble peptides which would permit the explicit identification of the three remaining cysteines in the A2 domain. On the basis of homology, it is likely that Cys_{589} is a free SH while a disulfide bridge exists between Cys_{579} and Cys_{660}. Thus, three major disulfide bonding patterns, characterized as "α", "β", and "γ" loops, are found in factor V. Each A domain contains a 26 residue "α loop at positions 139–165, 471–497, and 1684–1710. The A1 and A2 domains each contain 81 amino acid residue "β" loops at 220–301 and 579–660. The C domains contain 2 "γ" loops of 154 (C1) and 155 (C2) residues, respectively, 1866–2020 and 2025–2180.

Factor V is an M_r = 330 000 single-chain glycoprotein present in plasma and platelets which is an essential component of the blood coagulation cascade (Owren, 1947; Mann et al., 1981, 1988; Kane & Davie, 1988). Factor V is activated to its active form, factor Va, by thrombin and factor Xa (Nesheim & Mann, 1979; Nesheim et al., 1984; Suzuki et al., 1982; Foster et al., 1983; Monkovic & Tracy, 1990). Factor Va serves as a cofactor in the *prothrombinase* complex which catalyzes the activation of prothrombin to thrombin (Esmon, 1979; Nesheim & Mann, 1979; Krishnaswamy et al., 1989, 1993).

The complete amino acid sequences of human and bovine factor V have been deduced from the cDNAs of the molecules (Jenny et al., 1987; Kane & Davie, 1986, 1987; Guinto et al., 1992). Human and bovine factors V contain 2196 and 2183 residues, respectively. During the activation process, a central "B" domain which accounts for ~40% of the factor V mass is excised, and the resulting bovine factor Va product consists of a heavy chain (M_r = 94 000) containing two A domains (A1–A2) which is nonconvalently associated, in the presence of divalent cations, to a light chain (M_r = 74 000). The light chain contains one A domain and two C domains (A3–C1–C2). Bovine and human factors Va share approximately 85% identity. Factor V is also homologous to plasma factor VIII with respect to the organization of A, B, and C domains. There is ~40% sequence identity among the A and C domains of human factor Va and human factor VIIIa. In addition, the A domains of both factor Va and factor VIIIa exhibit ~36% identity with the three conserved A domains present in the copper-containing protein ceruloplasmin. Factor Va and factor VIIIa have been reported to contain copper (Mann et al., 1984; Bihoreau et al., 1993).

Bovine and human factors Va each contain 18 cysteine residues: 10 in their heavy chains and 8 in their light chains. In contrast, human factor VIIIa has 19 cysteine residues: 5 in each A domain and 4 in the C1 and C2 domains of the light chain (Toole et al., 1984; Vehar et al., 1984). Comparisons between human factor VIIIa and human ceruloplasmin (Ortel et al., 1984) have led to the suggestion of the locations of the disulfide bonds present in these proteins which, in turn, have led to the suggestion of disulfide bonds in factor Va. Recently we reported the bovine factor Va light-chain disulfide bridges and free cysteine locations. In the present work, we report the locations of cysteines and disulfide bonds in bovine factor Va heavy chain.

EXPERIMENTAL PROCEDURES

Materials

Staphylococcus aureus V-8 protease and trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone were obtained from the Worthington Biochemical Corp., Freehold, NJ. Solutions (1 mg/mL) of trypsin and V-8 protease were prepared in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, and 50 mM sodium phosphate buffer, pH 7.8, respectively. Human plasmin, a gift from Dr. Tom Orfeo, University of Vermont, Department of Surgery, was dissolved in 50 mM potassium phosphate, pH 7.0, at a concentration of 7.5 μM and stored at −20 °C. Iodo[1-^{14}C]-acetamide (57.6 mCi/mmol, 308 μCi/mg) was purchased from Amersham. Ammonium 7-fluoro-2,1,3-benzoxadiazole-

† This work was supported by Merit Award R37 HL34575 and Training Grant T32 HL07594 from the National Institutes of Health.

* To whom correspondence should be addressed.

‡ Present address: Howard Hughes Medical Institute, Research Laboratories, Washington University School of Medicine, Saint Louis, MO 63110.

§ Present address: Department of Medicine, Washington University School of Medicine, Jewish Hospital of Saint Louis, Saint Louis, MO 63110.

® Abstract published in *Advance ACS Abstracts,* October 1, 1994.

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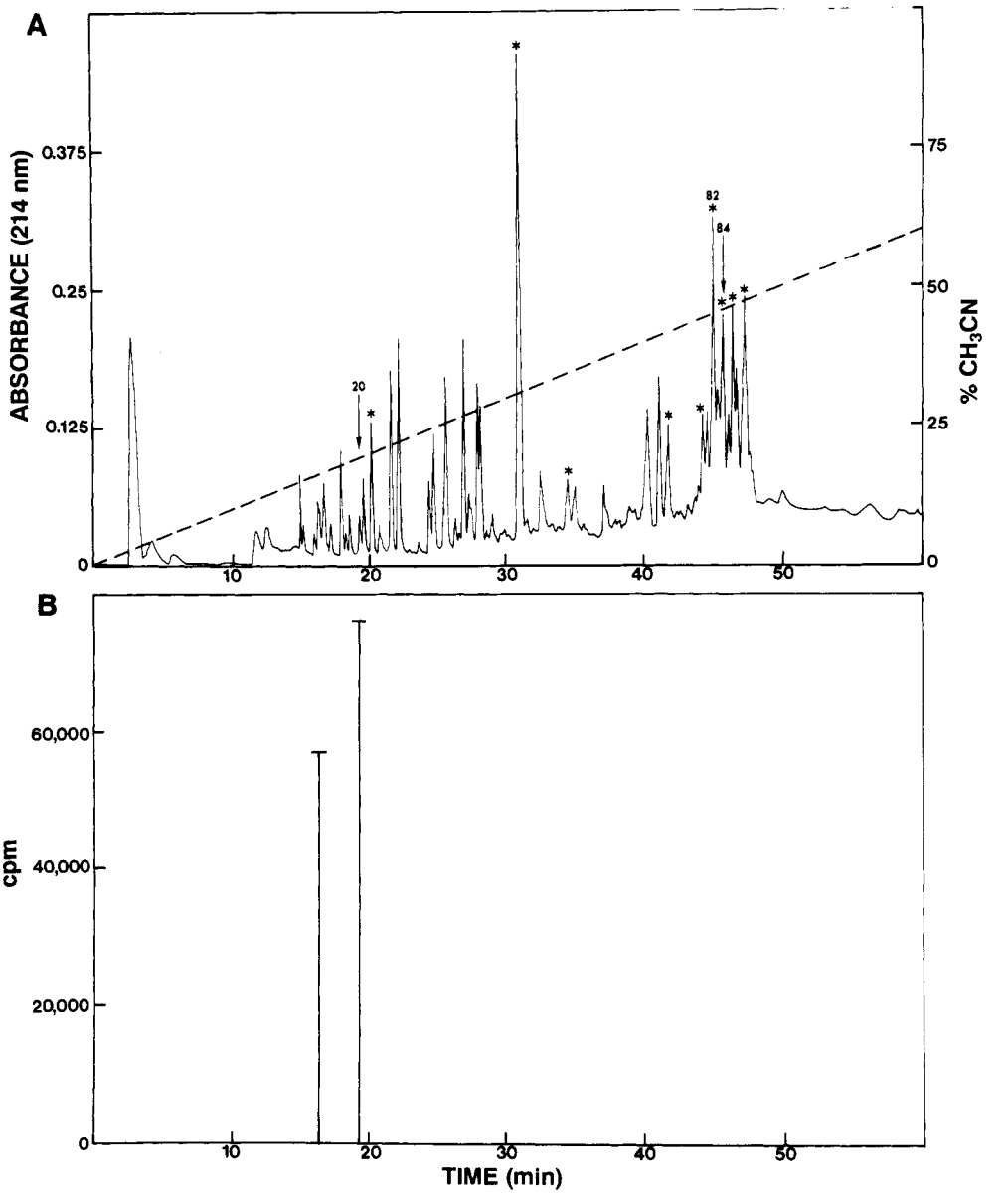

FIGURE 1: C-18 HPLC chromatogram of trypsin-digested ^{14}C-labeled bovine factor Va heavy chain (panel A) and the corresponding radioactive peaks (panel B). The HPLC linear elution gradient was from 0–60% solvent B over a period of 60 min. The asterisks indicate peaks that tested positive in the SBD-F fluorometric assay.

4-sulfonate (SBD-F) was purchased from Wako Chemicals, Inc., Dallas, TX. Oxidized glutathione, *N*,*N*-dimethylacetamide, and cyanogen bromide (CNBr) were obtained from Sigma Chemical Co., St. Louis, MO. Tributylphosphine was purchased from Johnson Mattey Electronics, Ward Hill, MA.

Ultrapore C-3 (4.6 mm × 75 mm) and reverse phase C-18 (4.6 mm × 250 mm) HPLC columns were purchased from Beckman and J. T. Baker Inc., respectively. TSK-Gel G2000SWxl (7.8 mm × 30 cm) HPLC columns were purchased from Tosohaas Inc., (Philadelphia, PA). HPLC-grade water and acetonitrile (CH_3CN) were obtained from J. T. Baker Inc. Reagents and solvents for amino acid sequence analyses were obtained from Applied Biosystems, Inc. All other commercial chemicals were of the highest grade commercially available.

Bovine factor V was isolated and activated by α-thrombin as reported (Nesheim et al., 1981; Krishnaswamy et al., 1986). Factor Va was isolated using a modified method of Odegaard and Mann (1987). The isolation of the heavy chain made use of an anti-light-chain immunoadsorbent column (Odegaard & Mann, 1987). Purified factor Va was applied to the column and the initial flow-through peak and buffer wash were collected. The buffer was changed to 5 mM ethylenediaminetetraacetic acid (EDTA) instead of $CaCl_2$, and the heavy chain was eluted. The column was then washed with 1.7 M NaCl to elute the light chain. The purified heavy and light chains were stored at −20 °C in 50% (v/v) 20 mM Hepes (pH 7.4)/glycerol. All proteins and their components were routinely characterized through SDS–PAGE analyses of the purified products. (Laemmli, 1970).

Methods

Cysteine Identification. [^{14}C]Carboxamidomethyl-labeled heavy chain was prepared by a modification of that of Crestfield et al. (1963). Heavy chain in 50% (v/v) glycerol/20 mM Hepes, pH 7.4, was dialyzed against 0.2 M acetic acid at 4 °C overnight and lyophilized. The lyophilized heavy chain was resuspended in 6 M guanidinium chloride/0.2 M Tris-HCl, pH 8.6, containing 2 μM iodo[^{14}C]acetamide (57.6 mCi/mmol, Amersham) for 15 min in the dark at room temperature. The pH was readjusted to 8.6 by the addition of 2.5 M Tris. Iodoacetic acid was added to 10 mM, and the

Table 1: Sequence of NH_2-Terminal Amino Acid Residues of the Tryptic Peptide Contained in Peak 20 of Figure 1

cycle no.	peak 20		heavy chain[c] 537–543
1	F	(174.5)[a]	F
2		ND[b]	C
3	E	(93)	E
4	N	(96.5)	N
5	P	(85.5)	P
6	E	(63.5)	E
7	K	(33)	K

[a] The number in parentheses indicates picomoles at the given cycle. [b] Not determined. [c] From Guinto et al. (1992).

mixture was incubated for another 15 min in the dark to ensure alkylation of free cysteine residues. The reaction was terminated by the addition of acetic acid to drop the pH to about 6. The labeled sample was dialyzed against 0.2 M acetic acid until no radioactivity could be detected in the dialysate. The dialyzed sample was lyophilized and stored at −20 °C.

SBD-F Assay and Peptide Preparation. Cystinyl peptides were detected by a modified procedure of Sueyoshi et al. (1985; Xue et al., 1993). In this assay, disulfide bridges are cleaved with tributylphosphine, and the thiol groups are coupled to the fluorescent reagent SBD-F. This simple and sensitive method is able to quantitatively detect peptide containing disulfides in the range of 20 pmol to 2 nmol. Under reduced conditions, aliquots of oxidized glutathione or unknown sample in 140 μL of 2.5 M borate buffer (prepared with boric acid and KOH), pH 9.5, containing 4 mM EDTA, 10 μL of SBD-F solution (0.4 mg/mL in water), and 2 μL of tributylphosphine (0.2 g/mL in dimethylacetamide) were vigorously mixed and heated at 60 °C for 1 h. The fluorescence intensities of the samples were measured at room temperature using an SLM-8000 photon counting fluorescence spectrophotometer. The excitation and emission wavelengths were set at 385 and 515 nm, respectively. For nonreduced conditions, the reducing agent tributylphosphine was replaced with borate buffer. All unknown samples were compared against a standard calibration curve, prepared using oxidized glutathione (20–2000 pmol), in order to determine the amount of cysteine present.

Trypsin and V-8 Protease Digestions. The ^{14}C-labeled heavy chain was dissolved in 1 mM HCl and the pH adjusted to 8 with the addition of 0.1 M Tris. The sample was digested with trypsin at an enzyme:substrate weight ratio of 1:100 at 37 °C for 24 h. The digested samples were applied to a HPLC C-18 column and eluted with a linear gradient of 0–60% solvent B over a 60 min period at 1 mL/min (where solvent A = 0.05% trifluoroacetic acid in H_2O and solvent B = 0.05% trifluoroacetic acid in CH_3CN). Each fraction was analyzed by the SBD-F fluorometric assay and counted using a scintillation counter (Beckman LS 6000IC). The radioactive and SBD-F positive peaks were further purified by C-18 HPLC column chromatography. Some assay positive peaks were further digested with *Staphylococcus aureus* V-8 protease in 0.1 M ammonium acetate, pH 4.0, at 37 °C for 24 h at an enzyme:substrate weight ratio of 1:100. The V-8-digested samples were applied to an HPLC C-18 column and eluted with a linear gradient of 0–55% solvent B over a 55 min period at 1 mL/min. After each peak was analyzed by the SBD-F fluorometric assay, the assay positive peaks were further purified by C-18 HPLC column chromatography. The absorbance of the effluent was monitored at 214 nm.

Table 2: Sequence of NH_2-Terminal Amino Acid Residues of the Tryptic Peptide Contained within Peak 82 of Figure 1

cycle no.	peak 82a		heavy chain[d] 485–498	peak 82b		heavy chain 456–474
1	D	(35)[a]	D		ND	W
2	L	(30.6)	L	N	(16.9)	N
3	A	(28.6)	A	I	(21.7)	I
4	S	(10.0)	S	L	(22.7)	L
5	G	(19.6)	G		ND	E
6	L	(26.4)	L	S	(6.1)	S
7	I	(21.2)	I	D	(10.8)	D
8	G	(15.4)	G	E	(4.5)	E
9	L	(15.4)	L	P	(6.8)	P
10	L	(20.2)	L	T	(2.5)	T
11	L	(20.6)	L	E	NQ	E
12	I	(14.0)	I	N	(4.0)	N
13		ND[b]	C	D	(5.3)	D
14	K	NQ[c]	K	A	(1.6)	A
15				Q	NQ	Q
16					ND	C
17				L	NQ	L
18					ND	T
19					ND	R

[a] The number in parentheses indicates picomoles at the given cycle. [b] Not determined. [c] Not quantitated. [d] From Guinto et al. (1992).

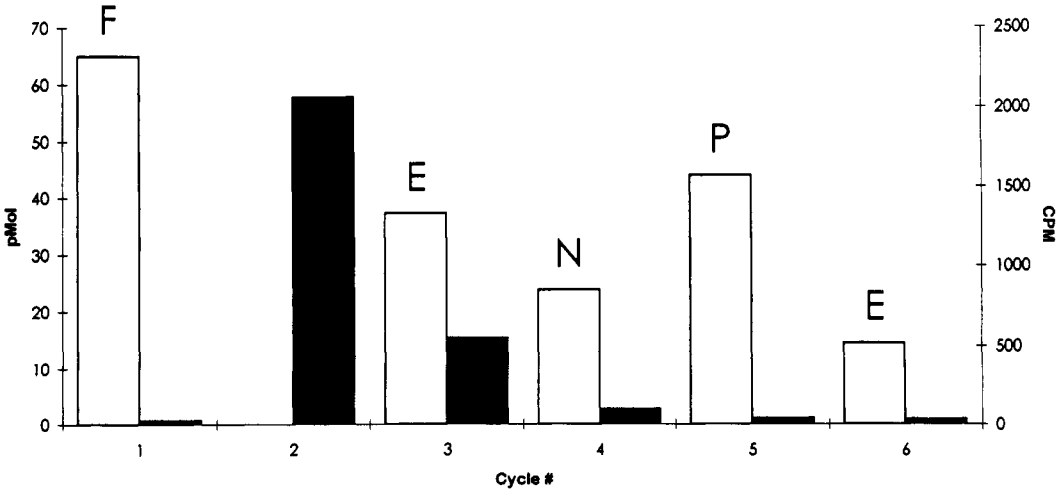

FIGURE 2: Sequence analysis of peptide 20 from Figure 1. NH_2-terminal sequence analyses were performed as described under Experimental Procedures. During sequencing, fractions were collected, and the radioactivity contained within each fraction was quantitated in a scintillation counter. *Open bars* indicate the picomoles of amino acid obtained per cycle whereas the *filled bars* represent the cpm found per fraction. The letters on the top of the *open bars* indicate amino acids identified after comparing the chromatogram obtained at each cycle with a standard chromatogram containing all amino acids except cysteine.

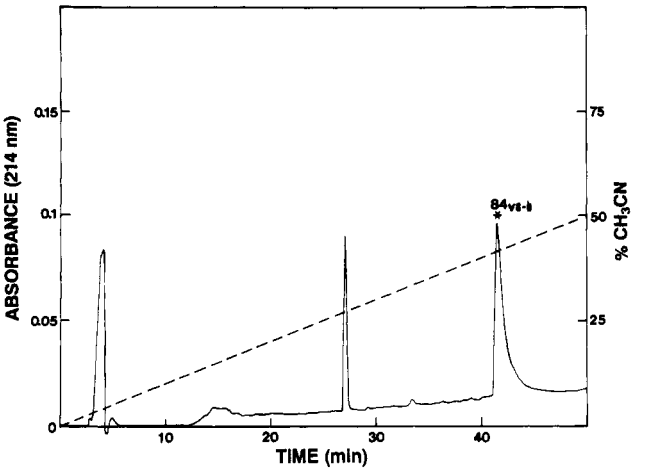

FIGURE 3: C-18 HPLC chromatogram of peak 84 from Figure 3 digested with V-8 protease. A linear gradient of 0–55% solvent B for 55 min was used to elute the column. The asterisk indicates the peak that tested positive in the SBD-F assay.

Plasmin, CNBr, and V-8 Protease Digestions. Bovine factor Va heavy chain was digested with plasmin at an enzyme: substrate molar ratio of 1:100 in 50 mM NH_4HCO_3, pH 8.0, at 37 °C. The appropriate digestion time was determined by SDS–PAGE analysis of the collected samples from a time course reaction; 2 mg of the heavy chain was digested with plasmin at 37 °C for 30 min. The reaction was stopped by freezing the reaction with a dry ice/methanol mixture prior to lyophilization overnight. The lyophilized material was then brought up directly in solvent A for HPLC, using a TSK-Gel G2000SWxl column equilibrated with 45% solvent B. After application of the sample, the column was eluted with the same solvent at a flow rate of 1 mL/min. The effluent was monitored at 280 nm for peptides. Each peak was analyzed by SDS–PAGE. Peaks of interest from the TSK column were digested with CNBr in 70% formic acid at room temperature for 24 h. The digested samples were repeatedly washed with solvent A and dried using a Speedvac (Savant). The samples were then brought up directly in solvent A for HPLC, and peptides were separated using a TSK-Gel G2000SWxl column as described above. SBD-F assay positive peaks were digested with V-8 protease as described above. The digested samples were adjusted to pH 8.0 with the addition of 2.5 M Tris, and then further digested with trypsin at 37 °C for 24 h. The samples were then applied to a C-18 column and eluted with a linear gradient of 0–70% solvent B over a 70 min period at 1 mL/min. The effluent absorbance was monitored at 214 nm. The SBD-F assay positive peaks were further purified by C-18 column chromatography.

V-8 Protease Digestion. Bovine factor Va heavy chain was digested with V-8 protease at an enzyme:substrate weight ratio of 1:50 in 50 mM potassium phosphate, pH 7.8, at 37 °C for 24 h. The reaction was stopped immediately by freezing as described previously. The fragments of the heavy chain were isolated using a C-18 HPLC column. The elution was carried out with a linear gradient of 0–70% solvent B over a 70 min period at 1 mL/min. The effluent was monitored at 214 nm. The SBD-F positive peaks were further purified using a C-18 HPLC column.

Peptide Sequencing. Amino acid sequence analyses of isolated peptides were performed with an Applied Biosystems Inc. (Foster City, CA) Model 470A gas-phase sequencer. The phenylthiohydantoin (PTH)-amino acids were separated on an Applied Biosystems Model 120A analyzer connected to the protein sequencer (Hunkapiller et al., 1983). To identify amino acids and calculate recoveries, PTH-amino acid standards were chromatographed prior to sequencing of each peptide. In some instance, the radioactivity of the PTH cycle product was determined (see Figure 2).

Table 3: Sequence of NH_2-Terminal Amino Acid Residues of the Peptide Isolated from Trypsin and V-8 Protease Digestions (Figure 3)

cycle no.	peak 84a V8-b		heavy chain[d] 153–166	peak 84b V8-b		heavy chain 129–148
1	D	(134)[a]	D		ND	H
2	F	(61.7)	F	S	(27.6)	S
3	N	(45.2)	N	G	(41.2)	G
4	S	(14.3)	S	P	(41.1)	P
5	G	(38.6)	G	T	(29.7)	T
6	L	(31.7)	L	H	(8.1)	H
7	I	(31.7)	I	D	(27.3)	D
8	G	(24.6)	G	D	(33.6)	D
9	P	(49.5)	P	P	NQ[c]	P
10	L	(19.4)	L	P	NQ	P
11	L	(23)	L		ND	C
12	I	(17)	I	L	NQ	L
13		ND[b]	C	T	(8.6)	T
14		ND	K		ND	H
15				I	(7.5)	I
16				Y	(5.2)	Y
17				Y	(8.3)	Y
18					ND	S
19				Y	(5.0)	Y
20				V	(1.6)	V

[a] The number in parentheses indicates picomoles at the given cycle. [b] Not determined. [c] Not quantitated. [d] From Guinto et al. (1992).

RESULTS

Intact factor Va heavy chain labeled with iodo[^{14}C]-acetamide incorporated 2.13 mol of ^{14}C/mol of heavy chain. The heavy chain was then digested with trypsin, and the cleavage products were separated using C-18 HPLC chromatography (see Figure 1). The chromatogram shown in Figure 1 contains about 100 peaks, and displays both highly radioactive peaks (panel B) and SBD-F positive peaks (denoted in panel A by asterisks). All radioactive and SBD-F positive peaks were further analyzed; however, only a limited number of fractions gave explicit information regarding the presence of free cysteines or disulfide bridges. In some cases, this was a consequence of peaks corresponding to partial digestion fragments. In other cases, no structural information was obtained from sequence analysis.

The radioactive peptide contained in peak 20 was further purified by C-18 HPLC chromatography and analyzed for NH_2-terminal sequence. The sequence obtained from peak 20 (Table 1) corresponds to the amino acid residues of bovine factor Va heavy chain numbering from 537 to 543. From the known sequence of factor V (Guinto et al., 1992), this sequence must contain one cysteine at position 538. In separate experiments, when sequencing the same peptide, fractions were collected and counted in a scintillation counter. Figure 2 displays the identity and quantity of amino acids found per cycle (*open bars*) compared to the radioactivity found in the corresponding fractions (*filled bars*). At cycle 2, radioactivity was identified, and no amino acid peak could be detected. In the factor V sequence, a cysteine appears at residue 538 which corresponds to residue 2 in the peptide contained in peak 20. As a result, this cysteine (Cys_{538}) must be a free cysteine in bovine factor Va heavy chain.

Peak 82 (Figure 1) was futher purified using a C-18 reverse phase HPLC column. A single peak was obtained which was analyzed by the SBD-F assay under reducing and nonreducing

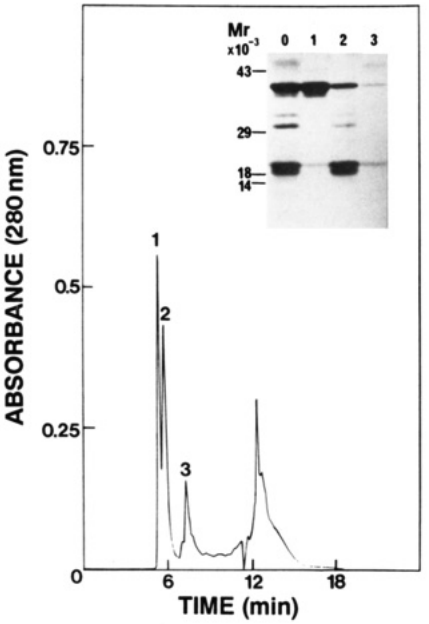

FIGURE 4: TSK-Gel G2000SWxl HPLC chromatogram of plasmin-digested bovine factor Va heavy chain. The fragments were eluted in 45% solvent B. The inset of the figure displays 8–18% gradient SDS–PAGE analysis of the peaks from the chromatogram as follows: lane 0, plasmin-digested factor Va heavy chain; lane 1, peak 1; lane 2, peak 2; and lane 3, peak 3. Two micrograms of protein was loaded per lane.

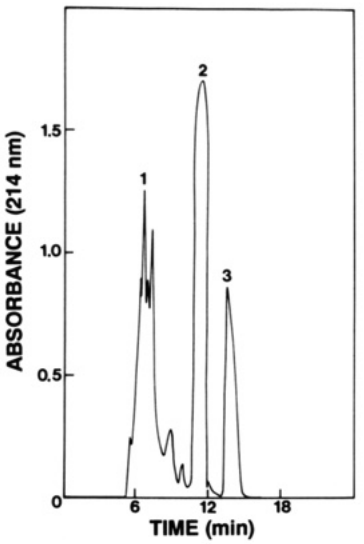

FIGURE 5: TSK-Gel G2000SWxl HPLC chromatogram of peak 1 from Figure 4 digested with CNBr. The peaks were eluted in 45% solvent B as described under Experimental Procedures.

conditions. No cysteines were detected under nonreducing conditions whereas the peptide was positive in the SBD-F assay when reduced with tributylphosphine. A double sequence, obtained from peak 82 (Figure 1), corresponds to the amino acid residues of factor Va heavy chain numbering from 456 to 474 and from 485 to 498 (Table 2). Since these two peptides are known to each contain one cysteine, the data suggest that Cys_{471} and Cys_{497} form a disulfide bond.

On the basis of the primary sequence of factor Va heavy chain, trypsin digestion would be expected to generate a peptide containing residues 110–166, in which there are two cysteine residues (Cys_{139}, Cys_{165}). The NH_2-terminal sequence obtained from the peptide contained in peak 84 (Figure 1) matched the amino acid sequence in bovine factor V spanning residues 110–149. As a result, only cysteine 139 was detected.

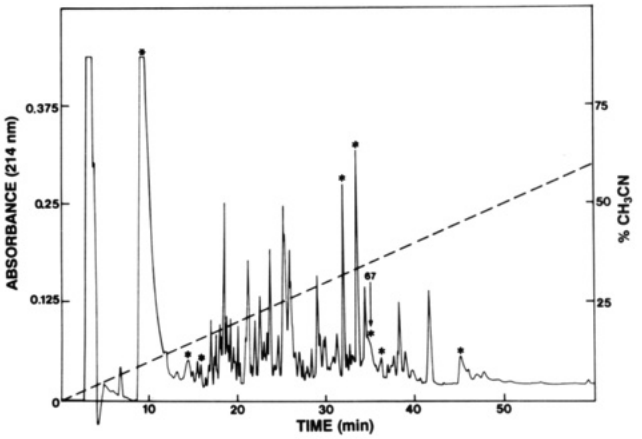

FIGURE 6: C-18 HPLC chromatogram of peak 1 from Figure 5 digested with V-8 protease and trypsin. A linear gradient of 0–60% solvent B for 60 min was used to elute the column. The asterisk indicates which peaks has been tested positive according to the SBD-F assay.

Table 4: Sequence of NH_2-Terminal Amino Acid Residues of the Peptide Contained in Peak 67 of Figure 6

cycle no.	peak 67a		heavy chain[c] 215–224	peak 67b		heavy chain 300–303
1	P	(75.3)[a]	P	N	(89.1)	N
2	D	(52.2)	D		ND	C
3	I	(28.6)	I	A	(34.3)	A
4	T	(18.3)	T	K	(86.5)	K
5	V	(15.0)	V			
6		ND[b]	C			
7	A	(15.3)	A			
8	H	(2.7)	H			
9	D	(22.4)	D			
10	H	(2.1)	H			

[a] The number in parentheses indicates picomoles at the given cycle. [b] Not determined. [c] From Guinto et al. (1992).

Since the peptide contained within peak 84 contains aspartyl residues, it was further digested with V-8 protease. The resulting peptides were separated using a C-18 HPLC column (see Figure 3). Each peak was analyzed by the fluorometric assay under reducing and nonreducing conditions. The peptide(s) contained in the SBD-F positive peak (after reduction) was (were) further digested with V-8 protease for another 24 h at 37 °C, and the resulting peptides were isolated by HPLC. SBD-F assay of peak 84_{v8-b} (Figure 3) was negative under nonreducing conditions. In contrast, after reduction with tributylphosphine, the assay was positive. NH_2-terminal sequence analysis of the peptide material resultant contained in peak 84_{v8-b} revealed the peptides that corresponded to amino acid residues 129–148 and 153–166 of bovine factor V (Table 3). Since each of the peptides contains only one cysteine, a disulfide bond exists between Cys_{139} and Cys_{165}.

In order to determine the remaining locations of the disulfide bonds in factor Va, the intact heavy chain was initially digested with plasmin, and the products were separated using TSK-Gel G2000SWxl column. Bovine factor Va heavy chain (M_r 94 000) is cleaved by plasmin to give major polypeptide products: M_r = 40 000, 20 000, and 18 000 (Omar et al., 1987). Under the conditions used here, over 90% of the intact heavy chain was digested after 30 min. The plasmin cleavage products were purified using TSK-Gel filtration HPLC column chromatography (Figure 4). The M_r = 40 000 fragment was eluted in the first peak. The next peak contained mixtures of the three fragments as shown by SDS–PAGE (see Figure 4 inset, lane 2). The third peak did not contain any detectable peptide material. Amino acid sequence analysis of the M_r =

40 000 fragment contained within peak 1 showed that this fragment represents the NH_2-terminal portion of the heavy chain. According to the amino acid sequence derived from the cDNA (Guinto et al., 1992), this fragment which most likely spans the region containing amino acids 1–306 (Kalafatis & Mann, 1993) contains four cysteine residues (residues 139, 165, 220, and 301). Two of the cysteines were found in a disulfide bridge (i.e., Cys_{139} and Cys_{165}). SBD-F analysis of the M_r = 40 000 fragment under nonreducing conditions was negative. Following reduction with tributylphosphine, the SBD-F assay was positive; thus, all four cysteines contained within the M_r = 40 000 fragment are engaged in disulfide bridges, suggesting that Cys_{220} and Cys_{301} form the other disulfide bond. In order to test this hypothesis, the M_r = 40 000 fragment was digested with CNBr, and the resulting peptide products were separated using TSK gel filtration HPLC chromatography (Figure 5). The SBD-F positive peaks were sequentially digested with V-8 protease and trypsin, and the resulting peptides were purified by C-18 HPLC chromatography (see Figure 6). Each SBD-F positive peak was further purified using a C-18 HPLC column. Peak 67 was negative when analyzed by the SBD-F assay under nonreducing conditions. After reduction, the assay was positive. Thus, the peptide contained within peak 67 contains a peptide possessing a disulfide bond. Amino acid sequence analyses performed on the peptide contained within peak 67 established the sequence which corresponds to heavy-chain residues 215–224 and 300–303, each of which contains a single cysteine (Table 4). Thus, a disulfide bond exists between Cys_{220} and Cys_{301}.

Numerous attempts were made to identify the remaining disulfide bond and free cysteine in the heavy chain of factor

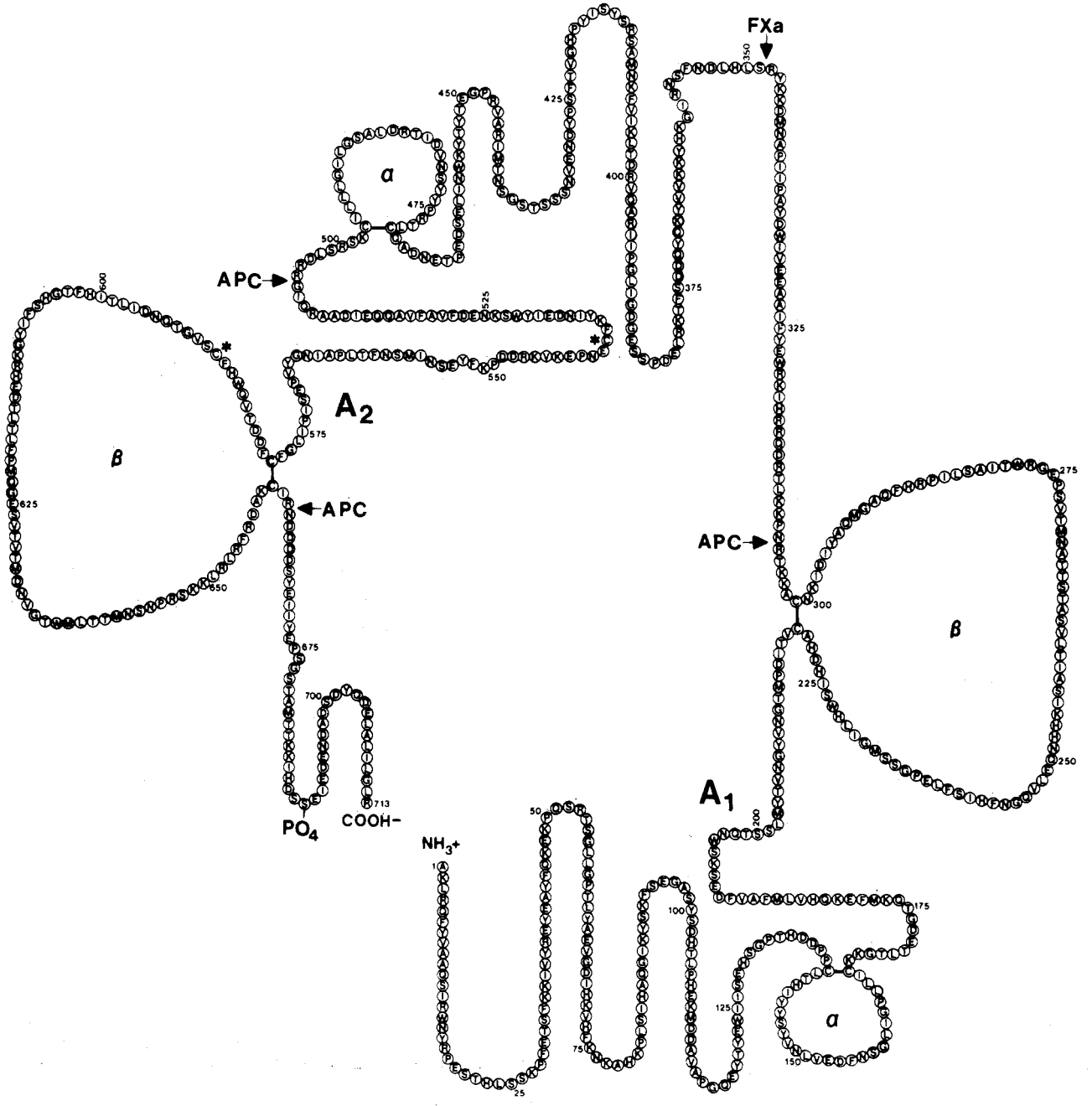

FIGURE 7: Organization of the factor Va heavy chain. The position of each disulfide loop structure is identified in the A1 and A2 domains of the factor Va heavy chain and with the locations of the free cysteine residues (asterisks) and the sites of cleavage of the factor Va heavy chain by activated protein C (Kalafatis & Mann, 1993) and by factor Xa (Odegaard & Mann, 1987). Also identified in the COOH-terminal of the heavy chain is the site of phosphorylation of factor Va by casein kinase 2 (Kalafatis et al., 1993).

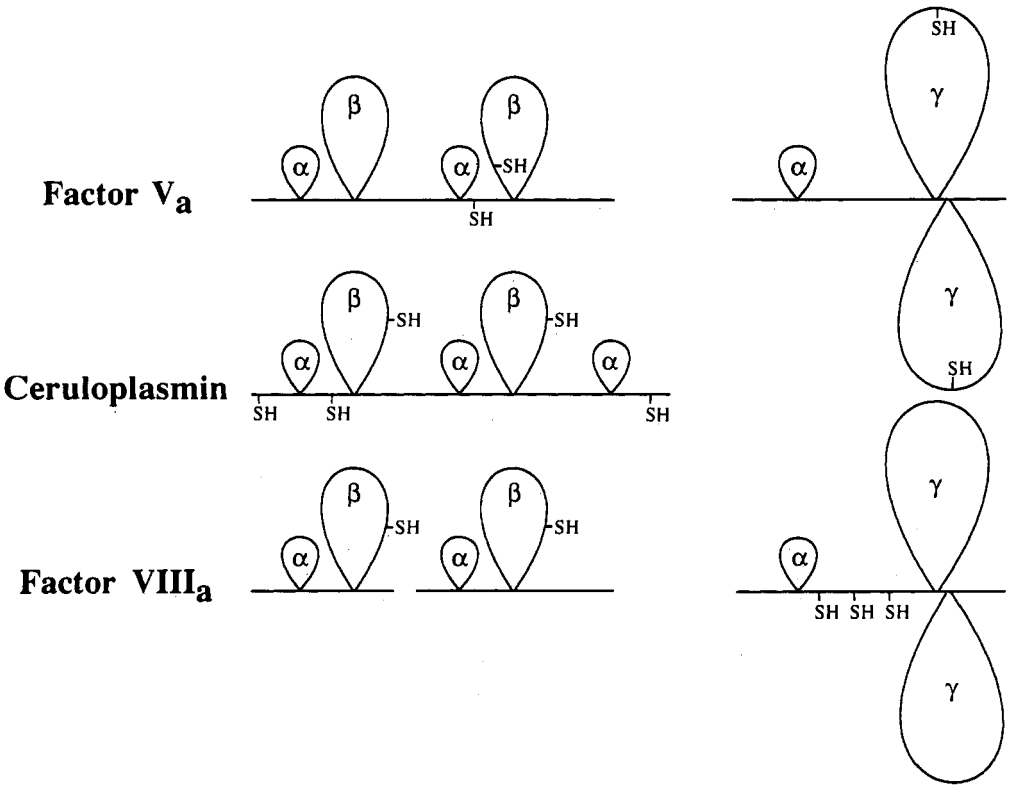

FIGURE 8: Comparison of the disulfide-bonded organized structure and free cysteines in factor Va, factor VIIIa, and ceruloplasmin. The repeated α, β, and γ loops are noted in each protein structure as are the locations of the remaining cysteines in the molecule.

Va. Bovine factor Va heavy chain was digested with V-8 protease under basic conditions (see Experimental Procedures). While this approach reduced the degree of product aggregation and insolubility generally occurring with more acidic digestion conditions, repeated attempts at resolution of these fragments failed owing to the consistent insolubility of the Cys-containing peptides. Because of the insolubility of the peptides, the chemical nature of these cysteines could not be placed with certainty. Soluble cysteine-containing peptides produced by additional proteolysis possessed insufficient structural information to be assigned in sequence. However, based upon homology with cysteine with Cys_{220}–Cys_{301}, we conclude that a bridge exists between Cys_{579} and Cys_{660}. This would make cys_{589} the remaining free Cys in the A2 domain.

DISCUSSION

Bovine factor Va heavy chain contains 10 cysteine residues. Quantitative [^{14}C]iodoacetamide incorporation studies suggest that eight are associated with disulfide bonds and two are present as free SH. The data presented in this paper explicitly place 7 of these 10 cysteines; the remaining 3 are placed by homology. We were not successful in obtaining peptides with the appropriate properties of composition and/or solubility to conduct explicit analyses required for the latter identifications. The organization of the cysteine residues in the bovine heavy chain is presented in Figure 7. Each of the cysteines which contribute to disulfide bridges in the bovine factor Va heavy chain have homologous counterparts in the human factor Va heavy chain, in the human factor VIIIa heavy chain, and in ceruloplasmin. The free cysteine residues present in the bovine factor Va heavy chain are conserved in the human factor Va heavy chain but do not have homologues in human factor VIII or in ceruloplasmin.

The data of the present paper when combined with data from our similar study on the bovine factor Va light-chain

Table 5

Proteins	α Loops	% Identity
	139	
bV-A1	CLTHIYYSYV NLVEDFNSGL IGPLLIC	
	167	89
hV-A1	CLTHIYYSHE NLIEDFNSGL IGPLLIC	
	172	78
hVIII-A1	CLTYSYLSHV DLVKDLNSGL IGALLCV	
	174	
hCER-A1	CVTRIYHSHI DAPKDIASGL IGPLIIC	
	CXTXXYXSHX XXXXDXXSGL IGXLXXC	
	471	
bV-A2	CLTRPYYSNV DITRDLASGL IGLLLIC	
	500	93
hV-A2	CLTRPYYSDV DIMRDIASGL IGLLLIC	
	547	74
hVIII-A2	CLTRYYSSFV NMERDLASGL IGPLLIC	
	534	
hCER-A2	CLAKMYYSAV DPTKDIFTGL IGPMKIC	
	CLXXXYXSXV XXXXDXXXGL IGXXXIC	
	1684	
bV-A3	CRAWAYYSAV NPEKDIHSGL IGPLLIC	
	1725	100
hV-A3	CRAWAYYSAV NPEKDIHSGL IGPLLIC	
	1851	74
hVIII-A3	CKAWAYFSDV DLEKDVHSGL IGPLLVC	
	874	
hCER-A3	CPIWAYYSTV DQVKDLYSGL IGPLIVC	
	CXXWAYXSXV XXXKDXXSGL IGPLXXC	
Overall consensus	CXXXXYXSXX XXXXDXXXGL IGXXXXC	

cysteine locations (Xue et al., 1993) provide instruction with respect to the overall organization of the disulfide bridges and free cysteines in the protein. A representation of the disulfide bonding pattern for the entire molecule is presented in Figure 8. Three, nearly identical, 26 amino acid residue "α" loops are present, one in each A domain in factor Va. In the A1 and A2 domains of the heavy chain, the "α" loops are each followed by a larger "β" loop, each of which contains 82 amino

acids. Only an "α" loop is found in the A3 domain of the light chain of factor Va, while two nearly symmetrical "γ" loops comprised of 154 and 155 residues are found in the C1 and C2 domains of the protein. In approximately the middle of each of the "γ" loops, a free cysteine is found. As illustrated in Figure 8, there is significant homology in the organization of the "α", "β", and "γ" loops in factor VIIIa heavy and light chains while there is no equivalent retention of other Cys residues. In factor Va, one heavy-chain free SH is easily modified by fluorescent maleimide labels (Krishnaswamy & Mann, 1988). Following EDTA dissociation of the two chains, one free cysteine is easily labeled in the factor Va heavy chain (Krishnaswamy & Mann, 1988; Krishnaswamy et al., 1989). These labeled residues have been useful in examining the chain–chain association in factor Va and in examining the binding of factor Va to membranes. A similar pattern of reactivity appears to exist in factor VIIIa and has been exploited in binding studies with that protein (Lollar et al., 1993). Ceruloplasmin is also composed of A1, A2, and A3 domains and has approximately 35% sequence identity with bovine factor Va (Ortel et al., 1984). As can be seen in Figure 8, the organization of the "α" and "β" loops in ceruloplasmin is virtually identical to the conserved organization of these disulfide-bonded structures in factor Va and factor VIIIa.

The residue conservation in the "α" loops of the A1, A2, and A3 domains of the three proteins is striking. The conserved sequences are identified in Table 5. The A1 "α" loops of human and bovine factor V share 89% identity while in human factor VIII 78% of the residues are identical to human factor V. The factor V A2 domain "α" loops are 93% conserved while the A3 domain "α" loop is totally conserved between species. The overall consensus sequence, for all "α" loops, in species of factor V, factor VIII, and ceruloplasmin is displayed in Table 5. The conservation of this structure is maintained to approximately to the same extent as the conservation of the "kringle", "EGF", and "apple" domains found in other coagulation proteins (Pathy, 1993; Ichinose & Davie, 1994). The significance of the "α" loops to the functions of these proteins has not been identified; however, the conservation of structure suggests an important role in the organization of these three proteins.

ACKNOWLEDGMENT

We thank Tim Valley for assistance in amino acid sequencing.

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