# Determination of the Disulfide Bridges in Factor Va Light Chain†

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ABSTRACT: The 74-kDa light chain of bovine factor Va is composed of three domains: the $NH_2$-terminal A3 domain and the COOH-terminal C1 and C2 domains. In total, the light chain has eight cysteines: two in the A3 domain and three in each C domain. To determine the locations of the disulfide bridges, peptides were obtained from factor Va and iodo[1-$^{14}$C]acetamide-labeled factor Va light chains by digestion with trypsin, activated protein C, lysylendopeptidase, and V8 protease. After HPLC purification, amino acid sequence and composition analyses showed that each domain of bovine Va light chain possesses a disulfide bond. The sites are $Cys_{1684}$–$Cys_{1710}$ (A3), $Cys_{1866}$–$Cys_{2020}$ (C1), and $Cys_{2025}$–$Cys_{2180}$ (C2). One free cysteine is located in each C domain, i.e., $Cys_{1953}$ and $Cys_{2100}$. The locations of the disulfide bonds in human Va and VIIIa light chains are anticipated to be similar to those of bovine Va light chain, because the cysteines involved are conserved.

Factor V is a 330-kDa single-chain procofactor essential in the blood coagulation cascade (Nesheim et al., 1979, 1981; Mann et al., 1981, 1988). When activated by thrombin, three peptide bonds are cleaved, and a 168-kDa heterodimeric protein called factor Va is formed. Factor Va is composed of an $NH_2$-terminal heavy chain (94 kDa) and a COOH-terminal light chain (74 kDa). These two chains are tightly held together through $Ca^{2+}$ ion mediated interactions ($K_d$ = 5.9 nM) (Krishnaswamy, 1989).

The structure of factor Va has been compared to other proteins which have similar amino acid sequences (Guinto et al., 1992; Jenny et al., 1987; Vehar et al., 1984; Kane & Davie, 1988). The heavy chain of factor Va consists of two domains, A1 and A2, while the light chain contains the regions A3, C1, and C2. These domains in bovine Va exhibit high levels of amino acid sequence identity (70–90%) to those present in human Va (Guinto et al., 1992). In addition, similar A-type regions have been found in the copper binding plasma protein ceruloplasmin, while related C-domain structures have been identified in a surface protein (66/53 kDa) of mouse mammary endothelial cells and discoidin I (Koshcinsky et al., 1986; Stubbs et al., 1990; Poole et al., 1981).

Factor VIIIa, the cofactor of factor IXa in the factor X activation complex, has the same domain substructure as factor Va (Vehar et al., 1984). The sequences of the A1, A2, and A3 domains of Va and VIIIa are 40% identical, while their C1 and C2 regions are 43% identical. In addition, between the three A domains of these two cofactors and ceruloplasmin, there is about a 40% sequence identity, which makes them triplicated domains (Jenny et al., 1987). The mouse surface protein is 70% identical to the C1 and C2 regions of factors Va and VIIIa (Stubbs et al., 1990).

From the amino acid sequence of Va, it is known that there are 18 cysteine residues present: 10 in the heavy chain and 8 in the light chain. In comparison, factor VIIIa has 19 cysteines: 5 in each A1 and A2 domain and 9 in the light chain. On the basis of their amino acid sequences, computer analyses of ceruloplasmin and factor VIIIa have resulted in predicted locations for their disulfide bonds (Takahashi et al., 1983, 1984; Vehar et al., 1984). In this investigation, we determined the disulfide bond sites for bovine factor Va light chain. Sequence and structural comparisons were also made with human factor Va, human factor VIIIa, and ceruloplasmin.

## REAGENTS

*Staphylococcus aureus* V8 protease and trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone were obtained from Worthington Biochemical Corp., Freehold, NJ. Stock solutions (1 mg/mL) of trypsin and V8 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. Lysylendopeptidase from *Achromobacter lyticus* was obtained from Wako Chemicals, Inc. (Dallas, TX), dissolved in 2 mM Tris-HCl, pH 8.0, at a concentration of 1.5 mg/mL, and stored at −20 °C.

Iodo[1-$^{14}$C]acetamide (57.6 mCi/mmol, 308 μCi/mg) was purchased from Amersham. Ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonate (SBD-F) was purchased from Wako Chemicals, Inc. Oxidized glutathione, *N*,*N*-dimethylacetamide, and Sephadex G-50 resin were obtained from the 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. HPLC-grade water and acetonitrile were obtained from J. T. Baker, Inc. Reagents for amino acid composition analyses were purchased from Pierce, Rockford, IL. Reagents and solvents for amino acid sequence analyses were obtained from Applied Biosystems, Inc. All other chemicals were of the highest grade commercially available. Deionized water prepared from house-distilled water using a Milli-Q reagent water system was routinely employed.

## EXPERIMENTAL PROCEDURES

*Isolation of Proteins.* Bovine activated protein C was purified according to Odegaard and Mann (1987). Bovine factor V was isolated as described (Nesheim et al., 1981). Factor V was activated by α-thrombin as described (Krishnaswamy et al., 1986). Factor Va was isolated using a modified method of Odegaard and Mann (1987).

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The procedure for the isolation of the heavy chain from the light chain required the use of a monoclonal immunoadsorbent column (Odegaard & Mann, 1987). Purified factor Va was applied to the column, and the initial flow-through peak and buffer wash were collected. At this point, the buffer was changed to contain 5 mM ethylenediaminetetraacetic acid (EDTA) instead of $CaCl_2$. The heavy chain was eluted with this EDTA buffer. After that, the column was washed with buffer made 1.7 M NaCl to elute the light chain. Both purified heavy and light chains were stored at −20 °C in 50% (v/v) glycerol/20 mM Hepes, pH 7.4. All proteins and their components were routinely characterized through SDS–PAGE analyses of the purified products.

*Preparation of [$^{14}$C]Carboxamidomethyl-Labeled Light Chain.* The procedure was a modification of that of Crestfield et al. (1963). Light 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 light chain was resuspended in 6 M guanidine hydrochloride/0.2 M Tris-HCl, pH 8.6, and incubated with 2 μM iodo[1-$^{14}$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 then added to 10 mM and the mixture incubated for another 15 min in the dark to saturate the alkylation of 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.

*Isolation of Proteolytic Fragments of Light Chain. (a) Activated Protein C Digestion.* The light chain of bovine factor Va was cleaved with activated protein C using a modified method of Odegaard and Mann (1987). The digestion of light chain by activated protein C (APC) was performed in 20 mM Hepes/0.15 M NaCl, pH 7.4, at 37 °C and at an enzyme:substrate weight ratio of 1:50. An appropriate digestion time was determined by screening a time course and SDS–PAGE analysis of the collected time points; 2.23 mg of the light chain was digested with APC at 37 °C for 5 h. The reaction was stopped immediately 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. The fragments of the light chain were isolated by reverse-phase HPLC using a C3 column. The elution was carried out with a linear gradient of 30–60% solvent B over a 25-min period at 1 mL/min (where solvent A = 0.05% trifluoroacetic acid in $H_2O$ and solvent B = 0.05% trifluoroacetic acid in $CH_3CN$).

*(b) Lysylendopeptidase and V8 Protease Digestions.* Bovine Va light chain was digested with lysylendopeptidase at an enzyme:substrate weight ratio of 1:100 in 50 mM $NH_4HCO_3$/2 M urea, pH 9.0, at 37 °C for 24 h. The digested material was applied to a Sephadex G-50 gel filtration column (1 cm × 120 cm), equilibrated with 10% formic acid. After application of the sample, the column effluent was eluted with the same solution at a flow rate of 5 mL/h. The effluent was monitored at 280 nm for peptides. Each peak was analyzed with a fluorometric assay (SBD-F assay, see below).

The positive peak from the assay was digested with *Staphylococcus aureus* V8 protease in 0.1 M ammonium acetate, pH 4.0, at 37 °C for 24 h at an enzyme:substrate weight ratio of 1:50. The V8-digested sample was applied to an HPLC C-18 column. The C-18 column was eluted with a linear gradient of 0–50% solvent B over a 50-min period at 1 mL/min. Each peak was analyzed with the 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. Amino acid composition and sequence analyses on the purified peaks were then performed as described below.

*(c) Trypsin Digestion.* The $^{14}$C-labeled light chain was dissolved in 1 mM HCl, and the pH was adjusted to 8 with the addition of 0.1 M Tris. The sample was then digested with trypsin at an enzyme:substrate weight ratio of 1:100 at 37 °C for 24 h. The digested sample was applied to a HPLC C-18 column. The C-18 column was eluted with a linear gradient of 0–60% solvent B over a 60-min period at 1 mL/min. Each fraction was analyzed by the fluorometric assay and counted on the scintillation counter (Beckman LS 6000IC). The radioactive and SBD-F-positive peaks were further purified by C-18 HPLC column chromatography. The purified peptides were subjected to amino acid sequence analyses.

*Amino Acid Composition Analysis.* Peptides from HPLC separations were dried down on the Speed Vac concentrator (Savant), following the addition of 1 nmol of norleucine as an internal standard. Gas-phase hydrolysis of 200–400 pmol of peptide was carried out for 24 h *in vacuo* with 6 N HCl (Pierce) and 2% w/v thiodiglycolic acid. The amino acids were resolved by HPLC analysis using an Interaction AA911 column (Interaction Chemicals, Los Altos, CA) eluted with the Buffelute buffer system (Pierce). Amino acids were detected via postcolumn derivatization with *o*-phthalaldehyde using a fluorescent detector. Data were analyzed using the Maxima 820 chromatography workstation (Waters, Milford, MA). Proline was detected with *o*-phthalaldehyde following post-column oxidation with NaOCl (Bohlen, 1983). Cysteine, in the form of cysteic acid, was determined from 6 N HCl hydrolysates of dried, oxidized peptide following room temperature performic acid oxidation of the intact peptide for 30 min with 90 μL of 99% (v/v) formic acid and 10 μL of $H_2O_2$ (Bohlen, 1983).

*Amino Acid Sequence Analysis.* Amino acid sequence analysis of selected peptides was 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.

*SBD-F (Ammonium 7-Fluorobenz-2-oxa-1,3-diazole-4-sulfonate) Fluorometric Assay.* Cystinyl peptides were detected by a modified procedure of Sueyoshi et al. (1985). In this assay, disulfide bridges were cleaved with tributylphosphine, and the thiol groups were coupled to the fluorescent reagent SBD-F. This simple and sensitive method was able to quantitatively detect peptide-containing disulfides in the range of 20 pmol–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. After 20 min, 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

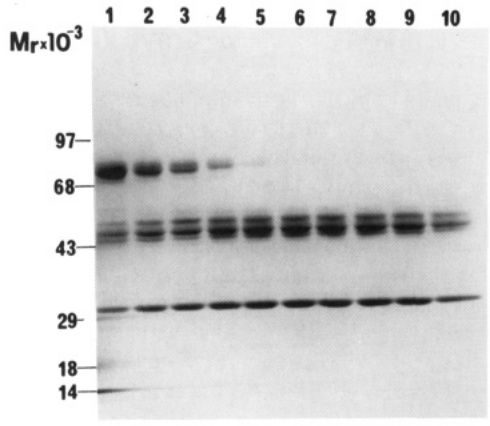


FIGURE 1: Time-dependent cleavage pattern of bovine factor Va light chain with activated protein C (APC) as analyzed by 5–15% gradient SDS–PAGE. Lanes 1–10 are samples taken from the digestion at the following time points: (1) 0, (2) 10, (3) 15, (4) 30, (5) 60, (6) 90, (7) 120, (8) 180, (9) 240, and (10) 360 min. The proteolysis was performed at an enzyme:substrate weight ratio of 1:50. Six micrograms of protein was loaded per lane. The high molecular weight markers are shown on the left side of the figure. All other experimental conditions are as stated under Experimental Procedures.

compared against a standard calibration curve, prepared using oxidized glutathione (20–2000 pmol), in order to determine the amount of cysteine present.

## RESULTS

*Va Light Chain: SH Analysis. (A) Isolation of Light-Chain Cleavage Products.* Bovine factor Va light chain (74 kDa) can be cleaved by factor Xa, APC, or plasmin to give the polypeptide products: 30 and 46/48 kDa (Foster et al., 1983; Odegaard & Mann, 1987; Omar & Mann, 1987). The time course for light-chain digestion with APC is displayed in Figure 1. It is noted that from intact light chain (74 kDa), the major products are as expected: an $NH_2$-terminal 30-kDa fragment and the COOH-terminal doublet of 46/48-kDa fragments. Under the conditions used here, over 90% of the intact light chain was digested after 1 h.

The cleavage products can be isolated and purified using C-3 HPLC column chromatography. A typical HPLC chromatogram of such a separation is shown in Figure 2. The $NH_2$-terminal 30-kDa fragment was observed to be eluted in the first major peak after the void volume peak. The heavier COOH-terminal 46/48-kDa fragments were eluted together in the second peak. These observations were confirmed by the SDS–PAGE gel of these two peaks as shown in the inset of Figure 2.

*(B) Identification of the Cysteine Residues in Va Light Chain. (i) The A3 Domain.* Iodo[1-$^{14}$C]acetamide-labeled Va light chain was analyzed by SDS–PAGE. From the autoradiogram of this gel, it was noted that only the intact Va light chain (74 kDa) and the COOH-terminal 46/48-kDa fragments were radioactively labeled. This provided strong evidence that the two cysteine residues in the $NH_2$-terminal 30-kDa fragment must form a disulfide bond. To confirm this conclusion, the HPLC-purified fragment was analyzed for the presence of a disulfide bond using the SBD-F fluorometric assay. This assay is specific for free sulfhydryl groups (Sueyoshi et al., 1985). Under nonreduced conditions, the assay was negative, indicating that no free cysteines were present. However, when the 30-kDa fragment was reduced with tributylphosphine, the assay was positive. This points to the presence of a disulfide bond in the 30-kDa fragment. In

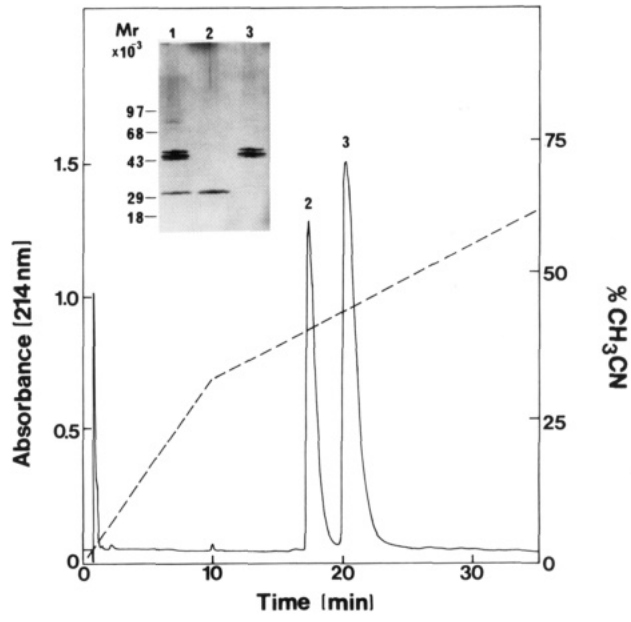


FIGURE 2: C-3 HPLC chromatogram of APC-digested bovine factor Va light chain. The solvent gradient extends from 30% solvent B to 60% solvent B over a period of 25 min after 10 min of a 0–30% solvent B linear gradient. The inset of this figure displays the 5–15% gradient SDS–PAGE analysis of the peaks from the chromatogram as follows: lane 1, APC-digested Va light chain; lane 2, peak 2; lane 3, peak 3. Two micrograms of protein was loaded per lane.

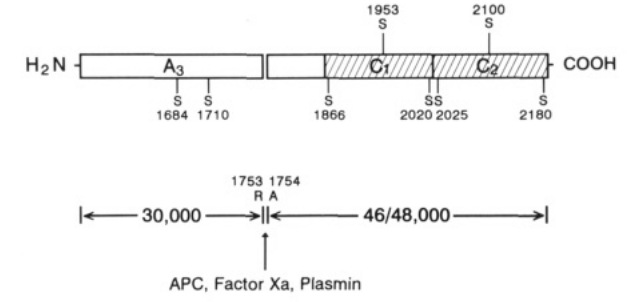


FIGURE 3: Diagram of bovine factor Va light chain denoting both the cysteine locations determined from amino acid sequence information as well as the cleavage sites for activated protein C, plasmin, and factor Xa.

accordance with the bovine Va light-chain sequence, the disulfide bond would be formed between $Cys_{1684}$ and $Cys_{1710}$ of the A3 domain as shown in Figure 3. Moreover, since under the conditions used here iodoacetamide reacts selectively with sulfhydryl groups, the free cysteines of the Va light chain must be located in the 46/48-kDa fragment of the light chain.

*(ii) The C1 and C2 Domains.* Intact Va light chain was labeled with iodo[1-$^{14}$C]acetamide, which resulted in the incorporation of 1.63 mol of $^{14}$C/mol of light chain. The light chain was then digested with trypsin, and the cleavage products were separated using C-18 HPLC chromatography (see Figure 4). The chromatogram shown in Figure 4 contains about 100 peaks, which were individually screened for radioactivity. The highly radioactive peaks are displayed in Figure 4B. Of these three peaks, peaks 35 and 71 were further purified by C-18 HPLC chromatography. After identification of the radioactive peaks, the peaks were then analyzed by amino acid sequence analysis. The results from the analyses are given in Table I.

In Table I, the sequence obtained from peak 35 corresponds to the amino acid residues of bovine light chain numbering from 2092 to 2101. In this sequence, a cysteine residue appears at location 2100. As a result, this cysteine must be a free

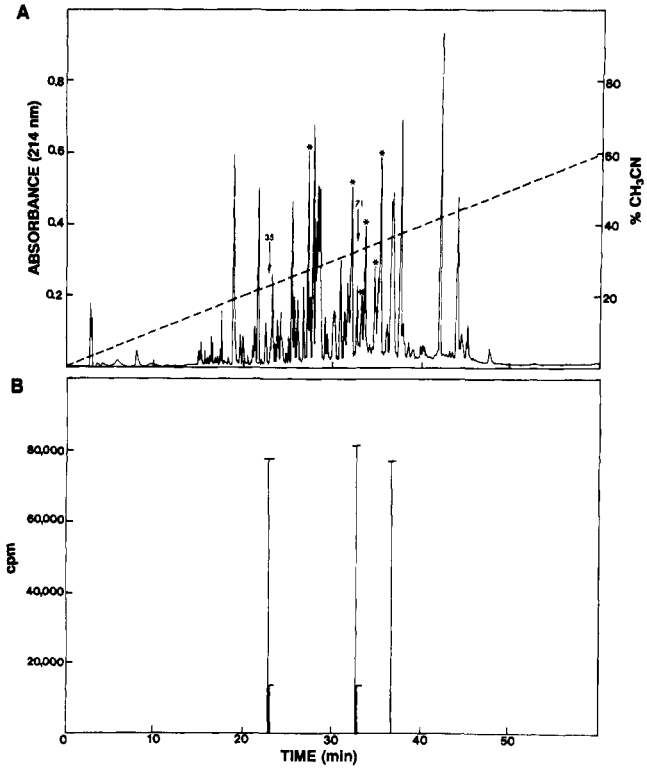


FIGURE 4: C-18 HPLC chromatogram of trypsin-digested, $^{14}C$-labeled bovine factor Va light chain (panel A) and the corresponding radioactive peaks (panel B). The HPLC linear elution gradient was from 0 to 60% solvent B over a period of 60 min. The asterisk (*) indicates which peaks have tested positive according to the SBD-F fluorometric assay.

Table I: Sequence of N-Terminal Amino Acid Residues of the Tryptic Peptide

| cycle | peak 35 | light chain[c] 2092–2101 | cycle | peak 35 | light chain[c] 2092–2101 |
|---|---|---|---|---|---|
| 1 | I (199)[a] | I | 6 | T (57) | T |
| 2 | T (93) | T | 7 | Q (45) | Q |
| 3 | A (148.5) | A | 8 | G (35) | G |
| 4 | I (93.5) | I | 9 | - ND[b] | C |
| 5 | V (72.5) | V | 10 | - ND | K |
| cycle | peak 71 | light chain 1942–1956 | cycle | peak 71 | light chain 1942–1956 |
| 1 | - ND | H | 9 | T (22.5) | T |
| 2 | - ND | Y | 10 | - ND | E |
| 3 | L (67) | L | 11 | F (27.5) | F |
| 4 | K (61.5) | K | 12 | - ND | C |
| 5 | P (52.5) | P | 13 | V (19) | V |
| 6 | Y (64) | Y | 14 | A (18) | A |
| 7 | Y (66.5) | Y | 15 | Y (12.5) | Y |
| 8 | T (23) | T | | | |

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

cysteine. The sequence obtained from peak 71 as shown in Table I corresponds to the amino acid residues of bovine light chain numbering from 1942 to 1956. In this sequence, a cysteine residue appears at position 1953. This cysteine is another free cysteine in the bovine Va light chain.

In order to determine the remaining locations of the disulfide bonds in Va light chain, the intact light chain was digested with lysylendopeptidase. The cleavage products were separated using a Sephadex G-50 column. The elution profile from this separation is shown in Figure 5. Each of these peaks was analyzed using the SBD-F fluorometric assay. The results from the assay under reduced conditions are given in the inset of Figure 5. Peak 4 of the chromatogram, which is highly positive, was further digested with V8 protease. The proteolysis products were separated by C-18 HPLC chromatography. The chromatogram of this separation is shown in Figure 6. Each peak was analyzed using the SBD-F fluorometric assay. From this analysis, the positive peak 28 was further purified by C-18 HPLC chromatography. Amino acid sequence analysis was then performed on peak 28. The sequence results as shown in Table II correspond to the bovine light-chain sequence running from position 2017 to position 2022. A cysteine residue appears at position 2020. It was also observed that the SBD-F fluorometric assay was only positive for peak 28 when reduced conditions were used. Therefore, $Cys_{2020}$ must be half of a disulfide bond.

Since the remaining undetected cysteine residues in the light chain form disulfide bonds, $Cys_{2020}$ must form a disulfide bond with one of these three cysteines: $Cys_{1866}$, $Cys_{2025}$, or $Cys_{2180}$. The probable configurations of the sequences relating to these proposed disulfide bonds are given in Figure 7 (as based on the cleavage patterns of lysylendopeptidase and V8 protease). To determine which one of these three is correct, the amino acid composition of the peptide corresponding to peak 28 was obtained. The composition was compared to the calculated compositions for the three possible sequence configurations given in Figure 7. Table III presents such a comparison. From this table, it is clear that the amino acid composition obtained from peak 28 only correlates with the sequence configuration for a disulfide bond between $Cys_{1866}$ and $Cys_{2020}$. As a result, the remaining two cysteine residues of the light chain, $Cys_{2025}$ and $Cys_{2180}$, must form the last disulfide bond. These last two disulfide bonds appear in the C1 and C2 domains of the light chain (see Figure 3).

## DISCUSSION

Bovine Va light chain (74 kDa) has 647 amino acids, of which 8 are cysteine residues (see Figure 3). The light-chain sequence begins at residue 1537 and ends at residue 2183. Contained within this sequence are the A3, C1, and C2 domains (Guinto et al., 1992). The function of the light chain of Va has so far been identified to be involved in Va interactions with phospholipid membranes and factor Xa (Krishnaswamy & Mann, 1988; Kalafatis et al., 1990; Ortel et al., 1992; Monkovic & Tracy, 1990; Pryzdial & Mann, 1991).

The work here has focused on the determination of the disulfide bond positions in bovine factor Va light chain. When intact denatured Va light chain was radiolabeled with iodo[1-$^{14}C$]acetamide, a 1.63 mol of $^{14}C$/mol of light-chain coupling ratio was obtained. This ratio predicts that there are two free cysteines in the light chain. In addition, this ratio differs from that obtained when Va light chain was labeled with acrylodan (i.e., 1.15 mol of label/mol of light chain) (Krishnaswamy et al., 1989), which may result from one of the sulfhydryl groups being less accessible than the other. Radiolabeling here was important since it prevented rearrangement and further oxidation of the free cysteines and immediately marked them for identification.

The free cysteines were found to be localized to the COOH-terminal C1 and C2 domains and were identified to be at $Cys_{1953}$ of the C1 domain and $Cys_{2100}$ of the C2 domain.

Autoradiography of $^{14}C$-labeled light chain showed that only the $NH_2$-terminal 30-kDa fragment remained unlabeled, indicating that the two cysteines present within this fragment must form a disulfide bond. This premise was supported by the positive result obtained from the SBD-F fluorometric assay of the 30-kDa fragment under reduced conditions. This

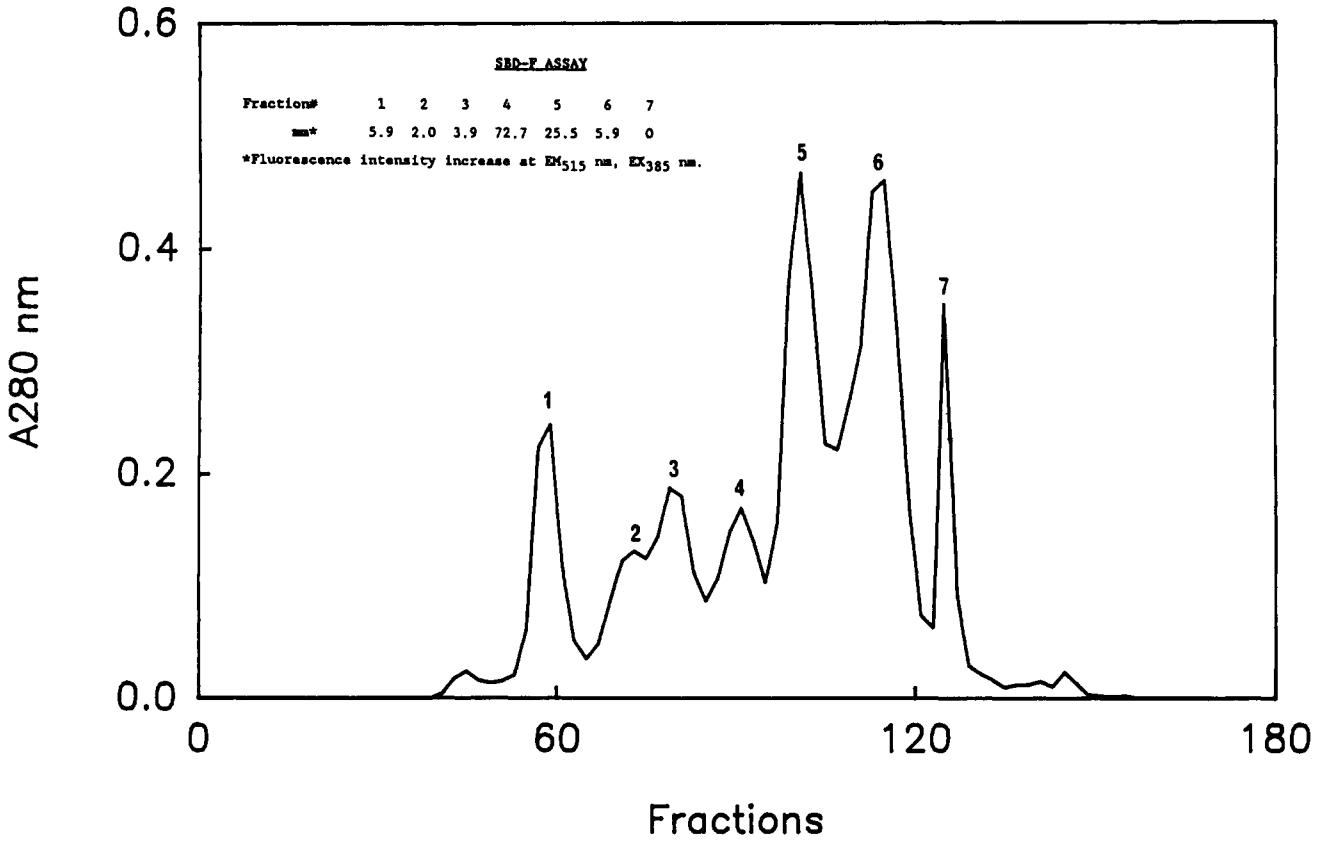


FIGURE 5: Sephadex G-50 chromatogram of lysylendopeptidase-digested bovine factor Va light chain. The inset displays the results obtained from the SBD-F fluorometric assay of each peak.

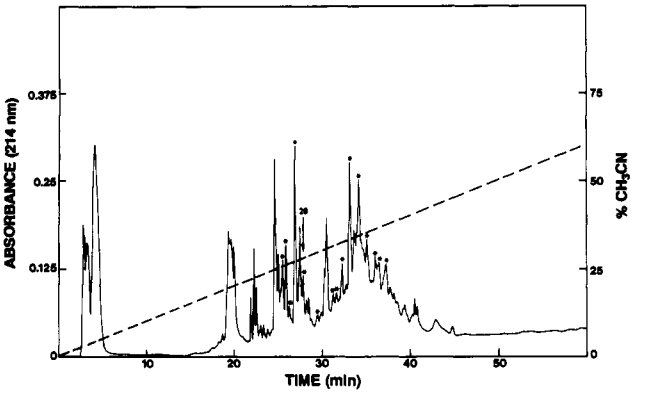


FIGURE 6: C-18 HPLC chromatogram of peak 4 from Figure 6. The asterisk indicates which peak has tested positive according to the SBD-F fluorometric assay. A linear gradient of 0–60% solvent B for 60 min was used to elute the column.

Table II: Sequence of N-Terminal Amino Acid Residues of the Peptide Isolated from Lysylendopeptidase and V8 Protease Digestions

| cycle | peak 28 | light chain[c] 2017–2022 | cycle | peak 28 | light chain[c] 2017–2022 |
|---|---|---|---|---|---|
| 1 | L (106.7)[a] | L | 4 | – ND[b] | C |
| 2 | Q (66.7) | Q | 5 | E (49.1) | E |
| 3 | G (69.7) | G | 6 | V (30.3) | V |

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

disulfide bond creates a small "loop" in the A3 domain, spanning residues 1684–1710 (27 amino acids).

The remaining disulfide bonds were determined on fragments obtained with the use of lysylendopeptidase and V8 protease. Lysylendopeptidase cleaves peptide bonds after lysine residues. Under the conditions used here, the V8

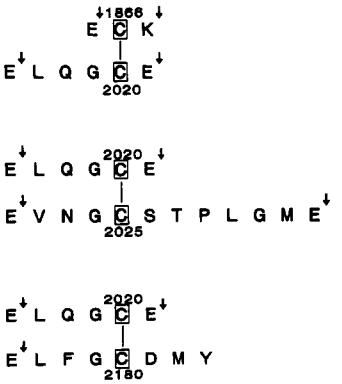


FIGURE 7: Schematic displaying the three possible disulfide bond combinations that $Cys_{2020}$ could form on the basis of V8 protease and lysylendopeptidase cleavage patterns. The solid and dashed arrows indicate the cleavage sites for V8 protease and lysylendopeptidase, respectively.

protease cleavage site was after glutamic acid residues. It was observed that some nonspecific cleavages occurred during V8 digestion (Bjorklind & Jornvall, 1974). Analyses of the peptides from sequential digestion permitted the identification of $Cys_{2020}$ (see Table II), which led to the determination of the disulfide bond formed between $Cys_{1866}$ and $Cys_{2020}$. This bond creates a "loop" of 155 amino acids in the C1 domain. The final disulfide bond identified as being between $Cys_{2025}$ and $Cys_{2180}$ forms a "loop" of 156 amino acids in the C2 domain. The complete disulfide bond pattern of bovine Va light chain is shown in Figure 8.

It is noted in Figure 8 that the A3 domain of bovine Va light chain spans residues 1537–1864. The C1 domain includes residues 1865–2023, and the C2 domain contains residues 2024–2183. Each domain (A3, C1, and C2) has a single disulfide bridge that forms a "loop-like" structure. A free

Table III: Amino Acid Compositions for Possible Disulfide Bond Configurations

| | amino acid[a] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cys–Cys | C | K | L | E+Q | G | V | D+N | Y | S | T | P | M | F |
| $C_{1866}$–$C_{2020}$ | 2 | 1 | 1 | 2 | 1 | (1) | (1)[b] | – | – | – | – | – | – |
| $C_{2020}$–$C_{2025}$ | 2 | – | 2 | 3 | 3 | 1 | 1 | – | 1 | 1 | 1 | 1 | – |
| $C_{2020}$–$C_{2180}$ | 2 | – | 2 | 2 | 2 | (1) | 1/(2) | 1 | – | – | – | 1 | 1 |
| AA found | 1.6 | 0.8 | 1.3 | 2 | 3.1 | 0.45 | 0.95 | – | – | – | – | – | – |

[a] From Guinto et al. (1992). [b] These amino acid residues are derived from nonspecific cleavage by V8 protease.

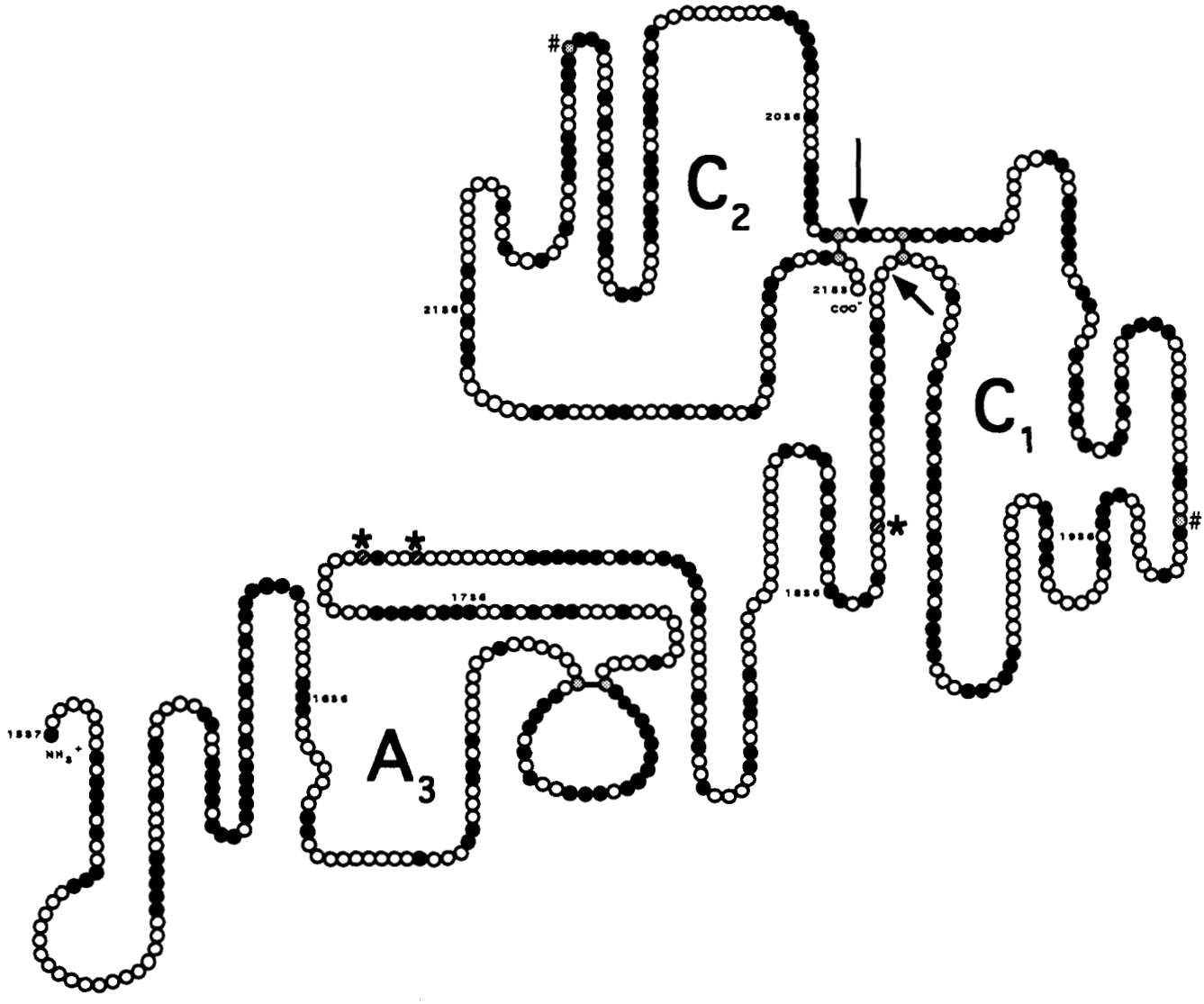


FIGURE 8: Diagram comparing the amino acid residues of bovine factor Va light chain with those in human factor Va and VIIIa light chains. The filled circles (●) indicate the residues which are identical among the three light chains while the open circles (○) show the ones that differ. Va and human VIIIa cysteines are indicated by the dot-filled (◉) and line-filled (⊘) circles, respectively. Bovine Va free cysteines are labeled by pound signs (#). Asterisks (*) indicate the cysteines of human VIIIa which differ from free cysteines in bovine Va. The arrows indicate where each domain (as labeled) meets.

cysteine appears in both C1 and C2 domains, near the midpoint of each "loop". The "loop" structures in the C1 and C2 domains are nearly symmetrical in both shape and size. The turns present in this diagram of bovine Va light chain arise from computer analysis of the light-chain amino acid sequence (Genetics Computer Group, 1989).

Comparing human Va light chain with bovine Va light chain, there is 85% amino acid sequence identity between A3 domains, 80% identity between C1 domains, and 70% identity between C2 domains (Guinto et al., 1992; Jenny et al., 1987). Overall, there is 86% sequence identity between both light chains. The predicted free cysteines in human Va light chain would be at residues 1960 (C1) and 2113 (C2). In addition, the anticipated disulfide bonds would be between cysteine residues 1697 and 1723 (A3), 1879 and 2033 (C1), and 2038 and 2193 (C2). After a 13 amino acid shift is accounted for, all cysteine residues are conserved between species except for $Cys_{1953}$ of bovine Va light chain. There is a seven amino acid difference in the human light chain since the corresponding cysteine appears at residue 1960.

The light-chain amino acid sequences of both species of Va and human factor VIIIa are compared in Figure 8. There exists a 40% sequence identity between the three proteins. In addition, a 78% sequence identity is found in the small "loop" of the A3 domain. The cysteines involved in disulfide bond formation appear to be conserved in all three light chains. Of

note are the differences in the locations of the free cysteines of bovine Va and the remaining three cysteines in human VIIIa. The cysteines in bovine Va light chain are localized to the C domain whereas those for VIIIa are found only in the A3 domain. This may be relevant as to why factors Va and VIIIa both perform similar function as cofactors, but are directed toward different enzyme complexes.

Recent CD studies and computer analyses by Bihoreau et al. (1992) of human factor VIIIa light chain have shown that the light-chain structure contains a fair amount of secondary structure. Approximately 55–58% of the light chain is ordered with about 31–36% $\beta$ sheet and 22–24% $\alpha$ helix. All three domains of the light chain have a mixture of $\alpha$ helix and $\beta$ sheet. Apparently, the A3 domain has a higher ratio of $\alpha$ helix to $\beta$ sheet (2.2) as compared to both C domains (0.5–0.8). The secondary structure of factor Va light chain is expected to be similar to that of factor VIIIa light chain.

Comparing the A3 domain of bovine Va light chain with the three A domains in bovine Va, human Va, and human VIIIa, a 22% sequence identity was observed between A domains. Of interest is the 60% sequence identity present in the small "loop" of the A3 domain. The disulfide bond in this "loop" is conserved in all A domains. It is also of interest to note that 75% sequence identity exists between regions 1679–1726 of the A3 domain of factor Va, 1814–1860 of factor VIIIa, and 356–403 of ceruloplasmin (Guinto et al., 1992). Furthermore, all disulfide bonds in Va light chain are localized within each domain and not formed between adjacent domains. A similar pattern of disulfide bond formation was predicted for both factor VIIIa and ceruloplasmin (Vehar et al., 1984; Takahashi et al., 1983; Ortel et al., 1984).

In summary, bovine Va light chain contains eight cysteine residues: two free cysteines and three disulfide bridges. The two free cysteines are located at $Cys_{1953}$ and $Cys_{2100}$ of the C1 and C2 domains, respectively. A disulfide bond is present in each domain as follows: $Cys_{1684}$–$Cys_{1710}$ (A3), $Cys_{1866}$–$Cys_{2020}$ (C1), and $Cys_{2025}$–$Cys_{2180}$ (C2). In addition, it is noted that since all cysteines involved in disulfide bridge formation are conserved in bovine Va, human Va, and human VIIIa, the corresponding disulfide bond locations are most likely similar in all three proteins.

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