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Complete Amino Acid Sequence of the Light Chain of Human Blood Coagulation Factor X: Evidence for Identification of Residue 63 as β-Hydroxyaspartic Acid†

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ABSTRACT: The complete amino acid sequence of the light chain of human blood coagulation factor X has been determined by automated Edman degradation of peptides isolated from chemical and enzymatic digests of the carboxymethylated light chain. The protein consists of 139 amino acid residues, which include 11 residues of γ-carboxyglutamic acid. The first 100 residues of the human factor X light chain exhibit approximately 80% homology when compared to the amino-terminal sequence of bovine factor X light chain. This homology decreases to approximately 50% in the remaining 39 residues of the carboxyl-terminal region of the protein. Proton nuclear magnetic resonance spectroscopy and mass spectrometry analyses of isolated residue 63 identified this residue as L-*erythro*-β-hydroxyaspartic acid, a hitherto unrecognized amino acid in proteins. Evidence is also presented for the presence of this residue in the corresponding regions of the light chains of bovine factor X and bovine protein C. The biological function of β-hydroxyaspartic acid in these proteins is unknown.

Human blood coagulation factor X is a vitamin K dependent protein that circulates in blood as a precursor of a serine protease. Factor X is a glycoprotein composed of a heavy chain (M_r 42 000) and a light chain (M_r 17 000) held together by a single disulfide bond. The amino-terminal portion of the light chain contains γ-carboxyglutamic acid residues that are instrumental in calcium and phospholipid binding. The molecule contains 15% carbohydrate associated exclusively with the heavy chain (DiScipio et al., 1977a).

During the coagulation process, factor X is converted to an enzyme, factor Xa, by either the intrinsic or extrinsic pathway of blood coagulation [see Davie et al. (1979) and Nemerson & Furie (1980) for reviews]. In the activation of factor X, a specific arginine–isoleucine bond is cleaved in the amino-terminal region of the heavy chain resulting in an activation peptide (M_r 14 000) and factor Xa (DiScipio et al., 1977b). Factor Xa participates in the common pathway of blood coagulation, converting prothrombin to thrombin in the presence of factor Va, phospholipid, and calcium ions (Davie et al., 1979).

The complete amino acid sequences have been reported for the heavy and light chains of bovine factor X (Titani et al., 1975; Enfield et al., 1975, 1980). A preliminary sequence analysis performed in this laboratory (DiScipio et al., 1977b) revealed extensive homology between human and bovine factor X in those regions compared, with the notable exception of the amino terminus of the heavy chain. This paper presents the amino acid sequence of the light chain of human factor X. In addition, evidence is presented for the existence of a previously unrecognized amino acid, β-hydroxyaspartic acid, in this peptide, as well as in the light chains of bovine factor X and bovine protein C.

Materials and Methods

Carboxypeptidases A and B, TPCK-trypsin, and α-chymotrypsin were obtained from Worthington. Before use, the TPCK-trypsin and α-chymotrypsin were purified further on benzamidine–agarose (Fujikawa & McMullen, 1983). Iodoacetic acid was purchased from Sigma and aminopeptidase from Boehringer Mannheim. L-[2,3-$^3H_2$]Aspartic acid was obtained from New England Nuclear. L-*threo*-β-Hydroxyaspartic acid and L-*erythro*-β-hydroxyaspartic acid were kindly provided by Drs. N. Izumiya and T. Kato, Faculty of Biochemistry, Kyushu University, Fukuoka, Japan (Okai et al.,

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0006-2960/83/0422-2875$01.50/0

1967). Cyanogen bromide, polybrene, fluorescamine, and micropolyamide sheets were obtained from Pierce. 2-Butanol was a product of Burdick and Jackson. Carboxypeptidase Y was a gift from Dr. M. Ottsen, Carlsberg Laboratory, Copenhagen, Denmark. Sephadex G-100, G-50, and G-25 and DEAE-Sephacel[1] were obtained from Pharmacia. The columns used in reverse-phase HPLC (μBondapak C_{18} and SynChropak RP-P) were purchased from Waters and SynChrom, respectively.

Bovine prothrombin was prepared according to Mann (1976). Bovine factor X_1 and bovine protein C were prepared according to Fujikawa et al. (1972) and Kisiel et al. (1976), respectively. Factor X was purified to homogeneity from human plasma by a combination of procedures described earlier (DiScipio et al., 1977a; Pepper & Prowse, 1977). Reduction and carboxymethylation of the intact human factor X was performed by a modified procedure of Crestfield et al. (1963), where β-mercaptoethanol and urea were replaced with dithioerythritol and 7 M guanidine hydrochloride. The heavy and light chains were separated by gel filtration in Sephadex G-100 equilibrated with 0.1 M ammonium bicarbonate. S-Carboxymethylated human factor X light chain was citraconylated according to Atassi & Habeeb (1972). Tryptic digestion of the citroconylated protein (pH 8.8, 37 °C, 6 h) was terminated by the addition of 1 M DFP to a final concentration of 5 mM DFP. Decitraconylation was accomplished by incubating the sample in 9% formic acid for 3 h at 37 °C. Chymotrypsin digestion of the carboxymethylated light chain was carried out for 2–6 h (pH 8.0, 37 °C). In these digestion mixtures, an enzyme-to-substrate mass ratio of 1:100 was employed. Cleavage of methionyl bonds by cyanogen bromide was performed essentially as described by Koide et al. (1978). The carboxyl-terminal sequence of the carboxymethylated (CM) light chain was determined with carboxypeptidase A, B, and Y according to Ambler (1967) and Hayashi (1977).

Peptides arising from tryptic digestion of the CM light chain were initially separated by gel filtration in Sephadex G-50 equilibrated with 0.1 M ammonium bicarbonate. Final purification of selected peptides was achieved by DEAE-Sephacel ion-exchange chromatography. Peptides were applied to a DEAE-Sephacel column (1.8 × 5 cm), equilibrated with 0.05 M ammonium bicarbonate, and were eluted from the column by a linear NaCl gradient (0–1 M) in the equilibrating buffer. Peptides isolated by DEAE-Sephacel subsequently were desalted in a Sephadex G-25 column equilibrated with ammonium bicarbonate prior to sequence analysis.

Peptides resulting from chymotrypsin digest of CM light chain were separated by HPLC. In the separation of peptides by HPLC (Varian liquid chromatograph Model 5000), the mobile phase consisted of 0.1% trifluoroacetic acid, and the mobile-phase modifier was acetonitrile containing 0.08% trifluoroacetic acid (Dunlap et al., 1978; Mahoney & Hermodson, 1980). The concentration of acetonitrile was increased linearly (0.5–5%/min) during 40 min at a flow rate of 2 mL/min.

Automated sequence analyses were performed with a Beckman sequencer (Model 890C) according to Edman & Begg (1967), by using modified programs described by Brauer et al. (1975) and Fernlund & Stenflo (1980). To reduce peptide washout, 3 mg of polybrene was added when the sample was applied to the cup (Tarr et al., 1978). Phenylthiohydantoin (Pth) derivatives of the amino acids were identified by two complementary systems of reverse-phase HPLC (Bridgen et al., 1976; Ericsson et al., 1977). Repetitive yields of 90–95% were routinely observed.

Amino acid analyses were performed on a Dionex Model D-500 amino acid analyzer according to standard procedures (Moore & Stein, 1963; Hugli & Moore, 1972). γ-Carboxyglutamic acid (Gla) analyses were carried out according to Kuwada & Katayama (1981) and were kindly performed by Dr. K. Katayama, Eisai Co., Tsukuba, Japan.

Proton nuclear magnetic resonance spectroscopy was performed at 500 MHz with a Bruker WM-500 spectrometer operating in the Fourier-transform mode. All spectra were recorded at 25 °C. Chemical shifts (δ) were measured from TSP in samples dissolved in ^2H_2O (99.996 atom %). Standard parameters used were a spectral width of ±500 Hz, a delay time of 2 s, and an acquisition time of 3.28 s. In order to ensure complete exchange of hydrogen with deuterium, the lyophilized samples were dissolved in ^2H_2O and taken to dryness three times.

Mass spectrometry was performed on N-acetylated, permethylated derivatives of peptides and methylated Pth-amino acids. Acetylation and permethylation of peptides was performed as follows. Approximately 50–100 nmol of peptide was dissolved in 200 μL of 1 M triethylamine acetate (pH 8), and acetic anhydride (20 μL) was slowly added in small aliquots over a 1-h period. At this point, the reaction mixture was lyophilized and subsequently permethylated essentially by the procedure of Leclercq & Desiderio (1971). Pth-amino acids were methylated in alcoholic hydrogen chloride essentially according to Fraenkel-Conrat & Olcott (1945). The resulting peptides and Pth-amino acid derivatives were dissolved in acetonitrile and chromatographed on a TSK C_{18} column (Toyosoda Co., Tokyo, Japan; 0.4 × 30 cm) in a Varian Model 5000 liquid chromatograph. Mass spectra were obtained by direct insertion probe analyses employing a VG 7070H mass spectrometer (VG Analytical, Manchester, England) operating in chemical ionization mode. On-line data acquisition and processing were performed on a VG 2035 data system. Methane was employed as the reagent gas. Reagent gas source pressure was held at 0.5 Torr, and source temperatures of 220 and 235 °C were employed. The higher temperature was for the analysis of the hexapeptide. In addition, the accelerating voltage of the instrument was lowered from 4 to 3 kV to achieve the extended mass range required for the analysis of the hexapeptide. Approximately 1 nmol of sample, dissolved in acetonitrile, was placed on the probe tip ($^1/_{16}$th-in. diameter; Vespel rod) and the solvent evaporated. The probe tip was then inserted into the ion source and its temperature elevated to source temperature. Under these conditions, the peptide yielded the characteristic sequence ions, as well as small amounts of a $(M + C_2H_5)^+$ addition ion. The methylated Pth-amino acids yielded characteristic fragmentation ions as well as MH^+, $(M + CH_3)^+$, and $(M + C_2H_5)^+$ addition ions.

Results

Amino-Terminal Sequence. Sequencer analyses of the intact carboxymethylated (CM) human factor X light chain provided assignment of the first 41 residues (Figure 1). Eleven glutamyl residues (at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, 32, and 39) showed yields much lower than anticipated. These residues were later identified as the modified amino acid γ-carboxyglutamic acid (see below). Presumably, glutamyl

[1] Abbreviations: Pth, phenylthiohydantoin; Atz, anilinothiazolinone; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; Bha, β-hydroxyaspartic acid; Gla, γ-carboxyglutamic acid; TSP, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt; DEAE, diethylaminoethyl; DFP, diisopropyl phosphorofluoridate.

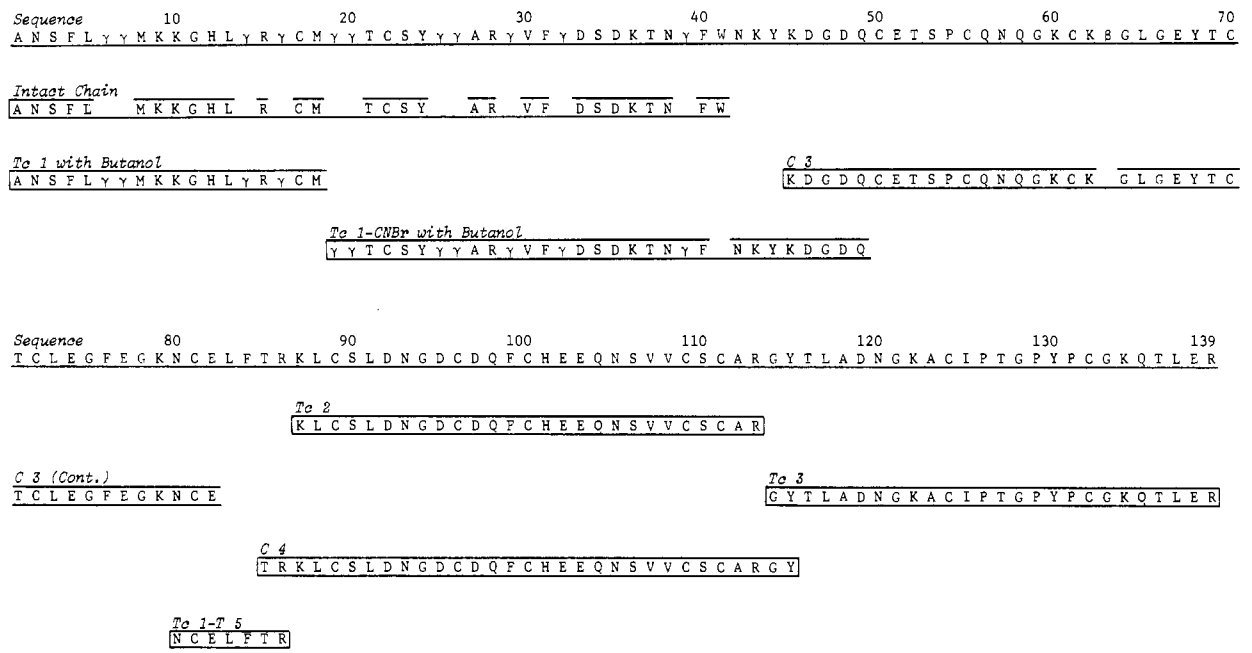

FIGURE 1: Summary of proof of sequence of light chain of human factor X. The one-letter code within the bars designates the amino acids identified after Edman degradation. The length of each bar indicates the number of residues analyzed in the peptide. Enclosed tops of the bars indicate the portion of the sequence that is proven; gaps in the upper enclosure indicate portions not identified in the sequence; an open end to the right indicates the fragment is longer than the indicated bar. Tryptic, chymotryptic, and cyanogen bromide peptides are designated by the letters T, C, and CNBr, respectively. Tryptic peptides obtained from citraconylated substrates are designated by the prefix Tc.

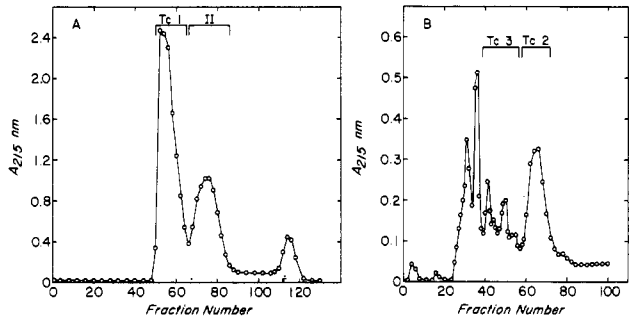

FIGURE 2: (A) Fractionation of a tryptic digest of citraconylated CM light chain (400 nmol). The Sephadex G-50 column was equilibrated with 0.1 M ammonium bicarbonate (pH 8.8). Fractions (2.2 mL) were collected at a flow rate of 6.6 mL/h. The elution was monitored by absorbance at 215 nm. (B) Separation of Tc2 and Tc3 on DEAE-Sephacel. The column (1.8 × 5 cm) was equilibrated with 0.1 M ammonium bicarbonate (pH 8.8). At fraction 20, a NaCl gradient was applied consisting of 200 mL of equilibrating buffer and 200 mL of equilibrating buffer containing 1 M NaCl. Three-milliliter fractions were collected.

residues were observed in these positions due to decarboxylation of γ-carboxyglutamic acid during the acidic phase of the Edman degradation.

Cleavage at Arginine. A tryptic digest of the citraconylated CM human factor X light chain was resolved into two peptide peaks by gel filtration in Sephadex G-50 (Figure 2A). Fraction I was composed of a single peptide (Tc1) that constituted the amino-terminal portion of the light chain. Fraction II contained two peptides, Tc2 and Tc3. Fraction III (fractions 110–120) did not contain any detectable peptide material as determined by amino acid analysis. Peptides Tc2 and Tc3 were separated by DEAE-Sephacel chromatography (Figure 2B). The first peak did not contain any detectable peptide material. The amino acid compositions of Tc1, Tc2, and Tc3 appear in Table I. Each tryptic peptide isolated was found to contain at least one arginine residue by amino acid analysis. Peptides Tc2 and Tc3 were each sequenced to their carboxyl-terminal arginine residues (Figure 1). Due to partial cyclization of an Asn-Gly sequence in each of the tryptic peptides, significant decreases in yield were observed following these residues. This did not interfere, however, with the assignments of subsequent residues.

Cleavage with Chymotrypsin. The peptides resulting from the digestion of the CM light chain with chymotrypsin were purified by HPLC on the μBondapak C_{18} column (Figure 3). One fraction, labeled fraction IV, was heterogeneous and was further fractionated into three peptides by chromatography on a Synchropak RP-P column (Figure 3, insert). The amino acid analyses of the peptides obtained from chymotrypsin digestion appear in Table I. Several peptides (C3a, C3b, C4a, and C4b) were found to be fragments of larger peptides (C3 and C4), indicating that the conditions used did not yield complete cleavage at all susceptible sites. Peptide C3 was sequenced to within two residues of its carboxyl terminus. A continuous sequence was observed with the exception of position 63 of the light chain. At this position, a small doublet peak was observed that eluted slightly ahead of Pth-aspartic acid by HPLC. No overlaps were observed with the tryptic peptides previously sequenced. Peptide C4, on the other hand, was sequenced completely and found to contain the tryptic peptide Tc2, extending it by two residues in both directions, and thus providing an overlap between Tc2 and Tc3 (Figure 1).

Trypsin Cleavage of Decitraconylated Tc1. The overlap between C3 and C4 was obtained by digesting peptide Tc1 with trypsin following decitraconylation of the peptide. A peptide (Tc1-T5) containing the carboxyl-terminal portion of Tc1 was isolated by HPLC on the μBondapak C_{18} column. This peptide was sequenced completely and established the overlap between C3 and C4 (Figure 1).

Carboxyl-Terminal Sequence of CM Light Chain. Treatment of 150 nmol of CM light chain with carboxypeptidase A failed to release any detectable amino acids. However, upon the addition of carboxypeptidase B to this incubation mixture, a total of 148 nmol of arginine was released in 3 h. In a similar experiment, digestion of 120 nmol

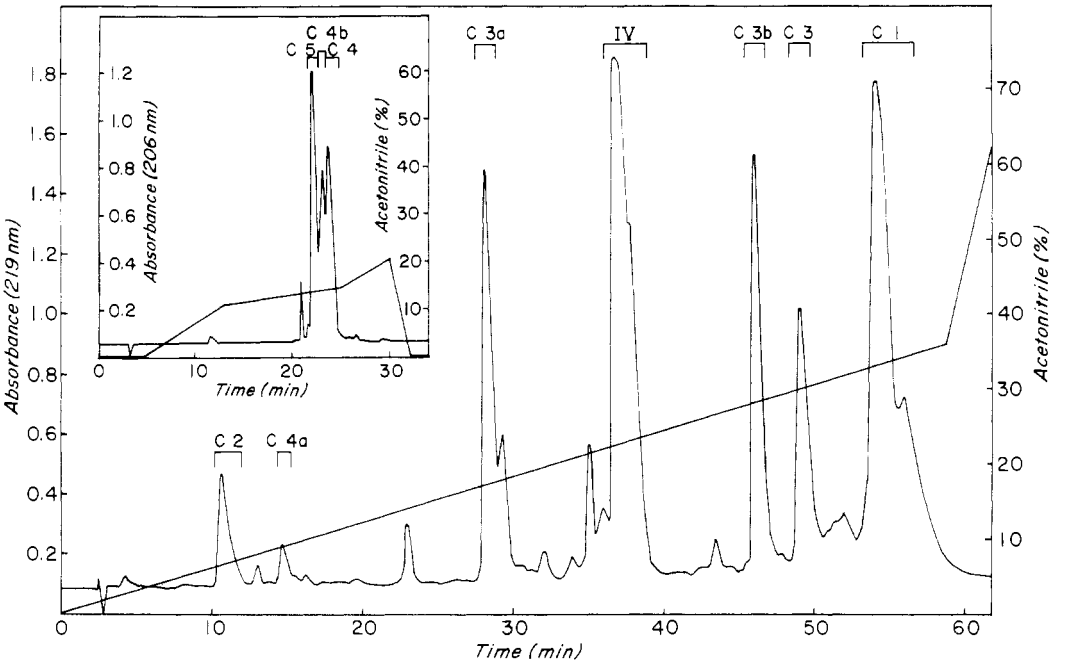

FIGURE 3: Separation of peptides obtained by cleavage of CM light chain by chymotrypsin. The digest was separated on a column of μBondapak C_{18} equilibrated with 0.1% trifluoroacetic acid in a 0–35% acetonitrile gradient. (Insert) Separation of fraction IV on a Synchropak RP-P column equilibrated with 0.1% trifluoroacetic acid. Separation was achieved with an 8-min 0–10% gradient in acetonitrile, followed by a 20-min 10–14% gradient in acetonitrile.

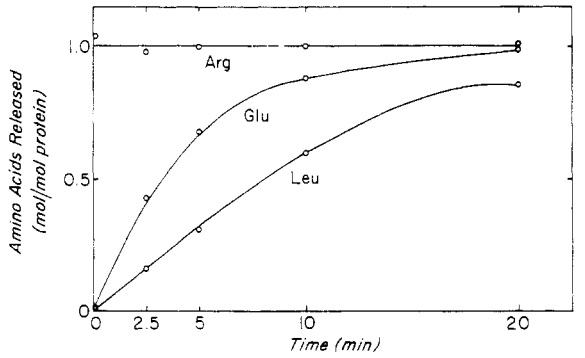

FIGURE 4: Time course of carboxypeptidase Y digestion of intact CM light chain after a 30-min preincubation with carboxypeptidase B. Aliquots of the digestion mixture were removed at 0, 2.5, 5, 10, and 20 min and subjected directly to amino acid analysis.

of CM light chain with carboxypeptidase B, 120 nmol of arginine was released in 30 min. Following lyophilization of this reaction mixture, a timed digestion with carboxypeptidase Y was performed (Figure 4). Glutamic acid and leucine were sequentially released, indicating that the carboxyl-terminal sequence of the factor X light chain was Leu-Glu-Arg. This sequence is identical with that observed for the carboxyl terminus of peptide Tc3.

Identification of γ-Carboxyglutamic Acid Residues. Authentic Pth-γ-carboxyglutamic acid (Pth-Gla) was prepared by sequencing bovine prothrombin, substituting 2-butanol for *n*-butyl chloride in the extraction step of the Edman degradation (Fernlund & Stenflo, 1980). The Pth-Gla obtained by this procedure served as a reference material for the identification of Pth-Gla from the intact CM light chain and its fragments. Sequencer analyses of the intact CM light chain were performed with the modified 2-butanol program, and a strong, continuous sequence was observed through residue 18. γ-Carboxyglutamic acid was positively assigned to positions 6, 7, 14, and 16. Cyanogen bromide cleavage of peptide Tc1 at room temperature yielded a large peptide isolated by gel filtration (Sephadex G-50), which began at residue 19 and overlapped with peptide C3 (Figure 1). Upon sequencing this peptide by the modified 2-butanol program, the remaining seven Gla residues were identified. Thus, by sequence analysis, human factor X contains 11 residues of Gla, which agreed nicely with the value of 11.1 observed for the intact CM light chain when assayed for Gla by the procedure of Kuwada & Katayama (1981).

Identification of Residue 63 as β-Hydroxyaspartic Acid. As mentioned earlier, automated Edman degradation of peptide C3 (residues 45–84) yielded a Pth derivative that eluted as a very small doublet in HPLC migrating ahead of Pth-Asp. The yield at this cycle was approximately 1%, while the yield of glycine in the following cycle increased to about 95%. In order to identify residue 63, peptide C3a (residues 45–68) was digested with trypsin, and the fragments were isolated by HPLC. Trypsin digest of C3a yielded two peptides, C3a-T1 (residues 45–60) and peptide C3a-T2 (residues 61–68). The composition of peptide C3a-T2 was X_1, Cys_1, Glu_1, Gly_2, Leu_1, Tyr_1, and Lys_1. Residue X migrated after cysteic acid and before (carboxymethyl)cysteine on the amino acid analyzer. The elution position of residue X corresponded closely to the known elution positions of either *erythro*-β-hydroxyaspartic acid or methionine sulfoxide (Hamilton, 1968). The elution position of residue X from acid hydrolysates of peptide C3a-T2 was unchanged by either prolonged acid hydrolysis or performic acid oxidation of the peptide prior to acid hydrolysis. This result suggested that the unknown residue was not methionine sulfoxide or (carboxymethyl)-cysteine. In addition, the elution position of residue X in the analyzer was constant regardless of whether the C3a-T2 peptide was derived from S-carboxymethylated light chain or S-pyridylethylated light chain as starting material. Prolonged digestion of peptide C3a-T2 with aminopeptidase and subsequent amino acid analysis of the digest yielded an identical composition with that observed by acid hydrolysis, which indicated that the elution position of residue X appeared to be unaffected by acid hydrolysis per se. While the elution position of residue X appeared unchanged by acid hydrolysis, the quantity of this residue progressively decreased with increasing

Table I: Amino Acid Compositions of Peptides Derived from Human Factor X Light Chain[a]

	intact, 1–139[b]	Tc1, 1–86	Tc2, 87–113	Tc3, 114–139	C1, 1–40	C2, 42–44	C3, 45–84	C3a, 45–68	C3b, 69–84	C4, 85–115	C4a, 85–88	C4b, 89–115	C5, 116–139
CMC[c]	14.8 (15)	8.6 (8)	4.8 (5)	1.9 (2)	2.1 (2)		5.8 (6)	3.3 (3)	2.8 (3)	5.1 (5)		4.7 (5)	2.1 (2)
Asx	16.1 (16)	9.7 (9)	4.8 (5)	2.1 (2)	4.0 (4)	1.0 (1)	4.2 (4)	3.0 (3)	1.2 (1)	5.1 (5)		4.8 (5)	2.2 (2)
Thr	8.5 (9)	5.3 (6)		3.0 (3)	1.9 (2)		2.8 (3)	0.9 (1)	1.8 (2)	0.9 (1)	0.9 (1)		2.8 (3)
Ser	7.1 (7)	3.9 (4)	2.3 (3)		2.6 (3)		1.3 (1)	1.0 (1)		2.7 (3)		2.6 (3)	
Glx[d]	25.3 (25)	18.6 (19)	4.0 (4)	2.1 (2)	11.0 (11)		7.6 (8)	4.9 (5)	3.1 (3)	4.1 (4)		4.0 (4)	2.2 (2)
Pro	4.9 (4)	1.2 (1)		3.1 (3)			1.3 (1)	1.0 (1)					2.9 (3)
Gly	12.0 (12)	7.2 (7)	1.3 (1)	3.7 (4)	1.3 (1)		6.0 (6)	3.9 (4)	2.3 (2)	2.2 (2)		2.2 (2)	3.2 (3)
Ala	5.5 (5)	2.4 (2)	1.2 (1)	2.0 (2)	2.0 (2)					1.1 (1)		1.1 (1)	2.0 (2)
Val	3.2 (3)	1.7 (1)	2.0 (2)		1.0 (1)					1.7 (2)		2.0 (2)	
Met	1.9 (2)	2.1 (2)			1.9 (2)								
Ile	0.9 (1)			1.1 (1)									0.9 (1)
Leu	9.0 (9)	5.0 (5)	2.0 (2)	2.0 (2)	2.0 (2)		2.9 (3)	1.0 (1)	2.0 (2)	2.0 (2)	1.0 (1)	1.0 (1)	2.0 (2)
Tyr	4.2 (5)	3.2 (3)		1.4 (2)	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)		0.9 (1)		1.5 (1)	0.8 (1)
Phe	5.6 (6)	4.6 (5)	0.9 (1)		3.0 (3)		2.0 (2)		1.7 (2)	1.1 (1)		1.2 (1)	
His	2.0 (2)	1.2 (1)	1.0 (1)		1.0 (1)					1.1 (1)		0.9 (1)	
Lys	10.5 (11)	7.8 (8)	1.3 (1)	2.4 (2)	2.9 (3)	1.0 (1)	4.0 (4)	2.9 (3)	1.1 (1)	0.9 (1)	1.0 (1)		2.8 (2)
Arg	4.9 (5)	2.9 (3)	1.0 (1)	0.8 (1)	2.0 (2)					2.1 (2)	1.0 (1)	1.0 (1)	0.8 (1)
Trp	1.2 (1)	ND (1)[e]											
Bha	ND (1)	ND (1)					ND (1)	ND (1)					
Gla	11.1 (11)	ND (11)			ND (11)								
no. of residues	139	86	27	26	40	3	40	24	16	31	4	27	24
yield (%)		95	54	56	25	81	26	77	80	30	44	23	31

[a] All values are from 24-h hydrolysates. [b] Residue numbers (see Figure 1). [c] S-(Carboxymethyl)cysteine. [d] Also contains the 11 residues of Gla. [e] ND, not determined.

hydrolysis time and paralleled the degradation rate normally associated with serine (~30–40% loss in 96-h hydrolysis time). Automated Edman degradation of peptide C3a-T2 yielded the sequence Cys-Lys-(X)-Gly-Leu-Gly-Glu-Tyr. As before, the Pth derivative at cycle 63 exhibited an extremely low yield (~1%) of a double peak that migrated ahead of Pth-Asp by HPLC. However, manual Edman degradation (Tarr, 1982) of peptide C3a-T2, where the Atz-amino acids are extracted with a heptane/ethyl acetate mixture (1:5) instead of *n*-butyl chloride, produced good yields (~95%) of a Pth derivative at cycle 63. HPLC analysis showed that this product migrated as a doublet at precisely the elution position observed in automated Edman degradation samples. Interestingly, when the HPLC effluent of this sample (Pth-X) was monitored at 313 nm, a significant peak was observed comigrating with Pth-alanine, suggesting that the Pth-X sample contained a small amount of material with an unsaturated carbon–carbon bond. Back-hydrolysis of the Pth-X obtained from automated Edman degradation and subsequent amino acid analysis failed to produce any detectable amino acid, suggesting that extraction of the Atz-X from the sequencer cup with *n*-butyl chloride was inefficient and accounted for the low yield observed at this cycle.

By process of elimination, the amino acid at position 63 appeared to correlate with residue X observed in amino acid analysis of peptide C3a-T2. A reexamination of amino acid analysis chromatograms of human factor X light chain revealed a peak with approximately the same area as isoleucine (one residue) that migrated with a retention time identical with that of residue X derived from peptide C3a-T2. This peak was also present in the amino acid analysis chromatograms of bovine factor X light chain and bovine protein C light chain but not in the heavy chains of these proteins. The unique elution position of residue X on Dowex columns provided us with the means to isolate milligram quantities of this residue from preparative-scale acid hydrolysates of human factor X light chain. In view of the fact that residue X eluted close to (carboxymethyl)cysteine in the analyzer, S-pyridylethylated human factor X light chain was chosen as the starting material. Accordingly, 60–70 mg of S-pyridylethylated human factor X light chain was hydrolyzed for 24 h (110 °C), evaporated to dryness twice, and redissolved in 3 mL of 0.1 M ammonium formate (pH 2.2). To facilitate localization of residue X in the effluent, 5 μCi of [^{3}H]aspartic acid was added to the hydrolysate prior to its application to a Dowex 50W-X8 (200–400 mesh) column (0.7 × 28 cm) equilibrated with 0.1 M ammonium formate (pH 2.2). After sample application, the column was eluted with 15 mL of 0.1 M ammonium formate (pH 2.9) followed by an ammonium formate gradient composed of 25 mL of 0.1 M ammonium formate (pH 2.9) and 25 mL of 0.2 M ammonium formate (pH 3.65). The elution position of residue X was determined as follows. Fifty microliters of each fraction eluting before [^{3}H]aspartic acid was lyophilized to remove ammonium formate. The dried residue was redissolved in 100 μL of water, and a 5-μL aliquot applied to Whatman No. 3 MM paper. The amino acid was then visualized with fluorescamine (0.01% in acetone). The remaining portion (95 μL) of those fractions that exhibited a positive reaction with fluorescamine was mixed with 25 nmol of norleucine and applied directly to the amino acid analyzer. Those fractions that contained pure residue X by amino acid analysis were then pooled, lyophilized, and stored at −20 °C.

Inasmuch as residue X exhibited a retention time on Dowex columns similar to *erythro*-β-hydroxyaspartic acid (EBha), our initial attempt to identify this residue focused on a com-

parison of residue X with authentic L-EBha. Two-dimensional thin-layer chromatography (Woods & Wang, 1967) of residue X on micropolyamide sheets indicated a mobility identical with that of authentic EBha. HPLC chromatograms of Pth-X and Pth-EBha were indistinguishable and eluted ahead of Pth-Asp. Both of the former Pth derivatives eluted as a major peak followed by a minor peak, but the area of the minor peak appeared to be slightly larger in the Pth-X sample. HPLC chromatography of authentic Pth-*threo*-β-hydroxyaspartic acid (Pth-TBha), a stereoisomer of EBha, showed a major peak with a retention time identical with that of the minor peak observed in the Pth-X and Pth-EBha samples. On the other hand, a small peak (roughly 5% of the area of the major peak) was observed to precede the Pth-TBha with a retention identical with the major peak observed for both Pth-X and Pth-EBha. Interestingly, by HPLC all three Pth derivatives revealed a very minor A_{254} peak with a retention time close to that of Pth-Ala. When the effluent was monitored at 313 nm, a significant peak was observed in this position in all three Pth samples in roughly the same amounts. By analogy with serine and threonine, it is not inconceivable that a certain amount of dehydration of these amino acids yielded an unsaturated β-elimination product with an increased hydrophobic character. Thus, from HPLC analyses, Pth-X appeared to correspond to roughly a 95%:5% erythro:threo mixture of L-β-hydroxyaspartic acid.

Further evidence to support the identity of residue X as *erythro*-β-hydroxyaspartic acid was derived from proton NMR spectroscopy. The 500-MHz NMR spectrum of residue X in ^2H_2O with TSP as the internal standard revealed a doublet corresponding to a methine proton at δ 4.03 (J = 3.66 Hz) and another doublet corresponding to an adjacent methine proton at δ 4.33 (J = 3.66 Hz) (Figure 5C). The chemical shift difference between the two sets of doublets is 0.30 ppm. Two additional minor doublet peaks are seen at δ 4.05 and δ 4.55, producing a chemical shift difference of 0.50 ppm. Irradiation of the doublet at δ 4.55 showed a slight nuclear Overhauser enhancement of the doublet at δ 4.05, indicating that these two methine protons are in the same molecule and adjacent to one another. The NMR spectra of authentic L-EBha and L-TBha are also presented in Figure 5. The NMR spectrum of L-EBha (Figure 5B) revealed two doublet peaks, which, relative to TSP, exhibited chemical shift differences of 0.29 ppm, while L-TBha (Figure 5A) revealed two doublet peaks with a chemical shift difference of 0.52 ppm. Thus, by this technique, residue X appeared to consist of L-EBha with a small amount of the isomeric L-TBha. If one assumes that residue X is indeed L-EBha, the apparent absence of L-TBha in the NMR spectrum of authentic L-EBha suggests that during isolation a small amount of residue X is converted to its stereoisomer. A quick calculation of the rotational bond energy differences between EBha and TBha revealed a difference of roughly 500 cal. Thus, it is not unreasonable, from an energetic standpoint, that a small amount of EBha (residue X) isomerizes to TBha during the isolation of this residue.

Final proof of the structure of residue X was achieved by MS. The methylated Pth derivatives of residue X, authentic EBha, and authentic TBha were chosen for this analysis. In addition, an acetylated, permethylated hexapeptide containing residue X at the amino terminus (residues 63–68) was prepared and subjected to sequence analysis by MS in order to obtain a confirmatory mass for residue X. The acetylated, permethylated hexapeptide was prepared by subjecting peptide C3a-T2 to two cycles of manual Edman degradation followed by acetylation and permethylation. Our initial mass spectrometry analyses were performed on derivatives purified on Waters μBondapak C_{18} columns. Both NMR and MS spectra of these derivatives revealed several unidentified impurities, presumably organic material leeching from the column support. After considerable trial and error, the TSK C_{18} column was found to yield acceptable levels of impurities that did not complicate the interpretation of the spectra. Consequently, all samples were purified on this column prior to mass spectrometry.

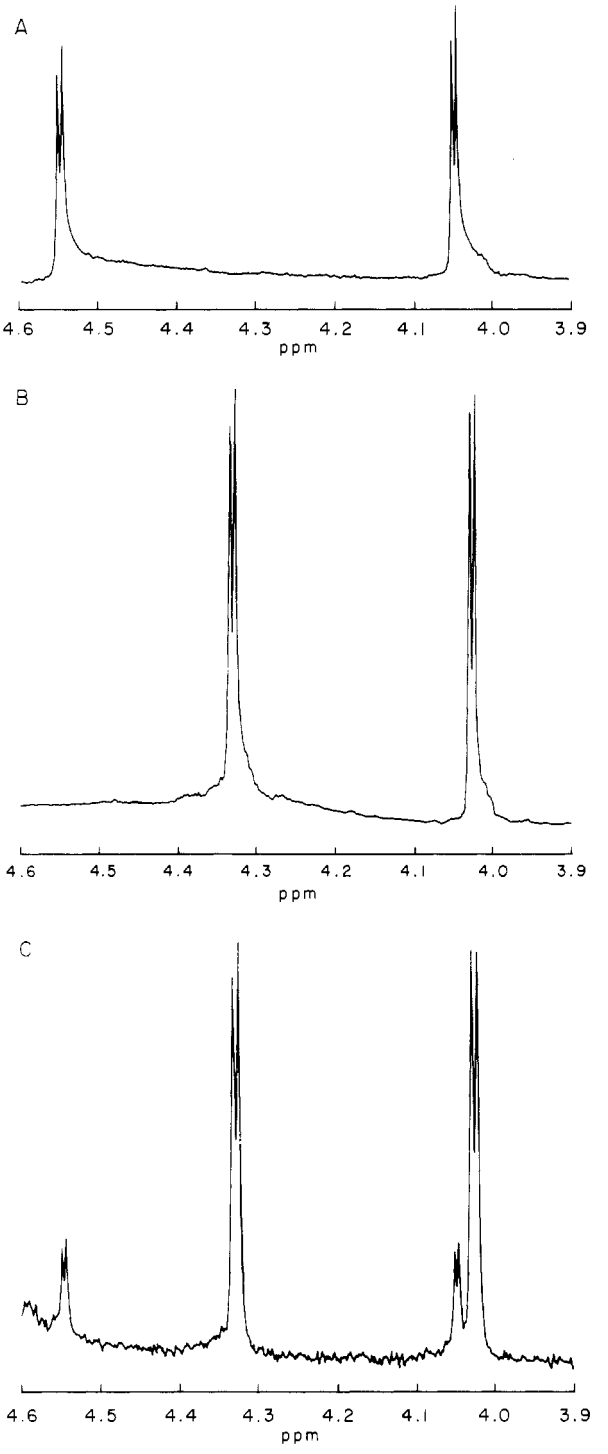

FIGURE 5: Proton nuclear magnetic resonance spectra (500 MHz) of authentic L-*threo*-β-hydroxyaspartic acid (A), authentic L-*erythro*-β-hydroxyaspartic acid (B), and isolated residue 63 (C). The scale is in ppm (δ) and relative to internal TSP.

The chemical ionization spectrum of methylated Pth-X is presented in Figure 6. This spectrum was indistinguishable from that observed for either methylated Pth-EBha or meth-

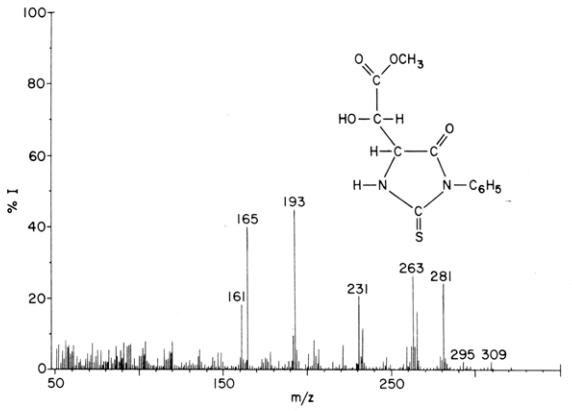

FIGURE 6: Chemical ionization mass spectrum of a methyl esterified phenylthiohydantoin derivative of residue 63 obtained at 200 °C.

ylated Pth-TBha prepared in the same manner as the unknown. Abundant ions at m/z 281, 263, 231, 193, 165, and 161 were consistently found in all three spectra. The ion at m/z 281 corresponds to the MH^+ ion of the methyl ester of Pth-Bha. Under the conditions chosen for the methylation of all three compounds, the β-hydroxyl group was not methylated. Dehydration of MH^+ produced $MH^+ - H_2O$ at m/z 263, while the ion at m/z 231 in all probability reflects the loss of a methoxy group from the Pth-dehydroBha, i.e., $MH^+ - (H_2O + CH_3OH)$. The fragment ion at m/z 193 is commonly observed in chemical ionization mass spectra of Pth derivatives (Fairwell & Brewer, 1980) and in this case represents $MH^+ - (COOCH_3 + CHO)$. The ions occurring at m/z 295 and 309 correspond to addition ions $(M + CH_3)^+$ and $(M + C_2H_5)^+$, respectively. The molecular rearrangements leading to fragment ions at m/z 165 and 161 have not been established. At this point, it is uncertain as to whether these species reflect fragmentation ions unique to β-hydroxyaspartic acid or arise from an impurity coeluting with the sample from either the column support or solvent systems. The relative abundancy of these ions, however, was similar in all three spectra.

Additional information regarding the mass of residue X was obtained by subjecting an acetylated, permethylated hexapeptide containing residue X to mass spectrometry. The chemical ionization mass spectrum of hexapeptide containing residues 63–68 is shown in Figure 7. As anticipated, both N-terminal and C-terminal sequence ions were observed (Morris, 1979). The spectrum was interpreted as follows: m/z 216 indicates N-terminal β-hydroxyaspartic acid and is verified by the expected loss of carbon monoxide and methanol to yield fragment ions of m/z 188 and 184, respectively. The fragment ion at m/z 156 indicates loss of CH_3OH and CO from m/z 216. The peak of m/z 146 is a protonated imine ion of β-hydroxyaspartic acid. The ion at m/z 116 probably results from the loss of CO and CH_3CONCH_3 from m/z 216. The weak peak at m/z 287 is in agreement with the second residue being glycine, and the fragment ion at m/z 255 reflects the loss of CH_3OH from m/z 287. The peak at m/z 227 is the combined loss of CO and CH_3OH from m/z 287. The intense peak at m/z 224 indicates C-terminal tyrosine. A mass difference of 127 from m/z 287 to 414 places leucine in the third position, giving the sequence of Bha-Gly-Leu. Loss of CH_3OH from m/z 414 yields a peak at m/z 382, and loss of CO from this ion produces a peak at m/z 354. Fragment ions at m/z 485 and 642 correspond to glycine and glutamic acid in positions 4 and 5 in the hexapeptide, respectively. The intense peaks at m/z 381, 452, 579, and 650 correspond to successive C-terminal cleavages producing fragments of Glu-Tyr, Gly-Glu-Tyr, Leu-Gly-Glu-Tyr, and Gly-Leu-Gly-Glu-Tyr, respectively. The intense peak at m/z 349 probably arises from the loss of CH_3OH from the m/z 381 ion. No MH^+ ion was observed for the hexapeptide. However, a $(M + C_2H_5)^+$ addition ion at m/z 893 was present (not shown).

Taken collectively, the chemical ionization mass spectral data of methylated Pth-X and an acetylated, permethylated hexapeptide containing residues 63–68 are consistent with a mass value of 149 for the underivatized unknown residue at position 63. This mass value is identical with that of Bha. Furthermore, the fragmentation pattern observed for the methylated Pth-X derivative was virtually identical in terms of mass ions and relative abundance with that observed for the methylated Pth derivatives of authentic EBha and TBha. Thus, on the basis of all the evidence presented, it is reasonable to conclude that residue 63 is indeed L-*erythro*-β-hydroxyaspartic acid, a heretofore unrecognized amino acid found in peptide linkage.

Localization of β-Hydroxyaspartic Acid in Light Chains of Bovine Factor X and Bovine Protein C. As noted above, amino acid chromatograms of the light chains of bovine factor X and bovine protein C indicated that these peptides also contained approximately one residue of Bha. In order to define the position of this residue in the sequence of these polypeptides, S-carboxymethylated or S-pyridylethylated derivatives of each polypeptide were digested sequentially with chymotrypsin and trypsin, and the peptides arising from each

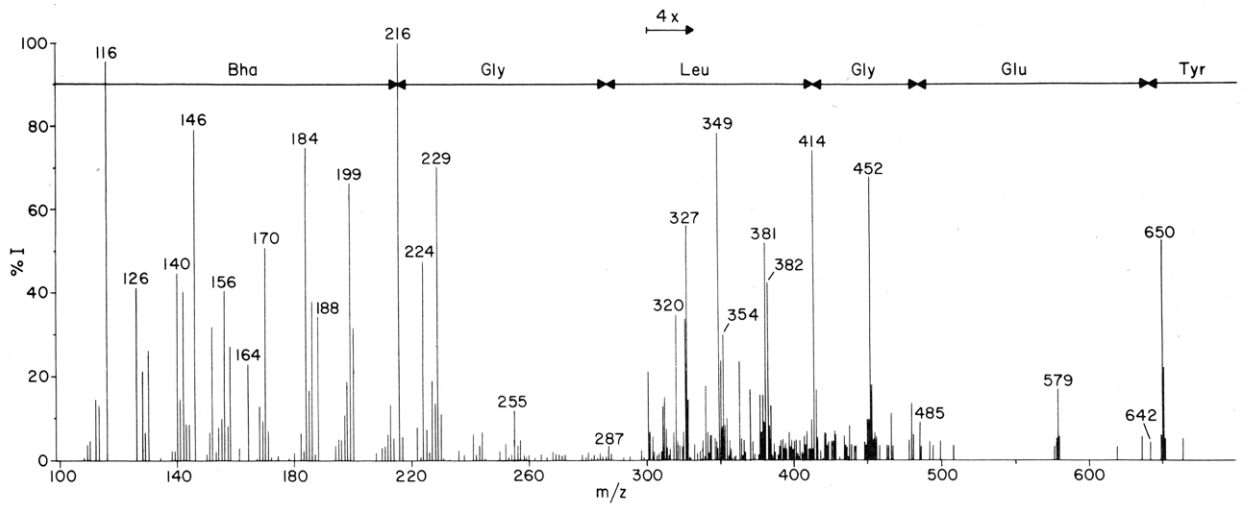

FIGURE 7: Mass spectrum of acetylated and permethylated hexapeptide (residues 63–68) obtained at 235 °C. The sensitivity was increased 4-fold beginning at m/z 300.

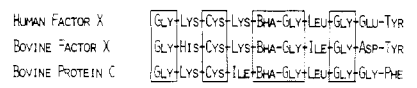

FIGURE 8: Amino acid sequences surrounding the β-hydroxyaspartic acid (Bha) residue in the light chains of human factor X, bovine factor X, and bovine protein C. Amino acids that are identical in all of the peptides are shown in blocks. The Bha residue corresponds to residue 63 in the light chains of human and bovine factor X and residue 71 in the light chain of bovine protein C.

digestion were isolated by HPLC. Those peptides containing Bha by amino acid analysis were then subjected to manual Edman degradation (Tarr, 1982). In the case of bovine factor X light chain, a Bha-containing peptide was isolated that yielded a sequence of Bha-Gly-Ile-Gly-Asp-Tyr. An inspection of the published sequence for bovine factor X light chain (Enfield et al., 1980) revealed that this peptide corresponded to residues 63–68 in the intact light chain. The presence of β-hydroxyaspartic acid at cycle 63 presumably accounts for the discrepancy in sequence vs. composition aspartic acid values, as well as the low yield of asparagine at cycle 63 reported by Enfield et al. (1980). Chymotrypsin and trypsin digestion of the S-carboxymethylated light chain of bovine protein C and subsequent HPLC yielded an octapeptide with a sequence of Cys-Ile-Bha-Gly-Leu-Gly-Gly-Phe, which corresponded to residues 69–76 in the intact light chain as reported by Fernlund & Stenflo (1982). In that study, residue 71 was tentatively identified as cysteine. However, as pointed out by these investigators, a poor yield was observed at this cycle, and the Pth derivative of cycle 71 yielded an unidentified doublet peak eluting approximately in the position of aspartic acid in HPLC. Our finding of Bha at position 71 is also consistent with the anomolously high negative charge observed by Fernlund & Stenflo (1982) for a peptide containing residue 69–77 (TR2a). The apparent homology of the sequences surrounding Bha in the light chains of human factor X, bovine factor X, and bovine protein C is presented in Figure 8. The most prominent feature of this alignment is the conservation of cysteine and glycine residues surrounding the β-hydroxyaspartic acid.

Discussion

The complete amino acid sequence of the light chain of human factor X is shown in Figure 9. The protein consists of 139 residues and the summation of the individual weights of the residues yields a molecular weight of 16 211 for the polypeptide. Eleven residues of γ-carboxyglutamic acid (Gla) were found in the amino-terminal region of the polypeptide at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, 32, and 39. The 11 Gla residues found in human factor X light chain is one less than observed in bovine factor X light chain (Enfield et al., 1980). In the bovine light chain, a Gla residue at position 35 is conservatively replaced by aspartic acid in the human light chain.

An overall homology of approximately 70% was observed between the amino acid sequence of the human and bovine factor X light chains. The homology is highest in the first 100 residues (~80%) and decreases to roughly 50% in the remaining 39 residues of the peptide. The substantial decrease in homology in the latter region was unexpected as both human and bovine prothrombin (Mann et al., 1981), as well as human and bovine factor IX (Kurachi & Davie, 1982; Katayama et al., 1979), exhibit homologies of 85–90% in the first 150 residues of the amino-terminal portions of these proteins.

By sequence analysis, human factor X light chain contains 15 half-cystinyl residues. In all likelihood, the half-cystine residues at positions 17 and 22 in the human factor X light

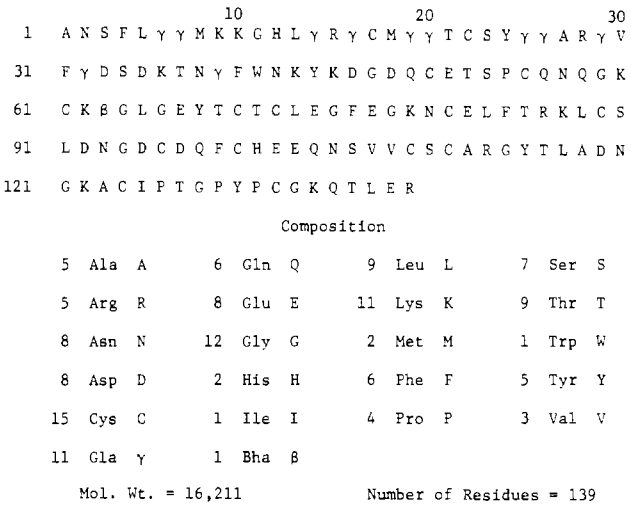

FIGURE 9: Amino acid sequence and composition of light chain of human factor X. The amino acid composition below the sequence defines the one-letter code notations.

chain form an intrachain disulfide bridge similar to that reported for the bovine molecule (Enfield et al., 1980). The remaining 13 half-cystinyl residues in the carboxyl-terminal region of the light chain (residues 50–139) suggest an extensively cross-linked domain not unlike that observed in the corresponding regions of the light chains of bovine factor X (Enfield et al., 1980) and bovine protein C (Fernlund & Stenflo, 1982). By analogy with the bovine molecule, one of these 13 half-cystinyl residues presumably is disulfide paired to another half-cystinyl residue in the heavy chain of the molecule.

In this study, a carboxyl-terminal sequence of Leu-Glu-Arg was observed for the human factor X light chain. By comparison, the bovine factor X light chain contains an additional residue and possesses a carboxyl-terminal sequence of Gly-Arg-Ser (Enfield et al., 1980). Current evidence suggests that factor X is synthesized as a single-chain precursor in both rat (Graves et al., 1982) and human (Fair & Edgington, 1982) hepatocytes. Considering that the amino-terminal residue of the heavy chain of human factor X is serine (DiScipio et al., 1977b), it is tempting to speculate that in the secretion process, single-chain factor X is cleaved by an elastase-like protease in bovine hepatocytes and a trypsin-like protease in human hepatocytes to produce the mature two-chain protein.

The proof of the structure of the light chain of human factor X presented no technical problems. Identification of residue 63, however, proved to be a more difficult task. On the basis of proton NMR and MS data, we are reasonably certain that this residue is *erythro*-β-hydroxyaspartic acid. To our knowledge, this represents the first instance where this amino acid has been reported in peptide linkage. In addition, β-hydroxyaspartic acid was subsequently found in the corresponding regions of the light chains of bovine factor X and bovine protein C. Inspection of the amino acid sequence surrounding this residue in these proteins revealed a high degree of homology with a predominance of conserved glycine residues in the vicinity of the β-hydroxyaspartic acid residue (Figure 8). In all likelihood, the conserved sequence surrounding this residue reflects a unique recognition site for the posttranslational hydroxylation of aspartic acid at position 63. Of particular interest is the glycine residue carboxyl to the β-hydroxyaspartic acid residue. In the hydroxylation of prolyl and lysyl residues in collagen and elastin, a glycine residue carboxyl to either proline or lysine is an absolute requirement for the action of the hydroxylating enzymes (Kivirikko &

Myllylä, 1980). Unlike the variable efficiency observed in the hydroxylation of susceptible prolyl and lysyl residues in collagen (Cardinale & Udenfriend, 1974), hydroxylation of aspartic acid at position 63 in the light chain of factor X appeared to be a quantitative reaction, as no detectable aspartic acid was observed at this cycle.

The biological function is presently unknown for β-hydroxyaspartic acid in both factor X and protein C. These proteins exist in plasma as two-chain precursors to serine proteases and have similar activation mechanisms. Furthermore, these proteins belong to a growing family of plasma proteins that require vitamin K for carboxylation of specific glutamic acid residues in the molecule. Presently, seven plasma proteins (prothrombin, factor VII, factor IX, factor X, protein C, protein S, and protein Z) are known to undergo posttranslational carboxylation of 10–12 glutamic acid residues in their amino-terminal regions. The mechanism that signals for the termination of carboxylation in the peptide backbone of these proteins remains an enigma. The possibility that β-hydroxyaspartic acid represents a unique recognition site for the carboxylating enzyme in factor X and protein C biosynthesis appears to be doubtful from several considerations. First, sequence and compositional data for other vitamin K dependent proteins including human and bovine factor IX (Kurachi & Davie, 1982; Katayama et al., 1979), as well as human and bovine prothrombin (Mann et al., 1981; Degen et al., 1983), do not suggest the presence of β-hydroxyaspartic acid in the entire molecule, let alone the corresponding regions of these proteins. Thus, β-hydroxyaspartic acid does not appear to be common to all the vitamin K dependent proteins. Second, temporal considerations of such a mechanism would obviously require the posttranslational hydroxylation of aspartic acid at position 63 either prior to or concurrent with the carboxylation reactions. Last, the light chains of factor X and protein C each contain a glutamic acid residue in position 51 that is not carboxylated. Thus, it would appear unlikely that β-hydroxyaspartic acid plays a significant role, if any at all, in the carboxylation of glutamic acid residues in the amino termini of these proteins.

The possibility that β-hydroxyaspartic acid may be instrumental in the physiological activation of these proteins stems from recent, provocative findings by Esmon and his co-workers. These investigators showed that removal of the Gla domain in the light chain of bovine protein C (residues 1–41) by chymotryptic cleavage resulted in a Gla-domainless protein C that still required calcium ions for its activation by a complex of thrombin and thrombomodulin (Esmon et al., 1983). Interestingly, the rate of activation of Gla-domainless and native protein C by this complex appeared indistinguishable. Thus, Gla residues do not appear to be essential for the physiological activation of protein C. Analysis of Gla-domainless protein C by fluorescence emission spectroscopy revealed a calcium-dependent conformational change in the protein that correlated with the ability of the ion to support the activation of the Gla-domainless protein C (Johnson et al., 1983). On the basis of these data, these investigators proposed that a tight calcium binding site exists in protein C, distal from the Gla domain, which plays a critical role in producing a functionally active conformation in protein C that is recognized by the thrombin–thrombomodulin complex. Given the acidic nature of β-hydroxyaspartic acid, it is not inconceivable that this residue facilitates the coordination of a calcium ion in a Gla-domainless protein C molecule. Demonstration of an analogous requisite nucleation center in the activation of Gla-domainless factor X would clearly lend credibility to this hypothesis. This idea is currently under active investigation in our laboratory. Hopefully, future research will not only elucidate the role of β-hydroxyaspartic acid in these proteins but also shed light on the mechanism on its posttranslational formation.

Acknowledgments

We are indebted to Dr. Earl W. Davie for his encouragement, support, and helpful discussions throughout these studies. We thank Drs. N. Izumiya and T. Kato, Kyushu University, for their generous gift of L-*threo*- and L-*erythro*-β-hydroxyaspartic acid. Special thanks go to Roger Wade for performing the amino acid analyses. The helpful discussions and technical assistance of Drs. K. Takio and K. Titani are greatly appreciated. Lastly, the excellent technical assistance of Kathleen Palmer is gratefully acknowledged.

Registry No. Blood coagulation factor X (human light chain reduced), 85628-96-2; blood coagulation factor X, 9001-29-0; L-*erythro*-β-hydroxyaspartic acid, 7298-98-8.

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Fluorescence Depolarization of Tryptophan Residues in Proteins: A Molecular Dynamics Study†

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ABSTRACT: A molecular dynamics simulation of lysozyme is used to examine the fluorescence depolarization of tryptophan residues on the picosecond time scale. The calculated time dependence of fluorescence emission anisotropy for the six tryptophans in lysozyme exhibits a wide variety of motional behavior that should correspond to the range expected more generally for tryptophan residues in proteins. It is found that some tryptophans are highly mobile with a large fluorescence anisotropy decay on the picosecond time scale while others are essentially rigid due to the presence of the protein matrix. Further, it is demonstrated that correlations among the internal degrees of freedom (e.g., dihedral angles) play an important role in the observed decay behavior; this suggests that care has to be used in interpreting experimental results in terms of simple motional models. Because the available experimental time resolution is limited to the nanosecond time scale, only the effective zero-time anisotropy can be compared with the calculated values. The results suggest that the study of fluorescence depolarization with femtosecond lasers would provide new insights into the short time dynamics of amino acid side chains in proteins.

A knowledge of the internal dynamics of proteins is important for an understanding of the physical properties and biological function of these molecules (Karplus & McCammon, 1981). At present, an increasing variety of experimental and theoretical methods are being used to study these motions. Of particular interest are experimental methods that can supplement X-ray temperature factors, which provide data on the magnitude of the fluctuations but no information on their time scales. Both nuclear magnetic resonance (NMR) relaxation and fluorescence depolarization experiments are useful in this regard since they provide data on the internal motions of proteins in the picosecond to nanosecond range. The parameters of interest in both types of experiments are related to time correlation functions whose decay is determined by reorientation of certain vectors associated with the probe (i.e.,

† From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138. *Received July 15, 1982; revised manuscript received February 28, 1983.* Supported in part by the National Science Foundation and the National Institutes of Health.

0006-2960/83/0422-2884$01.50/0