The *N*-Terminal Sequences of Blood Coagulation Factor X₁ and X₂ Light Chains

MASS-SPECTROMETRIC IDENTIFICATION OF TWELVE RESIDUES OF γ -CARBOXYGLUTAMIC ACID IN THEIR VITAMIN K-DEPENDENT DOMAINS

By HANS C. THØGERSEN,† TORBEN E. PETERSEN,† LARS SOTTRUP-JENSEN,†
STAFFAN MAGNUSSON† and HOWARD R. MORRIS*†

†Department of Molecular Biology, University of Aarhus, DK-8000 Aarhus C, Denmark, and

‡ Department of Biochemistry, Imperial College of Science and Technology, Imperial Institute Road,
London SW7 2AZ, U.K.

(Received 28 March 1978)

Peptides (residues 1–42) (bovine prothrombin numbering) from bovine Factors X₁ and X₂ have been separately purified and digested before mass-spectrometric sequence assignment and identification of γ -carboxyglutamic acid. Each *N*-terminal sequence was found to be identical, and 12 residues of γ -carboxyglutamic acid were unambiguously identified. The new data give conclusive evidence for the *N*-terminal primary structure of bovine Factor X and extend present knowledge to show (i) unambiguous assignment of γ -carboxyglutamic acid residues, including a previously unreported residue of γ -carboxyglutamic acid at position 40 in both Factor X₁ and Factor X₂, (ii) the physical difference between Factors X₁ and X₂ is not due to either different amino acid sequences or different γ -carboxyglutamic acid contents of the *N*-terminal 42 residues.

Factor X in blood plasma is the zymogen of a serine proteinase, Factor X_a (EC 3.4.21.6), that, by specific limited proteolysis, activates prothrombin in the final stages of intrinsic and extrinsic blood coagulation [see review by Davie & Fujikawa (1975)]§.

Bovine Factor X can be resolved into two distinct variants, Factor X₁ and Factor X₂, by anion-exchange chromatography (Jackson & Hanahan, 1968; Fujikawa *et al.*, 1972). However, no significant differences between the two variants were revealed by examination of amino acid compositions, carbohydrate composition or biological activities (Fujikawa *et al.*, 1972).

Bovine Factors X₁ and X₂ both consist of a heavy chain and a light chain (mol.wts. 39000 and 16000 respectively); the amino acid sequences of the Factor X₁ chains were reported by Enfield *et al.* (1975) (light chain) and by Titani *et al.* (1975) (heavy chain).

Factor X is one of the vitamin K-dependent proteins, that is, biosynthesis of biologically active Factor X is suppressed during treatment with vitamin K antagonists such as warfarin or dicoumarol (Reekers *et al.*, 1973). During the study of bovine prothrombin, the major vitamin K-dependent plasma protein (Dam, 1935), a specific vitamin

K-dependent biochemical modification was discovered and described (Magnusson *et al.*, 1974; Stenflo *et al.*, 1974; Nelsestuen *et al.*, 1974). By mass-spectrometric sequence determination of acetylated permethylated peptides, ten residues of a new amino acid, γ -carboxyglutamic acid, were identified in the *N*-terminal region of bovine prothrombin (Magnusson *et al.*, 1974; Morris *et al.*, 1976); the deduced structure of the new amino acid was proved by synthesis of γ -DL-carboxyglutamic acid (Morris *et al.*, 1975; Fernlund *et al.*, 1975). The synthesis and chemical characterization of γ -carboxyglutamic acid have been undertaken in several laboratories (Morris *et al.*, 1975; Fernlund *et al.*, 1975; Boggs *et al.*, 1975; Märki & Schwyzer, 1975; Bajusz & Juhasz, 1976; Märki *et al.*, 1976; Weinstein *et al.*, 1976; Märki *et al.*, 1977). The γ -carboxyglutamic acid residues arise from a post-translational enzymic γ -carboxylation of glutamic acid residues, which requires vitamin K (Esmon *et al.*, 1975), and the polypeptide region thus modified binds Ca²⁺ ions (Nelsestuen & Suttie, 1972; Stenflo & Ganrot, 1973). The metal ion- and phospholipid-binding properties of prothrombin and Factor X have been studied extensively (Nelsestuen *et al.*, 1976; Furie *et al.*, 1976; Prendergast & Mann, 1977; Bajaj *et al.*, 1977; Märki *et al.*, 1977), but the tertiary structure and function of the γ -carboxyglutamic acid-containing domains are still poorly understood.

Sequence homology between the *N*-terminal sequences of bovine prothrombin and bovine Factor X light chain (Magnusson, 1973; Fujikawa

Abbreviation used: Gla, γ -carboxyglutamic acid; Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one; Δ Ala, dehydroalanine.

* To whom requests for reprints should be addressed.

§ The nomenclature for various blood coagulation Factors is that recommended by an International Nomenclature Committee (Wright, 1959).

et al., 1974; Magnusson *et al.*, 1975; Enfield *et al.*, 1975) suggested the presence of γ -carboxyglutamic acid in Factor X and indirect evidence for the presence of γ -carboxyglutamic acid in bovine Factor X was presented by Howard & Nelsestuen (1975). Neal *et al.* (1976) claimed to have observed different quantities of γ -carboxyglutamic acid in the light chain of bovine Factors X₁ and X₂ by analysis of alkaline hydrolysates.

Amino acid sequence determination by Edman degradation of peptides or proteins containing γ -carboxyglutamic acid residues (either manual or automated) is not easily practicable. This is demonstrated in the work of Enfield *et al.* (1975) on the bovine Factor X₁ light-chain sequence, where automated sequencing of the *N*-terminal region was reported to be inconclusive with respect to identification of certain residues as γ -carboxyglutamic acid, glutamic acid or glutamine. Furthermore, asparagine 39 was reported as tentative. The remainder of the sequence was apparently determined without special difficulty. In our present work we have used the basic sequence of Enfield *et al.* (1975) to overlap our γ -carboxyglutamic acid-containing peptide sequences.

One of the major difficulties in Edman degradation of these polypeptides is partial and uncontrolled decarboxylation of the γ -carboxyglutamic acid side chains under the acidic conditions to which the samples are exposed at elevated temperature during the conversion of thiazolinone derivatives into thiohydantoin derivatives. Furthermore, tendency to cyclization of newly formed *N*-terminal γ -carboxyglutamic acid residues during the cleavage step decreases the repetitive yield. Partial reversal of this cyclization reaction during subsequent degradation steps generates the problem of carry-over from previous steps. It has been claimed (Fernlund *et al.*, 1975; Bucher *et al.*, 1976) that methyl esterification of the γ -carboxyglutamic acid thiazolinone derivative before conversion in acidic aqueous conditions circumvents these difficulties, but their results (Fernlund *et al.*, 1975) indicate that partial decarboxylation and carry-over from previous steps in the sequence are still major problems, as illustrated by their finding of phenylthiohydantoin derivatives of leucine, glutamic acid and γ -carboxyglutamic acid in step 15 of the bovine prothrombin sequence.

Determination of the primary structure of γ -carboxyglutamic acid-containing polypeptides by mass-spectrometric sequence determination of short (eight to ten residues) acetylated permethylated peptides provides definite assignment of γ -carboxyglutamic acid residues, since it operates on an exact physical property of the amino acid, its mass, which is directly revealed in the spectra. A practical difficulty in this approach is the relative resistance of γ -carboxy-

glutamic acid-rich peptides to enzymic digestion (Morris *et al.*, 1976; Fernlund *et al.*, 1975). However, at pH 1.0, pepsin digestion proceeds effectively (presumably due to the neutralization of the negative charges in the γ -carboxyglutamic acid side chains) (Magnusson *et al.*, 1974; Morris *et al.*, 1976; H. C. Thøgersen, unpublished work). Peptides suitable for mass-spectrometric sequencing of the *N*-terminal regions of the bovine Factor X₁ and X₂ light chains were obtained in this way.

The present work provides conclusive and detailed evidence for the amino acid sequences of the *N*-terminal region of the light chains of bovine Factors X₁ and X₂, by using the mass-spectrometric-sequencing strategy. During the present study, Bucher *et al.* (1976) published the results of sequenator studies of a pool of bovine Factor X₁ and X₂ light chains by using their methyl esterification method mentioned above. In view of the inherent problems of this method and the reported difference in γ -carboxyglutamic acid content between the two Factor X variants (Neal *et al.*, 1976), these results are not conclusive, and although eleven γ -carboxyglutamic acid residues were observed, there is no evidence as to whether these residues are γ -carboxyglutamic acid residues in both Factor X₁ and Factor X₂. The present work resolves this question and provides evidence for the presence of twelve γ -carboxyglutamic acid residues in both proteins.

Materials and Methods

Enzymes and reagents

Chymotrypsin (three-times-crystallized; 6064-7), pepsin (twice-crystallized from ethanol; 681) and soya-bean trypsin inhibitor (81 53M 457) were obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Thermolysin (declared activity 68 caseinolytic units/mg of protein, 92C-2500) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Russell's viper (*Vipera russeli*) venom (Stypven RV 738B) was purchased from the Wellcome Research Laboratories, Beckenham, Kent, U.K. Tos-Phe-CH₂Cl ('TPCK') and Kodirex film were from Eastman-Kodak, Rochester, NY, U.S.A. The substrate *p*-benzoyl-L-isoleucyl-L-glutamylglycylarginine *p*-nitroanilide was obtained from A.B. Kabi, Mölndal, Sweden. Iodo[1-¹⁴C]acetamide (specific activity 40 Ci/mol) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Sephadex A-50 ion-exchange resin and Sephadex G-25 (fine grade) were purchased from Pharmacia A.B., Uppsala, Sweden. DE-52 cellulose ion-exchange resin was from Whatman Ltd., Maidstone, Kent, U.K. Polyamide sheets were obtained from the Cheng-Chin Trading Co., Taipei, Taiwan. All other

reagents and materials were the best commercial grades.

Amino acid analysis

Peptide samples (0.5–1 nmol) were hydrolysed in 6M-HCl (110°C, 20–24 h, in sealed tubes under reduced pressure) and analysed essentially as described by Spackman *et al.* (1958) with a Beckman 121 MB amino acid analyser and a 16 cm column of the Locarte LA/28 ion-exchange resin in a 70 min program. All results represent single-sample analyses.

Alkaline hydrolysis was performed essentially as described by Hauschka (1977).

Detection of radiolabelled peptides

Portions of fractions collected in the chromatographic separation were pipetted on to glass-fibre discs, which were then dried in a heated vacuum oven. Radioactivity in the discs was measured in a Beckman liquid-scintillation counter LS-350, with toluene/PPO (2,5-diphenyloxazole) scintillation fluid. Radioactive peptides separated by high-voltage paper electrophoresis were detected by radioautography.

Electrophoresis

Electrophoretic separation of peptides was carried out on Whatman no. 1 or 3 MM chromatography paper at 3 kV (approx. 68 V/cm) in volatile buffers of pH 6.5 and 2.1 (Ambler, 1963). Peptides were detected by staining with cadmium/ninhydrin reagent (Heilman *et al.*, 1957) or by radioautography. Phenanthraquinone (Yamada & Itano, 1966) was used to detect arginine-containing peptides.

N-terminal analysis

N-terminal amino acids were identified manually by t.l.c. analysis of dansyl (5-dimethylaminonaphthalene 1-sulphonyl) derivatives on polyamide sheets (Gray, 1967; Woods & Wang, 1967).

Amino acid sequences

Amino acid sequences were determined by the mass-spectrometric method given below.

Reduction and S-alkylation of disulphide-bridge peptides

Reduction with a 4-fold molar excess of dithiothreitol over peptide thiol groups was performed in 0.3 M-Tris/10 mM-EDTA in freshly deionized (mixed bed MB-3 resin; Whatman) 6 M-urea (adjusted to pH 8.3 with glacial acetic acid) at a peptide concentration of 0.3 μ M. The reaction mixture was incubated

50 min at room temperature before addition of 20–100 μ Ci of iodo[1- ^{14} C]acetamide. After 5 min, unlabelled iodoacetamide was added (4-fold molar excess over dithiothreitol). After a reaction time of 25 min the alkylated peptide was passed through a Sephadex G-25 column (2.6 cm $\times$ 30 cm; eluted with 0.1 M-NH₄HCO₃, pH 8.3) and freeze-dried.

Preparation of derivatives for mass spectrometry

Peptides (50–150 nmol) were acetylated in acetic anhydride/methanol (1:4, v/v) (Thomas *et al.*, 1968). The acetyl derivatives were permethylated by the Hakamori (1964) reaction with a short reaction time of 75–90 s (Morris, 1972) to avoid salt formation with some amino acid residues. The above procedure was modified by using labelled methanol and methyl iodide (C²H₃O²H and/or C²H₃I) at the appropriate place in the reaction sequence for a few of the peptides studied. Arginine-containing peptides were treated with hydrazine (aq. 50%; 80°C, 15 min) before the standard derivatization procedure; this treatment converts arginine into ornithine residues (Morris *et al.*, 1973).

Mass spectrometry

Spectra were recorded on a GEC-AEI MS-50 mass spectrometer operating with an accelerator voltage of 8 kV and an electron beam energy of 70 eV.

Sample handling, temperature gradient and partial-fractionation techniques for mixtures have been described elsewhere (Morris *et al.*, 1971).

Interpretation of spectra was based on the most recent data on the fragmentation properties of acetylated permethylated peptides (Morris *et al.*, 1974; Dell & Morris, 1974; Morris *et al.*, 1976).

The nomenclature for describing sequences deduced from spectra and the derivatives of the common amino acids is as previously described (Morris *et al.*, 1974). The trivial codes for the derivatives of γ -carboxyglutamic acid, X, X_c, Y, Y_c were introduced in our previous studies (Magnusson *et al.*, 1974; Morris *et al.*, 1976).

Isolation of bovine Factors X_1 and X_2

Purification of bovine Factors X_1 and X_2 was performed essentially as described by Fujikawa *et al.* (1972). The yields from 460 litres of bovine blood were 750 and 500 mg of Factor X_1 and Factor X_2 respectively.

The preparations appeared to be homogeneous as judged by sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis (Weber & Osborn, 1969). After reduction with 2-mercaptoethanol both variants gave rise to two bands on the gels (apparent mol.wts. 20000 and 40000 respectively). The amino acid

Table 1. Amino acid compositions of Factor X_1 , X_1 fragment 1–42 and the peptides selected for mass-spectrometric sequencing. Values in parentheses have been deduced from the sequences published by Enfield *et al.* (1975) and Titani *et al.* (1975). Abbreviations used: CmCyS, carboxymethylcysteine; n.d., not determined.

	Composition (molar amounts relative to underlined values)									
	Factor X_1	X_1 fragment 1–42	X_{1a}	X_{1b}	X_{1c}	X_{1d}	X_{1e}	X_{1f}	X_{1g}	X_{1h}
CmCyS	—	—	—	—	—	—	—	—	—	—
Asx	41.7 (41)	3.67 (4)	0.97 (1)	0.27 (0)	0.95 (1)	0.61 (1)	0.75 (1)		1.04 (1)	0.95 (1)
Thr	30.1 (31)	0.94 (1)								0.88 (1)
Ser	28.5 (31)	1.67 (2)	0.84 (1)				0.75 (1)			
Glx	59.2 (59)	13.1 (14)		3.22 (3)	1.06 (1)	3.96 (4)	1.85 (2)	2.29 (2)	3.94 (3)	1.05 (1)
Pro	17.6 (18)									
Gly	40.3 (41)	1.00 (1)	0.29 (0)	0.21 (0)	1.03 (1)					
Ala	31.0 (31)	3.60 (4)	1.00 (1)				0.82 (1)	1.00 (1)	1.00 (1)	
$\frac{1}{2}$ -CyS	10.5 (24)	0.91 (2)								
Val	27.3 (26)	1.62 (2)		1.05 (1)	0.93 (1)			0.85 (1)	0.91 (1)	
Met	6.8 (5)									
Ile	11.6 (12)									
Leu	33.0 (30)	4.03 (4)		1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)		0.30 (0)	
Tyr	9.9 (10)									
Phe	19.3 (21)	2.87 (3)		0.97 (1)				1.11 (1)	0.98 (1)	1.00 (1)
Lys	23.4 (23)	0.99 (1)		0.99 (1)	1.01 (1)					
His	11.3 (12)									
Arg	26.8 (25)	1.87 (2)				0.98 (1)		1.04 (1)	0.17 (0)	
Trp	n.d. (7)	Present (1)								
Residues*	—	1–42	1–4	5–11	9–14	15–21	22–27	28–33	31–37	38–41
$m_{6.5}\dagger$	—	—	0.00	0.63	–0.43	0.63	0.67	n.d.	n.d.	0.84
$m_{2.1}\ddagger$	—	—	0.92	0.72	1.41	0.35	0.39	0.77	0.32	0.53

* Position of peptide in the factor X_1 sequence (prothrombin numbering; see Fig. 4).

$\dagger$ Electrophoretic mobility of peptide at pH 6.5 relative to that of asparagine ($m_{6.5}$ (Asp) = 1.00).

$\ddagger$ Electrophoretic mobility of peptide at pH 2.1 relative to that of serine ($m_{2.1}$ (Ser) = 1.00).

compositions of acid hydrolysates of both Factor X_1 and Factor X_2 were compatible with the composition that can be calculated from the amino acid sequence of bovine Factor X_1 (Enfield *et al.*, 1975; Titani *et al.*, 1975) (see Tables 1 and 2). After activation with Russell's viper venom (Jackson & Hanahan, 1968), both variants rapidly cleaved the Factor X_a substrate benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine *p*-nitroanilide (Magnusson *et al.*, 1976).

Isolation of the chymotryptic fragments 1–42 $\dagger$ from factors X_1 and X_2

Freeze-dried Factor X_1 or Factor X_2 was dissolved to 1 mg/ml in 150 mM-NH₄HCO₃, pH 8.3 and incubated (2 h; 37°C) with chymotrypsin (enzyme/substrate molar ratio 1:50) to which soya-bean trypsin inhibitor (inhibitor/enzyme molar ratio 1:10) had been added. At the end of incubation a 2-fold molar excess of Tos-Phe-CH₂Cl over chymo-

trypsin was added. The digest was then freeze-dried before separation of peptides.

The tryptic fragment 12–44 (Nelsestuen & Suttie, 1973) and the chymotryptic fragment 1–35 (Magnusson *et al.*, 1974) from bovine prothrombin (containing eight and ten γ -carboxyglutamic acid residues respectively) were isolated by specific adsorption to barium citrate. Essentially the latter procedure was followed to isolate the chymotryptic fragments 1–42 from Factors X_1 and X_2 , except that a final purification step was added in which each fragment was chromatographed on a column (1.6 cm $\times$ 20 cm) of DE-52 cellulose equilibrated in 10 mM-NH₄HCO₃, pH 8.3, and eluted with a linear gradient (500 ml of 10 mM-NH₄HCO₃, pH 8.3, + 500 ml of 1 M-NH₄HCO₃, pH 8.3; flow rate 15 ml/h). Fractions corresponding to the main peak of A_{230} , eluting at approx. 0.6 M-NH₄HCO₃ were pooled and freeze-dried (X_1 , fragment 1–42 and X_2 , fragment 1–42). The amino acid compositions of the two fragments were compatible with the composition of residues 1–42 than can be deduced from the Factor X_1 light-chain sequence (Enfield *et al.*, 1975) (see Tables 1 and 2).

$\dagger$ Residue numbering refers to bovine prothrombin; see Fig. 4.

Table 2. Amino acid compositions of factor X_2 , X_2 fragment 1–42 and the peptides selected for mass-spectrometric sequencingAll data given in parentheses have been deduced from the Factor X_1 sequences (Enfield *et al.*, 1975; Titani *et al.*, 1975).

Abbreviations used: CmCyS, carboxymethylcysteine; n.d., not determined.

Composition (molar amounts relative to underlined values)										
	Factor X_2	X_2 fragment 1–42	X_{2a}	X_{2b}	X_{2c}	X_{2d}	X_{2e}	X_{2f}	X_{2g}	X_{2h}
CmCyS	—	—				1.08 (1)	1.36 (1)			
Asx	40.7	3.77 (4)	0.94 (1)		0.92 (1)	0.13 (0)		0.28 (0)	1.00 (1)	0.98 (1)
Thr	28.8	1.01 (1)								0.99 (1)
Ser	27.5	1.78 (2)	1.02 (1)	0.68 (1)			0.87 (1)	0.14 (0)		
Glx	56.6	14.1 (14)		2.11 (2)	1.00 (1)	4.04 (4)	2.02 (2)	2.48 (2)	3.60 (3)	1.04 (1)
Pro	19.5									
Gly	40.5	1.00 (1)	0.10 (0)		0.96 (1)			0.24 (0)		
Ala	31.0	3.52 (4)	1.00 (1)				0.96 (1)	1.00 (1)	1.00 (1)	
$\frac{1}{2}$ -CyS	10.0	1.07 (2)								
Val	27.7	1.89 (2)			0.90 (1)			0.96 (1)	0.85 (1)	
Met	6.5									
Ile	10.9									
Leu	33.8	4.19 (4)	0.11 (0)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	0.32 (0)	0.15 (0)	
Tyr	10.4									
Phe	19.5	2.80 (3)	1.23 (1)	0.91 (1)				1.04 (1)	0.88 (1)	1.00 (1)
Lys	21.1	1.01 (1)			0.94 (1)			0.14 (0)		
His	11.1									
Arg	27.0	2.05 (2)				0.99 (1)		1.00 (1)	0.13 (0)	
Trp	n.d.	Present (1)								
Residues*	—	1–42	1–5	4–8	9–14	15–21	22–27	28–33	31–37	38–41
$m_{6.5}\dagger$	—	—	0.00	n.d.	–0.43	0.66	n.d.	0.60	n.d.	0.87
$m_{2.1}\ddagger$	—	—	0.83	0.41	1.23	0.40	0.40	n.d.	0.32	0.51

*†‡ Defined as in Table 1.

Thermolytic peptides from the X_1 fragment 1–42

A portion (8 μ mol) of fraction X_1 fragment 1–42 was digested (14 h, 37°C) with thermolysin (enzyme/substrate molar ratio 1:50) in 10 ml of 40 mM-*N*-ethylmorpholine adjusted to pH 8.0 with acetic acid. At the end of incubation, 2 ml of acetic acid was added and the digest was then freeze-dried.

The peptides were purified by chromatography on DE-52 cellulose by using the conditions described above (see under 'Isolation of the chymotryptic fragments'). Eleven A_{230} peaks emerged from the column; the corresponding fractions were pooled and freeze-dried. Measured portions were taken for amino acid analysis. From these pools, three peptides were chosen for further investigation, namely X_{1a} (residues 1–4, 7.4 μ mol), X_{1b} (residues 5–11, 2.7 μ mol) and X_1 fragment 5–42 (residues 5–42, 5.6 μ mol) (see Table 1).

Peptic peptides from the X_1 fragment 5–42 and the X_2 fragment 1–42

The samples were reduced and carboxamido-methylated and then dissolved in 0.1 M-HCl to a peptide concentration of 0.5 mg/ml. Pepsin (1 mg/ml

in 0.1 M-HCl) was added in two equal portions, the last of which was added after incubation for 2 h at 37°C (final enzyme/substrate molar ratio 1:35). Incubation was then continued overnight and followed by freeze-drying.

Peptides were separated on DE-52 columns (1 cm $\times$ 25 cm) as described above under 'Isolation of the chymotryptic fragments'. A_{230} , counting of ^{14} C radioactivity and electrophoretic analysis of portions from each fraction was used to decide on pooling of the fractions collected. Separation largely on the basis of net electrostatic charge was achieved, but further purification of the most acidic peptides was necessary. These pools were rechromatographed separately on a DE-52 column (1 cm $\times$ 25 cm) equilibrated with 130 mM-pyridine/5 mM-acetic acid, pH 6.5. The sample was loaded in the same buffer and the column was eluted with 10 ml of 130 mM-acetic acid/40 mM-formic acid, pH 2.1, followed by a 500 ml linear gradient (250 ml of 130 mM-acetic acid/40 mM-formic acid, pH 2.1, + 250 ml of 1.3 M-acetic acid/0.4 M-formic acid, pH 2.1). The column effluent was monitored by electrophoresis and ^{14} C radioactivity counting of portions from each 4 ml fraction. Most pools from these 'second dimension' ion-exchange separations contained pure peptides. The

rest were easily resolved by preparative electrophoresis at pH2.1.

The overall yields were low (in the order of 5%), but very pure peptides were obtained as judged by *N*-terminal analysis, amino acid analysis and analytical electrophoresis. All peptides isolated could be fitted into the amino acid sequence of the bovine Factor X_1 light chain, as determined by Enfield *et al.* (1975). Numerous split variants were isolated and could be aligned to account for the complete sequence; the amino acid compositions of the peptides selected for mass-spectrometric sequencing are shown in Tables 1 and 2.

Results and Discussion

In previous papers (Magnusson *et al.*, 1974; Morris *et al.*, 1976) we described the identification and sequence location of ten γ -carboxyglutamic acid residues in bovine prothrombin. Table 3 shows the masses and structures of γ -carboxyglutamic acid derived fragments that were deduced in the above work. These will be referred to in the spectra and in the text as X , X_c , Y , and Y_c and correspond to the two *N*-terminal mass numbers and two 'in-chain' mass differences respectively. In the discussion to follow it is important to realize that the inter-relationship of these fragments has been proved (Morris *et al.*, 1976), and the presence of just one of them is a sufficient diagnostic for the unambiguous assignment of γ -carboxyglutamic acid. Peptides from both the X_1 fragment 1–42 and the X_2 fragment 1–42 covering the entire sequence template of Enfield *et al.* (1975) for factor X_1 were sequenced by mass spectrometry. Of the data actually collected and interpreted, only the minimum necessary to prove the sequence assignment of γ -carboxyglutamic acid residues is presented below. The sequences deduced for peptides that do not contain γ -carboxyglutamic acid are discussed briefly.

Identification and sequence location of twelve γ -carboxyglutamic acid residues in the bovine factor X_1 light chain

Peptide X_{1a} (residues 1–4). This peptide did not contain γ -carboxyglutamic acid, the sequence deduced was:

Peptide X_{1b} (residues 5–11). Strong signals were obtained from this peptide, showing a clear *N*-terminal phenylalanine at m/e 204 and extending to Phe-Leu- Y_c - Y_c -Val via signals at m/e 331, 560, 789 and 902. Confirming this assignment, some signals 58 mass units lower were observed on the scans at higher source temperature (see Morris *et al.*, 1976) giving Phe-Leu- Y - Y -Val via signals at m/e 204, 331, 502, 673 and 786. Cyclized γ -carboxyglutamic

acid fragments were also abundant, and could be used to extend the sequence via signals at m/e 170 (X_c minus CO), 427, 540 and 738 assigned to X_c - Y_c -Val-Lys, and at m/e 170, 311, 509 and 679 to X_c -Val-Lys-Gln. The X - and Y -containing analogues of these sequences were also present, finally supporting the assignments made. The overall sequence deduced for this peptide is therefore:

Positioning with respect to the factor X_1 light-chain sequence of Enfield *et al.* (1975) shows that positions 7 and 8 are occupied by γ -carboxyglutamic acid residues.

Peptide X_{1c} (residues 9–14). This peptide did not contain Gla; the sequence deduced was:

Peptide X_{1d} (residues 15–21). Representative mass spectra obtained from derivatives of this peptide are reproduced in Fig. 1(a) and 1(b). In the low-source-temperature spectrum (Fig. 1a) we assign the intense signal at m/e 170 to X_c minus CO; note that m/e 142 is absent, which would otherwise have suggested the presence of leucine or isoleucine. The highest mass ion in the spectrum is a relatively low abundance m/e 458, which we assign to the molecular ion for X_c - Y_c -OCH₃. A loss of 59 mass unit to give an abundant signal at m/e 399 is fully consistent with this interpretation, and a plausible structure for m/e 399 is given below:

We find that the presence at low source temperature of m/e 458, 399 and 170 in the abundance ratios shown in Fig. 1(a) is diagnostic for a C-terminal Gla-Gla sequence.

A typical high-source-temperature scan of this peptide spectrum is shown in Fig. 1(b), and we assign it as follows. The presence of both m/e 170 and 112 is indicative of both X_c and X termini. Possible sequences assignable from the X_c terminus are X_c -Orn (arginine is converted into ornithine by hydrazinolysis) via signals at m/e 170 and 382, and X_c - Δ Ala-Leu (carboxamidomethylcysteine is often fully β -eliminated to give dehydroalanine) via signals at m/e 170, 281 and 408. Note that the presence of Δ Ala is causing some undermethylation of the leucine residue to give a signal at m/e 394.

Table 3. Structures, masses and common fragment ions for the products of derivative formation of γ-carboxyglutamic acid residues

Derivatives are formed by treatment of a peptide with acetic anhydride in methanol, followed by permethylation. For further details, see the text.

Code	Structure	Occurrence	N-Terminal mass	In-chain mass difference	Common fragment ion
X		At N-terminus	140 (Not observed)	—	(X minus CO) ⁺ (112 mass units)
X _c		At N-terminus	198 (Not observed)	—	(X _c minus CO) ⁺ (170 mass units)
Y		In-chain or at C-terminus	—	171	—
Y _c		In-chain or at C-terminus	—	229	—

The sequences assignable from the X-terminus are much stronger, and are interpreted as follows. A sequence X-Orn-Y-ΔAla-Leu is assigned via signals at *m/e* 112, 324, 495, 578 and 705. (Note the weaker 'carboxylated' versions of this sequence 58 mass units higher, e.g. *m/e* 636 and 763.) Another sequence X-ΔAla-Leu-Y is also assigned via signals at *m/e* 112, 223, 350 and 521, thus overlapping into the terminal X_c-Y_c-OCH₃ sequence assigned in the discussion of Fig. 1(a). The overall sequence of this peptide is therefore clearly:

and the spectra in Fig. 1 prove the presence of γ-carboxyglutamic acid at positions 15, 17, 20 and 21 in the factor X₁ light-chain sequence.

Peptide X_{1e} (residues 22–27). This peptide gave an identical spectrum to the equivalent peptide from the factor X₂ fragment 1–42 (X_{2e}), the mass spectrum of which is discussed in detail below. The factor X₁ peptide was sequenced as:

showing that positions 26 and 27 in the light chain of bovine factor X₁ are occupied by γ-carboxyglutamic acid residues.

Fig. 1. Mass spectra of peptide X_1d : (a) at an ion-source temperature of 190°C and (b) at an ion-source temperature of 300°C. For details, see the text.

Peptide X_1f (residues 28–33). The low-temperature spectra (210°C) were dominated by signals at m/e 170 (142 absent), 311, 472, 673, and 674 with a less

abundant signal at m/e 732. These data are readily assigned to a sequence $X_c-Val-Phe-Y_c-OCH_3$, m/e 673 arising from M^{++} minus $COOCH_3$ and m/e 674

Fig. 2. Mass spectra of peptide X_{1h} : (a) at an ion-source temperature of 150°C and (b) at an ion-source temperature of 250°C . For details, see the text.

corresponding to an X or Y analogue of this sequence. At 250°C , new signals are seen at m/e 128, 312, 483, and 596 assigned to Ac-Ala-Orn-Y-Val, and at m/e 112, 253, and 414 assigned to X-Val-Phe. The overall sequence is therefore deduced to be:

Ala-Arg-Gla-Val-Phe-Gla

proving the presence of γ -carboxyglutamic acid at positions 30 and 33 in the Factor X_1 light-chain sequence.

Peptide X_{1g} (residues 31–37). At low-source temperature the mass spectrum of this peptide

showed low abundance signals at m/e 170 (X_c minus CO) and at m/e 340 and 399, assigned to X_c -Gln-OCH₃ minus COOCH₃ and X_c -Gln-OCH₃ respectively. At a source temperature of 250°C , abundant signals were present showing the main sequence as Val-Phe-Y_c-Asp-Ala-Y_c via signals at m/e 156, 317, 546, 689, 774 and 1003. At 280°C all of these assignments are supported by the appearance of the corresponding Y-containing sequences 58 mass units lower. In addition, the sequences X_c -Asp-Ala-Y_c and ... Asp-Ala-Y_c (N-C cleavage product) can also be assigned to the spectra via signals at m/e 170, 341,

426 and 655 for the former, and m/e 113, 198 and 427 for the latter.

In a separate experiment on this peptide, $C^2H_3O^2H$ was used in place of methanol in the acetylation procedure (Morris *et al.*, 1976). The consequent mass shifts observed in the spectra fully corroborated all the assignments made above, suggesting an overall sequence of:

Val-Phe-Gla-Asp-Ala-Gla-Gln

and proving the existence of γ -carboxyglutamic acid at positions 33 and 36 in the Factor X_1 light-chain sequence.

Peptide X_{1h} (residues 38–41). The low-temperature (150°C) mass spectrum of this peptide (Fig. 2a) shows clear evidence of the presence of γ -carboxyglutamic acid via the strong signal for X_c minus CO at m/e 170. Beyond the terminal X_c residue, there are few signals of notable abundance, with the exception of m/e 256, 288, 331 and 390; none of these fits as amino acid mass differences from m/e 198 (X_c). Calculating possible C-terminal mass differences from m/e 198, only m/e 390 gives a meaningful amino acid ester difference, corresponding to a C-terminal phenylalanine residue. The structure X_c -Phe-OCH₃ thus assigned is strongly supported by the presence of a signal at m/e 331, corresponding to an expected loss of 59 mass units (CO₂CH₃). In addition to this, a '+10' signal frequently observed for molecular ions (Morris *et al.* 1976) is present at m/e 400 in Fig. 2(a).

At higher source temperatures, the spectrum seen in Fig. 2(b) is observed. A true N-terminal sequence is now apparent at m/e 172, corresponding to Ac-Thr, together with the expected loss of methanol at m/e 140. Signals rising together with m/e 172 are m/e 315, which we assign to Ac-Thr-Asp, and m/e 283 and 256, corresponding to losses of methanol and the entire threonine side chain respectively. Beyond this the next meaningful mass difference occurs via a signal at m/e 544, giving a mass difference of 229 mass units to m/e 315, and therefore assigned to Ac-Thr-Asp-Y_c, thus providing the overlap with the X_c -Phe-OCH₃ minus OCH₃.

The spectra shown in Fig. 2 are therefore derived from the sequence

Thr-Asp-Gla-Phe

thus identifying a new γ -carboxyglutamic acid residue at position 40 in the Factor X_1 light chain.

Summarizing the data for all the peptides discussed above, we are able to confirm the basic sequence of Enfield *et al.* (1975) for the Factor X_1 light-chain N-terminal fragment 1–42, and, more importantly, give conclusive evidence for the presence and sequence location of twelve residues of γ -carboxyglutamic acid. Residues nos. 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36 and 40 are γ -carboxyglutamic acid (N.B. the prothrombin numbering system is used; see Fig. 4).

Identification and sequence location of twelve γ -carboxyglutamic acid residues in the Factor X_2 light chain

With the exception of peptide X_{2b} (which will be discussed separately) the peptides used for mass-spectrometric assignment of γ -carboxyglutamic acid residues proved to be identical, on mass-spectrometric analysis, with their counterparts in the factor X_1 fragment discussion above.

Peptide X_{2a} (residues 1–5). This peptide contained no γ -carboxyglutamic acid residues; the sequence was:

Ala-Asn-Ser-Phe

Peptide X_{2b} (residues 4–8). Due to the variation in strategy for the isolation of the Factor X_1 and Factor X_2 peptides (see the Materials and Methods section), the peptide chosen for mass-spectrometric analysis and believed to contain γ -carboxyglutamic acid-7 and γ -carboxyglutamic acid-8, had an amino acid composition that was different from that of its factor X_1 counterpart (X_{1b}).

The low-temperature mass spectrum of this peptide contained m/e 170, 399 and 458 in the ratios previously described for peptide X_{1d} , and was thus assigned to a C-terminal X_c -Y_c-OCH₃ structure. At 250°C, the mass spectrum showed an abundant N-terminal sequence ion signal for Ac-serine at m/e 158, and a weaker signal for N-terminal Ac-phenylalanine at m/e 204 (showing the sample to be a mixture containing a minor component of an N-terminal phenylalanine sequence). The major sequence could easily be extended via strong sequence ion signals at m/e 319 (loss of methanol to m/e 287), 446 (414), 675 (643) and 903 (M^+ minus 32) to give Ac-Ser-Phe-Leu-Y_c-Y_c-OCH₃ minus methanol. The minor sequence could also be extended to Ac-Phe-Leu-Y_c via signals at m/e 331 and 560.

The major sequence in this peptide is thus assigned to

Ser-Phe-Leu-Gla-Gla

showing that positions 7 and 8 in the Factor X_2 light chain sequence are γ -carboxyglutamic acid residues.

Peptide X_{2c} (residues 9–14). The spectrum was identical with that of the derivative of peptide X_{1c} and the sequence deduced was:

Val-Lys-Gln-Gly-Asn-Leu

Peptide X_{2d} (residues 15–21). The spectra obtained from this peptide were identical with those from peptide X_{1d} (see Fig. 1a and 1b) and the sequence deduced was:

Fig. 3. Mass spectra of peptide X₂e: (a) at an ion-source temperature of 180°C and (b) at an ion-source temperature of 250°C. For details, see the text. Abbreviation used: CAM-Cys, carboxamidomethylcysteine.

showing that residues 15, 17, 20 and 21 in the Factor X₂ light chain are γ -carboxyglutamic acid residues.

Peptide X₂e (residues 22–27). The low-temperature (180°C) mass spectrum of this peptide contained only m/e 170 and 399 as abundant signals, together with

weak signals at m/e 202 and 458, thus showing the presence of a C-terminal $X_c-Y_c-OCH_3$ structure (Fig. 3a).

At higher source temperature (250°C) the spectrum changed considerably, as Fig. 3(b) shows. The mass

spectrum is interpreted as follows. The three abundant low mass signals at m/e 128, 211 and 326 can be readily assigned to Ac-Ala, Ac-Ala- Δ Ala (arising from alanylcarboxamidomethylcysteine) and Ac-Ala- Δ Ala-Ser respectively. The equivalent non- β -eliminated structures confirm these assignments by the presence of signals at m/e 330 and 445 corresponding to:

respectively. Indeed, the presence of a metastable peak at m/e 238.8 shows that m/e 326 is formed by β -elimination of the carboxamidomethylcysteine side-chain (probably thermal) from m/e 445 within the mass spectrometer. Beyond this the sequence is easily extended to:

via signals at m/e 572 (m/e 540 corresponds to a loss of methanol) and m/e 801. This assignment is supported by the presence of signals at m/e 453 and 682 arising from Ac-Ala- Δ Ala-Ser-Leu-Y_c, and by the presence of the 'decarboxylated' Y-analogues of the above two sequences at m/e 743 and 624 respectively. We note also the presence of signals corresponding to partial C-methylation of the carboxamidomethylcysteine residue at m/e 344, 459, 586 and 815. These signals do not interfere with the sequence assignment, and of course the C-methylation is not apparent for the dehydroalanine-containing sequence, where the carboxamidomethylcysteine side chain has been β -eliminated. The following overall sequence was deduced:

proving that positions 26 and 27 in the bovine factor X₂ light-chain sequence are occupied by γ -carboxyglutamic acid residues.

Peptide X₂f (residues 28–33). The spectra from this peptide were identical with those from peptide X₁f and the sequence is therefore:

thus proving that positions 30 and 33 in the factor X₂ light-chain sequence are γ -carboxyglutamic acid residues.

Peptide X₂g (residues 31–37). The spectra from this peptide were identical with those from peptide X₁g and its sequence is therefore

proving that residues 33 and 36 in the factor X₂ light-chain sequence are γ -carboxyglutamic acid residues.

Peptide X₂h (residues 38–41). The spectra produced by this peptide and peptide X₁h were identical, and the sequence deduced for this peptide was

proving that residue 40 in the factor X₂ light-chain sequence is a γ -carboxyglutamic acid residue.

In summary, the Factor X₂ data obtained can be used to account for a complete sequence of residues 1–42, which is identical with that of Factor X₁. Strictly speaking, there is not complete overlap evidence in the case of the factor X₂ fragment. However, considering that the two fragments (X₁ fragment 1–42 and X₂ fragment 1–42) have the same amino acid composition, give rise to the same enzymic peptide fragments having identical sequences, numbers and positions of γ -carboxyglutamic acid residues and also the extensive sequence homology between the γ -carboxyglutamic acid regions of prothrombin and Factor X₁ light chain, we conclude that the primary structures of fragment 1–42 of bovine Factor X₁ and Factor X₂ light chains are identical with regard to amino acid sequence and location of their twelve γ -carboxyglutamic acid residues.

The present work shows that both Factors X₁ and X₂ contain twelve γ -carboxyglutamic acid residues. The first ten (residues 7, 8, 15, 17, 20, 21, 26, 27, 30 and 33) correspond to the ten γ -carboxyglutamic acid residues in bovine prothrombin. The two additional γ -carboxyglutamic acid residues of factors X₁ and X₂ (residues 36 and 40) are located within the domain (residues 1–45) for which the amino acid sequence homology between prothrombin and factor X₁ is extensive.

The results of γ -carboxyglutamic acid determination in alkaline hydrolysates of factor X₁ and factor X₂ indicate that the twelve γ -carboxyglutamic acid residues found in the present sequence work are probably the only ones in bovine factors X₁ and X₂. In contrast with the data of Neal *et al.* (1976), we have observed no clear difference in the γ -carboxyglutamic acid contents of Factors X₁ and X₂ that could explain their different chromatographic behaviour. Therefore it would seem logical that this difference must be explained either by a difference in another part of the structure or by an easily dissociated cofactor or prosthetic group in the γ -carboxyglutamic acid region that could have escaped the techniques used in our present work and also that of others.

Between the first (residue 7) and the last (residue 40) γ -carboxyglutamic acid residues in the primary structures of the vitamin K-dependent domains of prothrombin and factors X₁ and X₂, asparagine

Fig. 4. Amino acid sequences of fragments 1-42 from bovine prothrombin (BPT) (Magnusson et al., 1974; Morris et al., 1976) and bovine Factor X₁ (Enfield et al., 1975; and the present paper) and bovine Factor X₂ (the present paper) BF.X

For clarity, γ-carboxyglutamic acid residues in the sequences are presented by their structures. The prothrombin numbering system is used despite the fact that residue 3 is deleted in the Factor X sequence.

occurs in one position (residue 13), aspartic acid in two (residues 34 and 39), and glutamine in two (residues 11 and 37). The fact that none of these residues have been postsynthetically carboxylated indicates that the vitamin K-dependent carboxylase (see Esmon *et al.*, 1975) is specific for glutamic acid residues.

The present results illustrate the ease and confidence of assignment of γ -carboxyglutamic acid by the mass-spectrometric method developed for general protein sequence analysis. This method is capable of solving the structures of impure γ -carboxyglutamic acid containing peptides or peptide mixtures, as shown in some of our original work on the identification and sequence location of γ -carboxyglutamic acid in prothrombin (Magnusson *et al.*, 1974; Morris *et al.*, 1976). In the present work, because of possible subtle differences between the Factor X₁ and X₂ light chains, it was considered best to isolate and purify peptides before γ -carboxyglutamic acid sequence assignment. The results of the mass-spectrometric analysis have clearly demonstrated both the γ -carboxyglutamic acid content and overall sequence assignment, including residues previously unassigned by other approaches (automated sequencer and amino acid analysis) used to study the structures of the Factor X₁ and X₂ light chains.

This work was supported by the Medical and Science Research Councils of Great Britain, the National Institutes of Health (HL-16238), Bethesda, MD, U.S.A. and the Danish Science Research Council. We thank Lisbeth Troels and Margit Skriver Jensen for excellent technical assistance and the Danish State Veterinary Serum Laboratory (Department for Jutland) for technical support during the preparative work. We also thank David Smith for maintenance of the mass-spectrometer facility.

References

- Ambler, R. P. (1963) *Biochem. J.* **89**, 349–378
- Bajaj, S. P., Byrne, R., Nowak, T. & Castellino, F. J. (1977) *J. Biol. Chem.* **252**, 4758–4761
- Bajusz, S., Juhasz, A. (1976) *Acta Chim. Acad. Sci. Hung.* **88**, 161–165
- Boggs, N. T., Gawley, R. E., Koehler, K. A. & Hiskey, R. G. (1975) *J. Org. Chem.* **40**, 2850–2851
- Bucher, D., Nebelin, E., Thomsen, J. & Stenflo, J. (1976) *FEBS Lett.* **68**, 293–296
- Dam, H. (1935) *Nature (London)* **135**, 652–653
- Davie, E. W. & Fujikawa, K. (1975) *Annu. Rev. Biochem.* **44**, 799–829
- Dell, A. & Morris, H. R. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1125–1132
- Enfield, D. L., Ericsson, L. H., Walsh, K. A., Neurath, H. & Titani, K. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 16–19
- Esmon, C. T., Sadowski, J. A. & Suttie, J. W. (1975) *J. Biol. Chem.* **250**, 4744–4748
- Fernlund, P., Stenflo, J., Roepstorff, P. & Thomsen, J. (1975) *J. Biol. Chem.* **250**, 6152–6133
- Fujikawa, K., Legaz, M. E. & Davie, E. W. (1972) *Biochemistry* **11**, 4882–4891
- Fujikawa, K., Coan, M. H., Enfield, D. L., Titani, K., Ericsson, L. H. & Davie, E. W. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 427–430
- Furie, B. C., Mann, K. G. & Furie, B. (1976) *J. Biol. Chem.* **251**, 3235–3241
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 469–475
- Hakamori, S. I. (1964) *J. Biochem. (Tokyo)* **55**, 205–213
- Hauschka, P. V. (1977) *Anal. Biochem.* **80**, 212–223
- Heilman, J., Barrollier, J. & Watzke, E. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219–220
- Howard, J. B. & Nelsestuen, G. L. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1281–1285
- Jackson, C. M. & Hanahan, D. J. (1968) *Biochemistry* **7**, 4506–4517
- Magnusson, S. (1973) *Thromb. Diath. Haemorrh. Suppl.* **54**, 31–35
- Magnusson, S., Sottrup-Jensen, L., Petersen, T. E., Morris, H. R. & Dell, A. (1974) *FEBS Lett.* **44**, 189–193
- Magnusson, S., Petersen, T. E., Sottrup-Jensen, L. & Claesys, H. (1975) in *Proteases and Biological Control* (E. Reich, D. Rifkin & E. Shaw, eds.), pp. 123–149, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Magnusson, S., Sottrup-Jensen, L., Petersen, T. E., Dudek-Wojciechowska, G. & Claesys, H. (1976) *Miami Winter Symp.* **11**, 203–235
- Märki, W. & Schwyzer, R. (1975) *Helv. Chim. Acta* **58**, 1471–1477
- Märki, W., Oppliger, M. & Schwyzer, R. (1976) *Helv. Chim. Acta* **59**, 901–902
- Märki, W., Oppliger, M. & Schwyzer, R. (1977) *Helv. Chim. Acta* **60**, 807–815
- Morris, H. R. (1972) *FEBS Lett.* **22**, 257–260
- Morris, H. R., Williams, D. H. & Ambler, R. P. (1971) *Biochem. J.* **125**, 189–201
- Morris, H. R., Dickinson, R. J. & Williams, D. H. (1973) *Biochem. Biophys. Res. Commun.* **51**, 247–255
- Morris, H. R., Williams, D. H., Midwinter, G. G. & Hartley, B. S. (1974) *Biochem. J.* **141**, 701–713
- Morris, H. R., Thompson, M. R. & Dell, A. (1975) *Biochem. Biophys. Res. Commun.* **62**, 856–861
- Morris, H. R., Dell, A., Petersen, T. E., Sottrup-Jensen, L. & Magnusson, S. (1976) *Biochem. J.* **153**, 663–679
- Neal, G. G., Prowse, C. V. & Esnouf, M. P. (1976) *FEBS Lett.* **66**, 257–260
- Nelsestuen, G. L. & Suttie, J. W. (1972) *Biochemistry* **11**, 4961–4964
- Nelsestuen, G. L. & Suttie, J. W. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3366–3370
- Nelsestuen, G. L., Zytkevich, T. H. & Howard, J. B. (1974) *J. Biol. Chem.* **249**, 6347–6350
- Nelsestuen, G. L., Broderius, M. & Martin, G. (1976) *J. Biol. Chem.* **251**, 6886–6893
- Prendergast, F. G. & Mann, K. G. (1977) *J. Biol. Chem.* **252**, 840–850
- Reekers, P. P. M., Lindhout, M. J., Kop-Klaassen, B. H. M. & Hemker, H. C. (1973) *Biochim. Biophys. Acta* **317**, 559–562
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206

- Stenflo, J. & Ganrot, P. O. (1973) *Biochem. Biophys. Res. Commun.* **50**, 98–104
- Stenflo, J., Fernlund, P., Egan, W. & Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2730–2733
- Thomas, D. W., Das, B. C., Gero, S. D. & Lederer, E. (1968) *Biochem. Biophys. Res. Commun.* **33**, 519–527
- Titani, K., Fujikawa, K., Enfield, D. L., Ericsson, L. H., Walsh, K. A. & Neurath, H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3082–3086
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Weinstein, B., Watrin, K. G., Loie, H. J. & Martin, J. C. (1976) *J. Org. Chem.* **41**, 3634–3635
- Woods, K. R. & Wang, K.-T. (1967) *Biochim. Biophys. Acta* **133**, 369–370
- Wright, I. (1959) *J. Am. Med. Assoc.* **170**, 325
- Yamada, S. & Itano, H. A. (1966) *Biochim. Biophys. Acta* **130**, 538–540