Carboxy-Terminal Amino Acid Sequence of a Human Fibrinogen γ-Chain Variant (γ′)†

Carlota Wolfenstein-Todel‡ and Michael W. Mosesson*,§

ABSTRACT: A normal human fibrinogen γ-chain variant, termed γ′, is larger than the γ chain (51 500 vs. 49 500) due to an extended COOH-terminal sequence. The extended COOH-terminal cyanogen bromide peptide (CNBr e′) was isolated by high-pressure liquid chromatography, and its amino acid sequence was determined. Comparison with the corresponding COOH-terminal γ-chain peptide (CNBr e) showed that the last four amino acids of the γ chain were replaced in γ′ chains by a 20-residue fragment rich in aspartic and glutamic acids, having the sequence Val-Tyr-Pro-Glu-His-Pro-Ala-Glx-Thr-Glx-Tyr-Asx-Ser-Leu-Arg-Pro-Glx-Asx-Asx-Leu. Mutant γ chains (γ Paris I) from a congenitally dysfunctional fibrinogen molecule (fibrinogen Paris I) express both γ and γ′ features, suggesting that both γ and γ′ chains are produced from a single gene. If this suggestion is correct, the observed differences in amino acid sequence could be explained by the existence of different mRNAs for γ and γ′ chains, respectively, which are transcribed from one gene by differential RNA splicing.

Recent reports indicate that a human fibrinogen γ-chain variant, termed γ′ (Mosesson et al., 1972), has a higher molecular weight than the γ chain (51 500 vs. 49 500) (Wolfenstein-Todel & Mosesson, 1980; Francis et al., 1980) due to an extended COOH-terminal amino acid sequence (Wolfenstein-Todel & Mosesson, 1980). The γ′ chains are more negatively charged than γ chains and are separable by gel electrophoresis at pH 8.6 or 2.7 (Mosesson et al., 1972), by isoelectric focusing, or by ion-exchange chromatography (Stathakis et al., 1978). There are no significant differences in their sialic acid or phosphorus contents (Finlayson & Mosesson, 1963; Mosesson & Finlayson, 1963).

The variant γ′ chains are functionally normal with respect to cross-linking in the presence of factor XIIIa and undergo this process nonselectively with respect to γ and γ′ chains (Wolfenstein-Todel & Mosesson, 1980). Analyses of *S*-carboxymethyl derivatives of γ and γ′ chains that had been labeled with dansylcadaverine indicated that the glutamine cross-linking receptor site and the lysine cross-linking donor site were the same in both types of chains. Available data also indicated that the sequence of the γ′ chain was the same as that of the γ chain from the NH_2 terminus to at least as far as the terminal five amino acids of the γ chain.

Earlier analyses of mutant γ chains (γ Paris I) from a congenitally dysfunctional fibrinogen molecule (fibrinogen Paris I) showed that both γ and γ′ features were expressed in the mutant γ chains (Stathakis et al., 1978), a finding suggesting that γ and γ′ chains had been produced from a single gene. Thus, our speculations regarding peptide processing events that could lead to the formation of these variant chains included the possibilities that γ′ chains represent (a) a naturally occurring "read through" protein (Korner et al., 1979) or (b) an incompletely processed precursor of the γ chain. In order to examine these possibilities and others, we determined the amino acid sequence unique to the γ′ chain.

Materials and Methods

Plasma fibrinogen fraction I-4 (Blombäck & Blombäck, 1956) (thrombin clottability >95%), the γ-chain population of which is comprised of approximately 7–10% γ′ chains (Mosesson et al., 1972), was prepared as previously described (Mosesson & Sherry, 1966). Reduction and S-carboxymethylation were carried out as previously reported (Galanakis et al., 1978), and the S-CM[1] γ and γ′ chains were isolated by DEAE-cellulose gradient elution chromatography in 8 M urea at pH 7.0 (Stathakis et al., 1978). CNBr cleavage of S-CM peptides was carried out as described by Gross & Witkop (1962). The CNBr digests were subjected to gel-sieving chromatography at room temperature on a column (1.5 × 90 cm) of Sephadex G-75 that had been equilibrated and developed with 10% formic acid. The flow rate was 6.2 mL/h, and fractions of 2.4 mL were collected.

HPLC was performed with equipment from Waters Associates (Milford, MA). It included two M-6000A pumps, one M-660 solvent programmer, a U6K universal liquid chromatography injector, and an M-450 variable wavelength detector attached to an Omni-Scribe recorder (Houston Instruments, Austin, TX). Peptide samples were subjected to chromatography at room temperature at a flow rate of 2 mL/min on a Waters μBondapak C-18 column (30 × 0.4 cm) using acetonitrile (HPLC grade; Fisher Scientific Co., Fair Lawn, NJ) for developing the column under isocratic or linear gradient conditions. All eluting solutions contained 0.1% phosphoric acid (Hancock et al., 1978), and the samples themselves were also dissolved in 0.1% phosphoric acid. The peptides eluting from the column were detected by their absorbance at 225 nm, pooled appropriately, freeze-dried, and then desalted by chromatography at room temperature on a column of Sephadex G-15 (1.8 × 27 cm) that had been equilibrated and developed with 10% formic acid at a flow rate of 36 mL/h.

† From the Department of Medicine, State University of New York Downstate Medical Center, Brooklyn, New York 11203. *Received May 4, 1981.* Supported by National Institutes of Health Grant HL-17419. This work was presented in part at the 65th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlanta, GA, April 1981 (Wolfenstein-Todel & Mosesson, 1981).

‡ Present address: Facultad de Farmacia y Bioquimica, Universitâ de Buenos Aires, Buenos Aires, Argentina.

§ Address correspondence to this author at the Department of Medicine, Mt. Sinai Medical Center, Milwaukee, WI 53201.

[1] Abbreviations used: CM, carboxymethyl; CNBr, cyanogen bromide; HPLC, high-pressure liquid chromatography; PTH, phenylthiohydantoin; AUFS, absorbance unit full scale; dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; DEAE, diethylaminoethyl; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

0006-2960/81/0420-6146$01.25/0

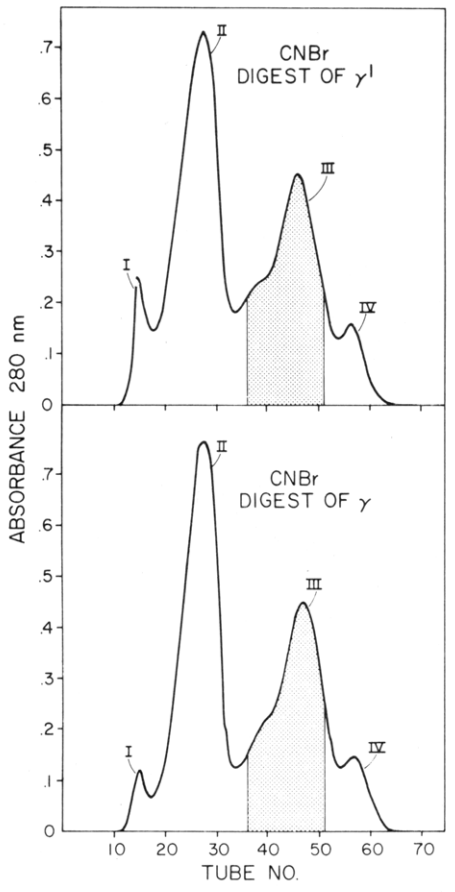

FIGURE 1: Sephadex G-75 chromatography (1.5 × 90 cm column) of a CNBr digest (25 mg) of γ and γ′ chains, respectively. The samples were applied to the column in 2 mL of 10% formic acid, and the column was then developed with the same solvent at a flow rate of 6.2 mL/h; fractions of 2.4 mL were collected. The pooling of fractions of interest (i.e., peak III) is indicated by the shaded areas.

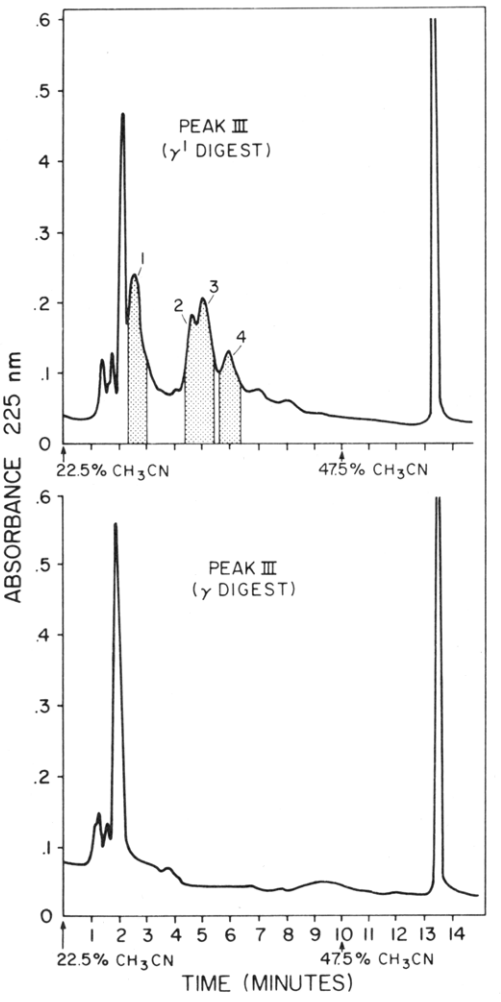

FIGURE 2: HPLC of chromatographic peaks III from experiments shown in Figure 1. Eighty microliters of sample (5 mg/mL) was applied to a μBondapak C-18 column. The mobile phase was 22.5% acetonitrile and 0.1% phosphoric acid, followed by a wash of 47.5% acetonitrile and 0.1 phosphoric acid. The pooling of fractions of interest is indicated by the shaded areas. Pressure, 1400 psi; flow rate, 2 mL/min; UV detector, 225 nm; AUFS, 1.

Tryptic digestion of peptides was performed with TPCK-treated trypsin (Worthington Biochemical Corp., Freehold, NJ) in 0.2 M ammonium bicarbonate buffer, pH 8.0, for 6 h at 37 °C, at an enzyme:substrate ratio of 1:50. The digest was then freeze-dried.

Amino acid analysis was carried out with a JEOL amino acid analyzer, Model JLC-6AH, on peptides that had been hydrolyzed in evacuated and sealed tubes for 20 h with 5.7 N HCl containing 0.1% phenol. Numerical values for each amino acid were generally expressed in terms of residues per peptide.

NH_2-terminal analysis was performed by an adaptation (Mosesson et al., 1973) of the dansyl chloride method of Gray & Hartley (1963). NH_2-Terminal sequence analysis by Edman degradation was performed manually (Gray, 1972) with dansylation of aliquot fractions to label and identify the NH_2-terminal residues at each step. PTH-glutamic acid and PTH-glutamine were distinguished by thin-layer chromatography as described by Summers et al. (1973).

Results

There were no apparent differences in the elution profiles obtained by Sephadex G-75 chromatography of CNBr digests of γ and γ′ chains, respectively (Figure 1). The peak containing the COOH-terminal peptide of the γ chain (peak III) (Sharp et al., 1972; Henschen et al., 1976) and the corresponding peak obtained from the γ′ chain were freeze-dried. The peptides contained in these fractions were first compared by HPLC by using a linear gradient of acetonitrile (10–45%) containing 0.1% phosphoric acid (data not shown). From this preliminary procedure we selected 22.5% acetonitrile and 0.1% phosphoric acid as the eluant for isocratic chromatography of the COOH-terminal CNBr peptide of the γ′ chain (Figure 2). As assessed by comparison with the chromatogram of peak III of the CNBr γ chains, peaks 1–4 were unique to γ′. Since amino acid analyses of material from each peak were virtually the same, we have designated them collectively as CNBr e′ (Table I). (Sequence analysis was performed on pools of peaks 2 and 3, whereas tryptic digestion was carried out on a pool of all four peaks.)

The sequence of the first 30 amino acids of CNBr e′ was determined by dansyl–Edman degradation (we observed a "gap" at position 25). The first 23 residues were identical with those of CNBr e (Sharp et al., 1972; Takagi & Doolittle, 1975). Amides in these first 23 residues of CNBr e′ were assigned by analogy with the established sequence of CNBr e and from the observation that tryptic peptide Tc (Table I) from γ′ chains behaved in the same way in fingerprint analysis as the corresponding γ-chain peptide (Wolfenstein-Todel & Mosesson, 1980).

CNBr e′ was digested with trypsin, and the resulting peptides were subjected to HPLC by using gradient elution from 12.5% to 45% acetonitrile (Figure 3). The peptides that emerged were labeled in order of their elution and were designated Ta, Tb, etc. Peptide Tb was not pure; peptides Td

Table I: Amino Acid Composition of CNBr e′ and Tryptic Peptides[a]

		peptide					
amino acid	CNBr e′	Ta	Tc	Tf	Tg	Th	Ti
Lys	1.9		1.0		1.0		
His	3.0		2.0	1.0		1.2	
Arg	1.9	1.0		1.0		0.8	
Asp	4.0	1.0		2.7		3.0	
Thr	2.0		0.9	0.9		0.9	
Ser	1.1			1.2		1.3	
Glu	8.1		3.0	4.6		4.9	
Pro	3.8			2.9	1.2	3.0	1.2
Gly	4.3		3.9				
Ala	2.1		1.0	1.0		0.9	
Val	0.9			0.9		1.0	
Ile	1.7[b]		0.7		1.1		0.9
Leu	3.5		1.8	1.7		1.6	
Tyr	1.9			1.6		1.6	
Phe	0.9				0.8		1.0
N	43	2	15	21	5	21	4
NH_2-terminal residue	Lys	Asx	Leu	Glx	Lys	ND[c]	ND

[a] Values for each residue are expressed as the number per peptide; N = total residues per peptide. [b] Due to incomplete hydrolysis under the conditions used, values for this residue are lower than expected. [c] ND = not determined.

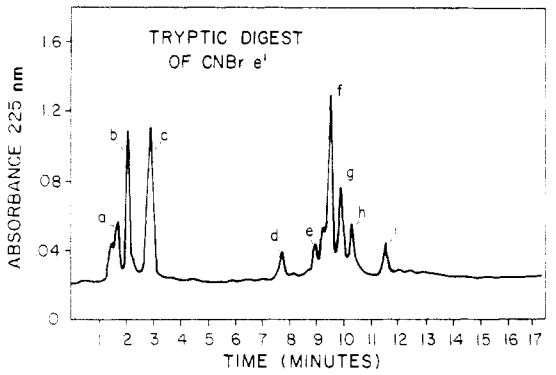

FIGURE 3: HPLC of a tryptic digest of CNBr e′ (pool of peaks 1–4, Figure 2). Sixty-five microliters of sample (1 mg/mL) was applied to a μBondapak C-18 column. Separation of peptides was obtained by using linear gradient elution from 12.5% to 45% acetonitrile over a period of 15 min. Both solvents contained 0.1% phosphoric acid. Pressure, 1400 psi; flow rate, 2 mL/min; UV detector, 225 nm; AUFS, 0.4.

and Te were obtained in too low a yield to give satisfactory amino acid compositions. The amino acid composition of the other tryptic peptides is shown in Table I. Ta, Tg, and Ti probably were formed as a result of contaminating chymotryptic activity in the trypsin preparation. Peptide Ti is evidently included in Tg. Peptide Th had the same amino acid composition as Tf, and we therefore presume that they have essentially the same sequence. Most likely the two peptides differ in the degree of amidation of Asp and/or Glu residues (see paragraph at end of paper regarding supplementary material).

The sequence of Tf was established by dansyl–Edman analysis (Figure 4). The majority of the steps were confirmed by amino acid analysis after back-hydrolysis of the PTH derivatives. Gln-23 and Glu-27 were determined by thin-layer chromatography of the PTH-amino acids. Since the dansyl derivatives of His and Arg could not be readily distinguished by thin-layer chromatography, His was identified in position 2 by back-hydrolysis and amino acid analysis of the PTH derivative; Arg-38 was positioned by exclusion.

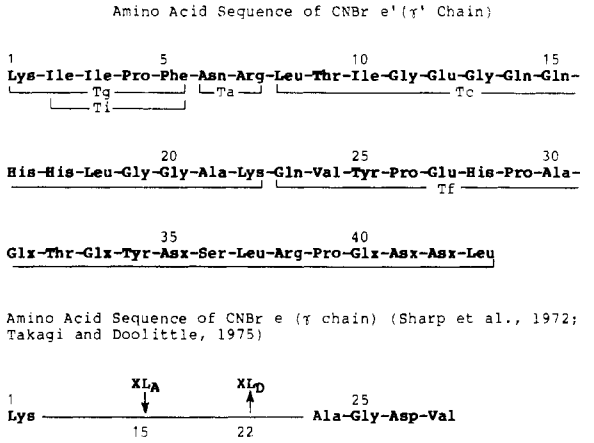

FIGURE 4: Comparison of amino acid sequences of CNBr e′ and CNBr e. Residues 1–23 of CNBr e are the same as shown for CNBr e′. The known cross-linking acceptor (XL_A) and donor (XL_D) sites are indicated on CNBr e.

Discussion

Our previous studies (Wolfenstein-Todel & Mosesson, 1980) showed that the γ′ chain had an extended COOH-terminal sequence relative to γ chains and suggested that the γ′ sequence was the same as that of the γ chain from NH_2-terminal tyrosine to at least as far as the lysine donor cross-linking site that is located five residues from the γ chain COOH-terminus. In this study we have determined by sequence analysis of CNBr e′ that the γ′ chain is identical with the γ chain up to four residues from the γ chain COOH-terminus (corresponding to residue 23 of CNBr e′; Figure 4). Beyond this point the sequence is unique to the γ′ chain and is rich in aspartic acid and glutamic acid (some of these residues may be amidated). The presence of these acidic residues accounts for the difference in charge between γ and γ′ chains (Mosesson et al., 1972; Stathakis et al., 1978).

Analysis of mutant γ chains (γ Paris I) from a congenitally dysfunctional fibrinogen molecule (fibrinogen Paris I) showed that both γ and γ′ features were expressed in the mutant γ chains (Stathakis et al., 1978). This finding implies that both chains are produced from a single gene, since otherwise it would be necessary to postulate that an identical mutation had occurred in two separate genes (e.g., γ and γ′), a less likely possibility.

If the "single gene" hypothesis is assumed to be correct, there are several possible explantations for the occurence of γ′ chains. On the basis of our present sequence data, it seems highly improbable that the γ′ chain represents a naturally occurring "read through" protein (Korner et al., 1979) or an unprocessed "precursor" of γ chains, since in both cases the COOH-terminal sequence of the γ chain would be completed and then followed by additional amino acids. This is not the case since the last four amino acids of the γ chain are replaced in γ′ chains by a unique 20-residue fragment. We suggest from these findings that different mRNAs for γ and γ′ chains, respectively, are transcribed from a single gene by differential RNA splicing (Darnell, 1979). This explanation corresponds to that which has been proposed to account for the existence of membrane-bound and secreted forms of immunoglobulin μ chains, which differ from one another only by their COOH-terminal sequences (Singer et al., 1980; Early et al., 1980; Alt et al., 1980). In view of the apparently widespread occurrence of γ/γ′ variants among mammalian species (Finlayson & Mosesson, 1964), we believe that this heterogeneity reflects important physiological differentiation of γ chains, although their exact nature remains to be discovered.

Acknowledgments

We thank Joseph Maniscalco for expert technical assistance and Nancy Mingoia for excellent secretarial assistance. We are also grateful to Drs. J. Koehn, J. D. Broome, and E. Gaetjens for the use of their HPLC equipment.

Supplementary Material Available

A table summarizing the basis for amino acid assignments in peptide Tf (1 page). Ordering information is given on any current masthead page.

References

Alt, F. W., Bothwell, A. L. M., Knapp, M., Siden, E., Mather, E., Koshland, M., & Baltimore, D. (1980) *Cell (Cambridge, Mass.) 20*, 293–301.

Blombäck, B., & Blombäck, M. (1956) *Ark. Kemi 10*, 415–443.

Darnell, J. E., Jr. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 327–353.

Early, R., Rogers, J., David, M., Calame, K., Bond, M., Wall, R., & Hood, L. (1980) *Cell (Cambridge, Mass.) 20*, 313–319.

Finlayson, J. S., & Mosesson, M. W. (1963) *Biochemistry 2*, 42–46.

Finlayson, J. S., & Mosesson, M. W. (1964) *Biochim. Biophys. Acta 82*, 415–417.

Francis, C. W., Marder, V. J., & Martin, S. E. (1980) *J. Biol. Chem. 255*, 5599–5604.

Galanakis, D. K., Mosesson, M. W., & Stathakis, N. E. (1978) *J. Lab. Clin. Med. 92*, 376–386.

Gray, W. R. (1972) *Methods Enzymol. 25*, 333–344.

Gray, W. R., & Hartley, B. S. (1963) *Biochem. J. 89*, 59.

Gross, E., & Witkop, B. (1962) *J. Biol. Chem. 237*, 1856–1860.

Hancock, W. S., Bishop, C. A., Prestidge, R. L., Harding, D. R. K., & Hearn, M. T. W. (1978) *J. Chromatogr. 153*, 391–398.

Henschen, A., Lottspeich, F., Sekita, T., & Warbinek, R. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 605–608.

Korner, A. M., Feinstein, S. I., & Altman, S. (1979) in *Transfer RNA* (Altman, S., Ed.) pp 105–135, MIT Press, Cambridge, MA.

Mosesson, M. W., & Finlayson, J. S. (1963) *J. Lab. Clin. Med. 62*, 663–674.

Mosesson, M. W., & Sherry, S. (1966) *Biochemistry 5*, 2829–2835.

Mosesson, M. W., Finlayson, J. S., & Umfleet, R. A. (1972) *J. Biol. Chem. 247*, 5223–5227.

Mosesson, M. W., Finlayson, J. S., & Galanakis, D. K. (1973) *J. Biol. Chem. 248*, 7913–7929.

Sharp, J. J., Cassman, K. S., & Doolittle, R. F. (1972) *FEBS Lett. 25*, 334–336.

Singer, P. A., Singer, H. H., & Williamson, A. R. (1980) *Nature (London) 285*, 294–300.

Stathakis, N. E., Mosesson, M. W., Galanakis, D. K., & Mênachê, D. (1978) *Thromb. Res. 13*, 467–475.

Summers, M. R., Smythers, G. W., & Oroszlan, S. (1973) *Anal. Biochem. 53*, 624–628.

Takagi, T., & Doolittle, R. F. (1975) *Biochim. Biophys. Acta 386*, 617–622.

Wolfenstein-Todel, C., & Mosesson, M. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5069–5073.

Wolfenstein-Todel, C., & Mosesson, M. W. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 778.

Effects of Temperature and pH on Prothrombin Fragment 1 Conformation As Determined by Nuclear Magnetic Resonance†

Carol H. Pletcher,‡ Elene F. Bouhoutsos-Brown, Robert G. Bryant, and Gary L. Nelsestuen*

ABSTRACT: The effects of temperature and pH on the solution conformation of native prothrombin fragment 1 were examined with ^1H NMR spectroscopy. A calcium-dependent quenching of the intrinsic protein fluorescence was used to monitor calcium binding to fragment 1 as an indicator of functional protein. The native fragment 1 NMR spectrum contained several features indicative of a folded protein: (a) nonequivalent histidyl C-2 resonances at 7.9 and 8.1 ppm, (b) two resonances of nearly equal intensity at 7.26 and 7.32 ppm, and (c) a resonance at −1.04 ppm. Temperature studies showed that thermal unfolding of fragment 1 (even at 80 °C) was reversible; however, there was an irreversible inactivation step which occurred subsequent to the unfolding. The basis for this inactivation appeared to include disulfide exchange reactions. On the basis of NMR spectra, fragment 1 retained its conformation from pH 7.0 to pH 11.5. From pH 7.0 to pH 5.0, the protein showed a reversible conformational change, and below pH 5, the protein self-associated. The pH dependence of the chemical shift of the tyrosyl resonances indicated a pK_a of approximately 10 for the tyrosyl residues. These data suggest that the tyrosyl residues are accessible to solvent in the native protein.

Prothrombin is one of a group of extracellular calcium-binding proteins which undergo a vitamin K dependent carboxylation of glutamyl residues (Stenflo & Suttie, 1977). The resulting γ-carboxyglutamyl residues have been implicated as ligands for calcium in these proteins. Prothrombin fragment 1[1] (prothrombin residues 1–156) contains all the γ-carboxy-

† From the Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55108 (C.H.P. and G.L.N.), and the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455 (E.F.B.-B. and R.G.B.). *Received April 2, 1981.* This work was supported in part by Grants HL 15728 (to G.L.N.) and GM-25757 (to R.G.B.) from the National Institutes of Health.

‡ National Institutes of Health Postdoctoral Fellow No. 05927.

[1] Abbreviations used: NMR, nuclear magnetic resonance; TSP, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4; EDTA, ethylenediaminetetraacetic acid; fragment 1, prothrombin residues 1–156; HPLC, high-performance liquid chromatography.

0006-2960/81/0420-6149$01.25/0