Location of γ-Carboxyglutamyl Residues in Partially Carboxylated Prothrombin Preparations†

DeAnn J. Liska‡ and John W. Suttie*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received April 4, 1988; Revised Manuscript Received July 15, 1988

ABSTRACT: Prothrombin contains 10 γ-carboxyglutamyl (Gla) residues in the N-terminal (fragment 1) domain of the protein. Following anticoagulant administration, a spectrum of undercarboxylated, physiologically less active forms of prothrombin is secreted into bovine or human plasma. The sites of undercarboxylation in these prothrombin species have now been investigated. Plasma containing a mixture of partially carboxylated forms of prothombin was obtained from a dicoumarol-treated bovine, and three pools of partially carboxylated (four, six, or eight Gla) species were purified by adsorption onto barium citrate and barium oxalate, ammonium sulfate fractionation, and chromatography. Fragment 1 obtained from these variants was equilibrated with ^3H_2O and heated in a dry state to decarboxylate Gla and incorporate ^3H into the resulting Glu residues. This peptide was then sequenced by Edman degradation, and the specific radioactivity of PTH-Glu was determined for each potential Gla-containing site. Data obtained from normal prothrombin fragment 1 fit a linear model when the log of specific activity of PTH-Glu was plotted against the cycle number. Analysis of the 80% variant showed a decrease in carboxylation only in the last two Gla residues, while data obtained from the 60% variant indicated a general decrease in carboxylation from the most amino- to the more carboxyl-terminal Gla residues. In the 40% Gla variant, all but the most amino-terminal of the Gla residues appeared to be undercarboxylated. These data indicate that the γ-carboxylation of glutamyl residues in prothrombin does not occur randomly but instead with preferential carboxylation of the most amino-terminal Gla residues. When carboxylation is limited, the impairment of carboxylation is more severe at the more carboxyl-terminal residues.

The posttranslational carboxylation of specific glutamyl residues to γ-carboxyglutamyl (Gla)[1] residues in a limited number of proteins is a vitamin K dependent event required for their normal physiological function (Suttie & Jackson, 1977). These Gla-containing proteins include the well-characterized plasma coagulation factors, prothrombin (factor II), factors VII, IX, and X, and proteins of the anticoagulant system, protein C and protein S (Walz et al., 1986; Davie, 1987). The most abundant of the vitamin K dependent coagulation factors in plasma, prothrombin, is present as a single-chain glycoprotein of 72 000 molecular weight containing 10 Gla residues in its most amino-terminal (fragment 1) domain.

The enzyme responsible for γ-carboxylation requires the reduced form of vitamin K as a substrate, and the vitamin is converted to vitamin K 2,3-epoxide by the enzyme (Suttie, 1988). Oral anticoagulants such as warfarin and dicoumarol inhibit vitamin K action in vivo by inhibiting the recycling of vitamin K 2,3-epoxide to the enzymatically active hydroquinone form of the vitamin (Matschiner et al., 1970; Bell & Matschiner, 1972). This antagonism results in the secretion of biologically inactive, undercarboxylated forms of prothrombin into the plasma of the human and bovine (Ganrot & Nilehn, 1968; Josso ett al., 1968; Stenflo, 1970). Although early studies of these proteins concentrated on species lacking any Gla residues (Nelsestuen & Suttie, 1973; Stenflo, 1974), it has been shown that the functionally abnormal prothrombin secreted in the presence of coumarin anticoagulants consists of a pool of partially carboxylated prothrombin as well as some completely des-γ-carboxyprothrombin (Prowse et al., 1976; Friedman et al., 1979). These multiple forms of partially carboxylated prothrombin have been extensively investigated by Malhotra (1981). By utilizing a technique of differential adsorption to barium citrate, barium oxalate, and alumina C-γ gel, it has been possible to isolate plasma prothrombin preparations which contain seven, five, two, or one Gla residue per mole (Malhotra, 1979a,b,c, 1982). Neither the degree of heterogeneity within these fractions nor the location of the carboxylated residues within each fraction has been determined.

We have now investigated the specific location of Gla residues present in preparations of partially carboxylated prothrombin obtained from the plasma of an anticoagulant-treated bovine. These partially carboxylated prothrombins were studied to determine if specific residues are undercarboxylated or if the effect of vitamin K antagonism on the carboxylase is nonspecific, resulting in a random carboxylation of the 10 potential Gla sites.

Experimental Procedures

Prothrombin Activity Assays. Prothrombin assays utilizing the chromogenic substrate S-2238 (Kabi Diagnostics, Stockholm, Sweden) were performed essentially as described by Shah et al. (1984). Calcium-dependent (physiological) prothrombin activity was assayed by measuring the thrombin generated from normal or variant prothrombin using a Sigma

† Supported by the College of Agricultural and Life Sciences of the University of Wisconsin–Madison and in part by Grants AM-14881 and HL-29586 from the National Institutes of Health.

‡ Present address: Department of Biochemistry, University of Washington, Seattle, WA 98195.

[1] Abbreviations: Gla, γ-carboxyglutamic acid; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography.

0006-2960/88/0427-8636$01.50/0 © 1988 American Chemical Society

(St. Louis, MO) thromboplastin preparation to initiate the reaction. Total prothrombin was quantitated by monitoring the thrombin formed upon incubation of normal or variant prothrombin with *Echis carinatus* (Sigma) snake venom. The buffer used for both assays was 0.1 M Tris-HCl, pH 7.2.

Purification of Normal and Partially Carboxylated Prothrombins. Normal prothrombin was purified from normal bovine plasma as described by Ingwall and Scheraga (1969), and partially carboxylated prothrombin was purified from plasma obtained from a female calf administered 1.85 mg/kg dicoumarol on days one, three, and five, fasted for 24 h, and then sacrificed. This protocol resulted in a decrease in the ratio of physiologically active prothrombin to total prothrombin of from 98%, as seen in normal plasma, to 25–35% at the end of 7 days. Blood was collected into 0.1 volume of 3.8% sodium citrate containing 50 mM benzamidine. Plasma was obtained and was fractionated with barium citrate and barium oxalate as described by Malhotra (1979a,b). After removal of the barium salts, alumina C-γ gel was added at one-tenth the volume of plasma, the solution was stirred overnight at 4 °C, and the gel was collected by centrifugation (4000*g*, 20 min). The various salt precipitates were washed, and prothrombin was eluted from them as described by Malhotra (1979a,b,c) and the eluate fractionated by ammonium sulfate precipitation from 0–30%, 30–50%, 50–67%, and 67–80% saturation. The ammonium sulfate precipitates were dialyzed extensively against water and assayed for the presence of prothrombin. The fractions from the 0–30% and 67–80% precipitations did not contain prothrombin and were discarded. The 30–50% and 50–67% ammonium sulfate fractions were concentrated with poly(ethylene glycol) 20 000, dialyzed against 2 mM sodium phosphate, and subjected to isoelectric precipitation of prothrombin by the dropwise addition of cold 0.025% HCl until the pH of the solution was 4.7. After 20 min, the suspension was centrifuged (10000*g*, 30 min), and the pellets were redissolved in 0.02 M sodium citrate, pH 7.5, and stored frozen at −4 °C. The prothrombin was further purified by chromatography on a heparin–Sepharose column as described by Bajaj et al. (1981). The Gla content of prothrombin preparations was quantitated as described by Kuwada and Katayama (1983) and protein concentration determined by the method of Lowry et al. (1951).

Preparation of Fragment 1. The amino-terminal, Gla-containing region of prothrombin fragment 1 was prepared from the variant prothrombins by incubation of the prothrombin preparation with thrombin (1:40 wt/wt) in 0.02 M Tris-HCl/0.1 M NaCl, pH 7.4, at 37 °C for 12 h. Fragment 1 from the barium citrate adsorbable variant prothrombins was purified by chromatography on a DEAE-Sephadex A-25 column as described by Mann et al. (1981). Fragment 1 from the barium oxalate and alumina C-γ variant prothrombins was purified by preparative disc gel electrophoresis in a 1.4 cm × 6.5 cm gel. The stacking gel contained 3.7% acrylamide and 0.09% bis(acrylamide), and the resolving gel contained 8.5% acrylamide and 0.2% bis(acrylamide). Gels and buffers contained 0.1 M, pH 8.3, Tris–borate/3 mM EDTA. The fragment 1 was eluted with 0.1 M, pH 8.0, Tris-HCl, and the eluate was monitored by the absorbance at 280 nm.

Tritium Labeling of Gla Residue Sites. Cysteine residues of the fragment 1 peptides were carboxymethylated as described by Allen (1981) followed by extensive dialysis against 0.1 M ammonium bicarbonate and lyophilization. Tritiated water (20 μL, 5 Ci/ml) was added to the lyophilized peptide, the sample was evacuated for 10 min at room temperature and then sealed in vacuo. A series of traps containing a dry ice/ethanol bath, phosphorus pentoxide, and Dririte were used as an interface between the lyophilizer and the sample containing tritiated water. The sample was heated at 110 °C for 4 h to facilitate decarboxylation of Gla to Glu residues and to incorporate ^3H into the resulting Glu residues (Tuhy et al., 1979; Poser & Price, 1979; Hauschka, 1979). After being heated, the sample was dissolved in 6 M guanidine hydrochloride/0.1 M ammonium bicarbonate and dialyzed against 25 mL of the guanidine/ammonium bicarbonate for 2 h. The sample was then dialyzed extensively against 0.1 M ammonium bicarbonate and lyophilized. Samples were redissolved in ammonium bicarbonate for sequence analysis.

Sequence Analysis. Automated Edman degradation was performed on a Beckman Model 890C spinning cup sequenator. Fragment 1 (20–200 nmol) was loaded with 4 mg of Polybrene added as carrier. A standard precycle program which omitted the addition of the phenyl isothiocyanate was used to condition the peptide. The peptide was double-coupled in the first degradation cycle and then degraded for an additional 34 cycles using a 0.1 M Quadrol program. Conversion of removed residues to PTH derivatives was performed in methanolic HCl as described by Tarr (1975), and PTH derivatives were identified and quantitated on a HPLC μBondapak column as described by Bhown et al. (1978). The HPLC column effluent was collected in 0.75-mL fractions, and the fractions containing PTH-Glu were mixed with 4.5 mL of Aquasol (New England Nuclear, Boston, MA), and the amount of ^3H was determined by liquid scintillation spectrometry. Specific activity, ^3H/PTH-Glu, was determined from these values and the amount of PTH-Glu in each fraction.

RESULTS

Isolation and Characterization of Partially Carboxylated Variant Prothrombins. Treatment of a calf with dicoumarol for 7 days resulted in a decrease of physiologically active prothrombin to about 25% of normal with a very small decrease in total prothrombin concentration. Fractionation of this dicoumarol-treated bovine plasma by sequential adsorption to barium citrate, barium oxalate, and alumina C-γ gel removed all of the prothrombin from the plasma. Almost all of the physiologically activatable prothrombin was associated with the barium citrate adsorbable prothrombin fraction (Table I). Prothrombin was purified from the various fractions as described under Experimental Procedures. The barium citrate adsorbable prothrombin was fractionated into two pools of variant prothrombins, one of which precipitated at 50–67% ammonium sulfate and was fully active under physiological conditions and one which precipitated at 30–50% ammonium sulfate and was 57% active under physiological conditions. The Gla content of these prothrombin fractions, the barium oxalate and alumina C-γ adsorbable prothrombin variants, and also the fragment 1 peptides derived from these preparations is also presented in Table I. The two pools of barium citrate adsorbable prothrombin variants and the barium oxalate variant were subsequently designated as 80%, 60%, and 40% variant prothrombin and were used for the determination of the distribution of Gla residues. The combined recovery of total plasma prothrombin in these three fractions following purification was 30%.

Localization of Gla Residue in Fragment 1 Obtained from Normal Prothrombin. The 10 Gla residues in bovine prothrombin are located at residues 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33. Standard Edman degradation techniques cannot be used to quantitate Gla residues due to irreproducible decarboxylation to Glu during the sequencing procedure and poor

Table I: Characterization of Undercarboxylated Prothrombins in Dicoumarol-Treated Bovine Plasma[a]

adsorbed to	$(NH_4)_2SO_4$ fraction (%)	% of plasma prothrombin	physiological act. (%)	Gla residue/mol	
				prothrombin	fragment 1
barium citrate	50–60	28	98	7.7 ± 1.7	8.0 ± 2.0
barium citrate	30–50	22	57	5.8 ± 1.3	6.5 ± 0.6
barium oxalate	30–50	20	16	3.5 ± 1.3	4.6 ± 0.6
alumina C-γ	30–50	30	14	0.3 ± 0.1	ND[b]

[a] The fraction of the total plasma prothrombin pool in each fraction was determined by activation with *E. carinatus* venom. No prothrombin was detectable in the alumina C-γ supernate. The physiological activity is expressed as a ratio of the activity obtained following physiological activation to that obtained following activatin with *E. carinatus* venom. The Gla contents of prothrombin and fragment 1 are means ± SD for analysis of four preparations. The normal prothrombin used as a control had a physiological activity of 98% and 10 ± 2.7 and 10 ± 1.5 residues of Gla per mole of prothrombin and fragment 1. [b] ND, not determined.

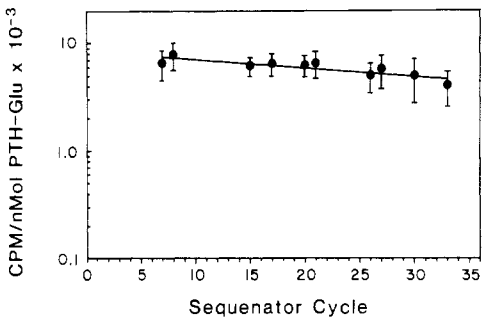

FIGURE 1: Specific activity of PTH-Glu derivatives obtained from the Gla sites of normal bovine prothrombin fragment 1. Preparations were subjected to ^3H equilibration and thermal decarboxylation as described under Experimental Procedures. Values plotted are mean ± SD for eight independent determinations.

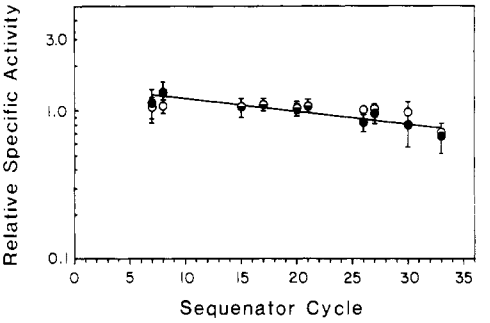

FIGURE 2: Relative specific activity of PTH-Glu derivatives obtained from the Gla sites of prothrombin preparations. The specific activity at each site was divided by the mean of the specific activity at all 10 sites. Preparations used were fragment 1 obtained from normal prothrombin [(●) $n = 8$] or from partially heat-decarboxylated preparations (see text) of normal prothrombin [(○) $n = 4$]. Values plotted are mean ± SD. For many points, the SD did not exceed 10% of the mean, and the plotted range is smaller than the plotted data point.

recovery of the phenylthiohydantoin derivative of Gla (Nelsestuen, 1984; Fernlund et al., 1975). Decarboxylation of Gla proteins in the presence of ^3H_2O (see Experimental Procedures) results in ^3H incorporation into the resulting Glu residues, and Gla residues in the partially carboxylated prothrombin preparations were, therefore, located by an assessment of the specific radioactivity of PTH-Glu residues following thermal decarboxylation of Gla in the presence of tritium. Specificity of ^3H incorporation for the identification of Gla residues was verified by subjecting a protein which does not contain Gla, myoglobin, and a previously thermal decarboxylated sample of fragment 1 to the same procedure. The specific activity (^3H/PTH-Glu) of the Glu residues from both of these control samples was less than 1% that of the specific activity of Glu residues resulting from the decarboxylation of fully carboxylated fragment 1 obtained from normal prothrombin.

Fragment 1 from normal prothrombin was used as a control to determine the specificity of labeling and recovery of label in each Gla residue. Eight samples of normal, fully carboxylated fragment 1 were independently decarboxylated in the presence of tritium and sequenced. Sequencing of thermally decarboxylated bovine fragment 1 resulted in repetitive yields of 88–94% with an average repetitive yield of 92% and lag of 3%. The specific activity of PTH-Glu from each Gla site was not equivalent, but the data (Figure 1) could be fitted to a linear semilog model with very little nonlinear component ($p < 0.001$). This first-order loss of ^3H suggested that some ^3H was lost from the γ-position of Glu at each cycle under the conditions of peptide sequencing. The specific activity of cycle 7, the first Gla residue, ranged from 4420 to 9590 cpm/nmol of PTH-Glu, in the eight samples analyzed, indicating that the specific activity of the equilibrated ^3H was not the same in all samples. This most likely occurred because of traces of water still associated with the protein even after extensive lyophilization. To decrease the variation in the data, the relative specific activity of PTH-Glu from each Gla site was obtained by normalizing the data to the average specific activity of all PTH-Glu derivatives for that sequence run (Figure 2).

Quantitative sequence analysis demonstrated that thermally decarboxylated bovine fragment 1 could be sequenced by Edman degradation through cycle 35. To determine if some of the Gla residues in prothrombin were more stable to thermal decarboxylation than others, samples of normal prothrombin were decarboxylated for 1–3 h prior to ^3H equilibration. Tritiated water was then added; the sample was evacuated and sealed and heated for the remainder of the 4 h. The complementary experiment was also performed. A tritium-equilibrated prothrombin preparation was decarboxylated for 2 h, the remaining ^3H removed by dialysis, and decarboxylation continued for a total of 4 h. Data from the sequence analysis of these samples are also presented in Figure 2. Whether the tritium was present initially or at the end of the decarboxylation procedure, the results were the same. The slope was not significantly different from that of the normal, control fragment 1. These data indicate that there is no significant difference in the thermal stability of the Gla residues located at different sites of carboxymethylated prothrombin fragment 1.

Localization of Gla Residues in Fragment 1 Obtained from Partially Carboxylated Variant Prothrombins. The 40%, 60%, and 80% variant prothrombins constituted approximately 70% of the total pool of dicoumarol-induced prothrombin recovered from bovine plasma. The remainder of the prothrombin recovered (Table I) appeared to be almost completely devoid of carboxylation and contained an average of less than one Gla per mole. The distribution of Gla residues in the three pools of partially carboxylated prothrombins was determined. If undercarboxylation were random, the deficiency of Gla at each of the 10 Gla sites in prothrombin should be equivalent, and

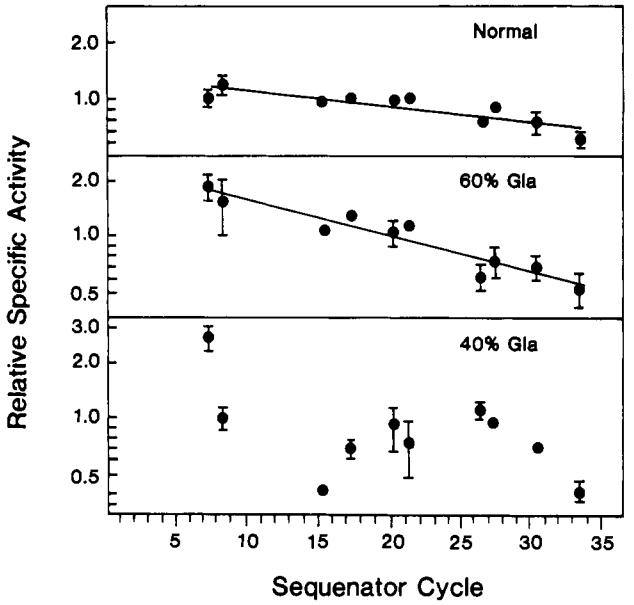

FIGURE 3: Relative specific activity of PTH-Glu derivatives obtained from the Gla sites of prothrombin variants. Preparations used were fragment 1 obtained from normal prothrombin (n = 8), from the 60% Gla variant (n = 4), or from the 40% Gla variant (n = 4). Values plotted are mean ± SD.

the specific activity of PTH-Glu would show a first-order decrease with the same slope as that seen with normal fragment 1. Four samples of 60% Gla variant fragment 1 were decarboxylated in the presence of tritium and analyzed as described for the normal prothrombin fragment 1 (Figure 3). As was seen with the normal prothrombin preparation, the data from the 60% variant fragment 1 could be approximated by a first-order decrease. However, the slopes were found to be significantly different by a F test ($p < 0.001$), suggesting that the Gla sites more distant from the amino terminus in this variant were carboxylated to a lesser extent than those closer to the amino terminus. In the 80% Gla variant fragment 1, only the last two Gla sites (30 and 33) were significantly undercarboxylated (data not shown).

Four samples of the 40% Gla variant fragment 1 also showed a significant decrease in Gla content as the sequence progressed from the amino terminus (Figure 3). However, in contrast to the data obtained from the 60% Gla variant, the decrease in Gla content of the 40% variant fragment 1 was not linear on a semilog graph. Except for cycle 7, the most amino-terminal Gla site, all of the Gla residues in this variant appear to be undercarboxylated with cycles 15 and 33 having the lowest Gla content. Also in contrast to the 60% and 80% variants, the Gla content at cycle 26 was higher than at residues 15, 17, 20, and 21.

The extent of undercarboxylation at each Gla site of the variant prothrombins was quantitated by correcting the data for the decrease in specific activity of PTH-Glu observed in normal prothrombin over the course of the sequence. This correction assumes that the Glu residues at each Gla site in decarboxylated normal fragment 1 have the same specific activity and that the first-order decrease of PTH-Glu specific activity is due to nonspecific "wash-out" of tritium during sequencing. The correction was performed by dividing the relative specific activity from each cycle of the variant prothrombins by the average relative specific activity from the corresponding cycle of normal fragment 1. The first Glu residue (cycle 7) in the variant fragment 1 preparations contained the highest specific activity and was assumed for the purpose of this calculation to by 100% carboxylated. The

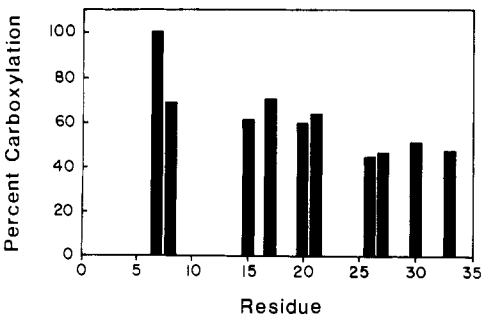

FIGURE 4: Extent of γ-carboxylation of Glu residues in the 60% Gla variant prothrombin. The fraction of carboxylation of each potential Gla residue is shown. This variant prothrombin adsorbed to barium citrate and was only 57% active under physiological conditions. The data were calculated as described in the text from the four independent sequence analyses of this variant shown in Figure 3.

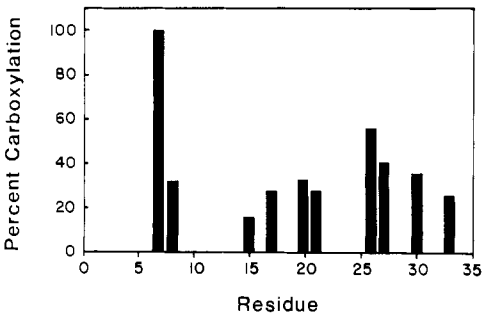

FIGURE 5: Extent of γ-carboxylation of Glu residues in the 40% Gla variant prothrombin. This variant prothrombin adsorbed to barium oxalate and was only minimally active under physiological conditions. The data were calculated from the four independent sequence analyses of this variant shown in Figure 3.

fraction of carboxylation at each potential Gla site in the 60% and 40% Gla variants is shown in Figures 4 and 5. In the 80% Gla variant, the fraction of carboxylation at the first eight Gla sites averaged 93% while residues 30 and 33 were 48 and 50% carboxylated. On the basis of the assumptions used in these calculations, the amount of Gla per molecule of the three variants, as determined from the sequence data, is 8.6, 6.2, and 4.0 which is consistent with the values obtained from Gla analysis of the base hydrolysates, 8.0 ± 2.0, 6.5 ± 0.6, and 4.6 ± 0.6, respectively. The similarity of Gla content calculated by this method and the results of direct analysis suggest that the first residue in the partially carboxylated prothrombins is nearly or fully carboxylated and that in the 40% variant this is the only residue which is substantially fully carboxylated.

Discussion

This study has provided additional information on the nature of the partially carboxylated forms of prothrombin previously studied by other investigators (Esnouf & Prowse, 1977; Friedman et al., 1979; Malhotra, 1981). The data in this study have clearly indicated that the carboxylation of partially carboxylated prothrombin is not random but that the extent of carboxylation is greatest at the most amino-terminal residue. A mildly undercarboxylated preparation (80% Gla content) appeared to be deficient in Gla residues only at the more carboxy-terminal sites. This preparation has nearly normal physiological activity (Table I). Malhotra et al. (1985) have demonstrated that a seven-Gla variant is activated very poorly, and this preparation appears to be similar to the 60% Gla content preparation studied here. In this moderately undercarboxylated preparation, the most amino-terminal site was nearly completely carboxylated, the next five sites 60–70% carboxylated, and the last four about 50% carboxylated. In

a more severely undercarboxylated preparation (40% Gla content) which has very low physiological activity, there was a sharp decrease in carboxylation following the most amino-terminal residue, and an indication of enhanced carboxylation at residues 26 and 27.

Borowski et al. (1986) have reported the distribution of Gla residues in three pools (four, six, or eight Gla) of undercarboxylated prothrombin variants from a human patient with an apparent hereditary defect in the vitamin K dependent carboxylation. The nature of the defect has not been defined, but, in contrast to the effects of vitamin K antagonism, the undercarboxylation is largely limited to prothrombin and factor X. The spectrum of barium citrate adsorbable undercarboxylated prothrombins in this patient's plasma also appears to differ immunoelectrophoretically from that seen in warfarin-treated patients (Goldsmith et al., 1982). All three variants analyzed by Borowski et al. (1986) showed Gla sites 6, 14, 19, and 20 (7, 15, 20, and 21 of bovine prothrombin) to be mostly, if not fully, carboxylated. These were the only sites substantially carboxylated in a four-Gla variant, and residue 16 was fully carboxylated in an eight-Gla variant but almost completely undercarboxylated in a six- or four-Gla variant. Residue 29 was near fully carboxylated in the six-Gla variant but poorly carboxylated in the other two. These data are in contrast to the general decrease in carboxylation from the amino-terminal residue noted in the dicoumarol-induced variants studied here. One common feature is the nearly complete carboxylation of the most amino-terminal Gla site noted in both studies. The basis for the different carboxylation patterns cannot be determined without a better understanding of the molecular defect in the patient studied by Borowski et al. (1986). Bovill et al. (1985) have presented preliminary data on the specificity of carboxylation of anticoagulant-induced prothrombin variants containing five or seven Gla residues. The results of that study appear to differ from both the data presented here and the Borowski et al. (1986) data, but little experimental data have been made available.

It is clear from both in vivo (Jorgensen et al., 1987; Foster et al., 1987) and in vitro (Knobloch & Suttie, 1987) studies that an amino-terminal propeptide region of vitamin K dependent proteins is an important element in recognition of these proteins by the carboxylase, but the order of carboxylation of the various Gla sites is not known. The carboxylase requires the reduced form of vitamin K as a cosubstrate, and coumarin anticoagulants inhibit the carboxylation in vivo. The available data indicate that the enzyme(s) sensitive to the presence of these anticoagulants is (are) the vitamin K epoxide reductase and quinone activities rather than the carboxylase itself (Suttie, 1987). The result of oral anticoagulation would therefore be a deficiency in the cellular concentration of reduced vitamin K, and identification of the sites of γ-carboxylation in the undercarboxylated protein products might then reflect the in vivo order of carboxylation.

Three general models for the order of carboxylation of the substrate glutamyl residues in prothrombin can be outlined: (1) the carboxylation of the 10 glutamyl residues proceeds randomly, and all sites have equal affinity for the enzyme; (2) the carboxylation proceeds randomly, but some sites have a higher affinity for the enzyme; (3) the carboxylation proceeds sequentially in a definite order. The data presented in this paper show that the most amino-terminal Gla site in all of the variants studied was the most completely carboxylated. The distribution of Gla in human genetic variants (Borowski et al., 1986) also showed the first residue to be fully carboxylated. These data suggest that carboxylation of glutamyl residues is not random but occurs with preferential carboxylation of the first Glu residue. The data are also consistent with an increased impairment of carboxylation of residues more carboxy terminal. Although a sequential mechanism is suggested from the data obtained with the 60% Gla variant, a more random pattern was apparent with the 40% Gla variant. A more detailed understanding of the properties of the enzyme will be required to fully understand the mechanism of its multisite action.

Registry No. Gla, 53861-57-7; prothrombin, 9001-26-7.

REFERENCES

Allen, G. (1981) *Sequencing of Proteins and Peptides* (Work, T. S., & Burdon, R. H., Eds.) p 327, Elsevier/North-Holland Biomedical, Amsterdam.

Bajaj, P., Rapaport, S. I., & Prodanos, C. (1981) *Prep. Biochem. 11*, 397.

Bell, R. G., & Matschiner, J. T. (1972) *Nature (London) 237*, 32.

Bhown, A. S., Mole, J. E., Weissinger, A., & Bennett, J. C. (1978) *J. Chromatogr. 148*, 532.

Borowski, M., Furie, B. C., & Furie, B. (1986) *J. Biol. Chem. 261*, 1624.

Bovill, E. G., Malhotra, O. P., Nesheim, M. E., & Mann, K. G. (1986) in *Advances in Coagulation testing: Interpretation and Application* (Triplett, D. A., Ed.) p 397, College of American Pathologists, Skokie, IL.

Davie, E. W. (1987) in *Haemostasis and Thrombosis, Basic Principles and Clinical Practice* (Colman, R. W., Hirsh, J., Mardar, V. J., & Salzman, E. W., Eds.) p 242, Lippincott, Philadelphia, PA.

Fernlund, P., Stenflo, J., Roepstorff, P., & Thomsen, J. (1975) *J. Biol. Chem. 250*, 6125.

Foster, D. C., Rudinski, M. S., Schach, B. G., Berkner, K. L., Kumar, A. A., Hagen, F. S., Sprecher, C., Insley, M., & Davie, E. W. (1987) *Biochemistry 26*, 7003.

Friedman, P. A., Rosenberg, R. D., Hauschka, P. V., & Fitz-James, A. (1977) *Biochim. Biophys. Acta 494*, 271.

Ganrot, P. O., & Nilehn, J. E. (1986) *Scand. J. Clin. Lab. Invest. 22*, 23.

Goldsmith, G. H., Pence, R. E., Ratnoff, D. D., Adelstein, D. A., & Furie, B. (1982) *J. Clin. Invest. 69*, 1253.

Hauschka, P. V. (1979) *Biochemistry 18*, 4992.

Ingwall, J. S., & Scheraga, H. A. (1969) *Biochemistry 8*, 1860.

Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., & Furie, B. (1987) *Cell (Cambridge, Mass.) 48*, 185.

Josso, F., Lavergne, J. M., Gouault, M., Prou-Wartelle, O., & Soulier, J. P. (1968) *Thromb. Diath. Haemorrh. 20*, 88.

Knobloch, J. E., & Suttie, J. W. (1987) *J. Biol. Chem. 262*, 15334.

Kuwada, M., & Katayama, K. (1983) *Anal. Biochem. 131*, 173.

Lowry, O. H., Rosebrough, N. J., Farrand, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Malhotra, O. P. (1979a) *Thromb. Res. 15*, 427.

Malhotra, O. P. (1979b) *Thromb. Res. 15*, 439.

Malhotra, O. P. (1979c) *Thromb. Res. 15*, 449.

Malhotra, O. P. (1981) *Ann. N.Y. Acad. Sci. 370*, 426.

Malhotra, O. P. (1982) *Biochim. Biophys. Acta 702*, 185.

Malhotra, O. P., Nesheim, M. E., & Mann, K. G. (1985) *J. Biol. Chem. 260*, 279.

Mann, K. G., Elion, J., Butkowski, R. J., Downing, M., & Nesheim, M. E. (1981) *Methods Enzymol. 80*, 286.

Matschiner, J. T., Bell, R. G., Amelotti, J. M., & Knauer, T. E. (1970) *Biochim. Biophys. Acta 201*, 309.
Nelsestuen, G. L. (1984) *Methods Enzymol. 107*, 503.
Nelsestuen, G. L., & Suttie, J. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3366.
Poser, J. W., & Price, P. A. (1979) *J. Biol. Chem. 254*, 431.
Prowse, C. V., Mattock, P., Esnouf, M. P., & Russell, A. M. (1976) *Biochim. Biophys. Acta 432*, 265.
Shah, D. V., Swanson, J. C., & Suttie, J. W. (1984) *Thromb. Res. 35*, 451.
Stenflo, J. (1970) *Acta Chem. Scand. 24*, 3762.
Stenflo, J. (1974) *J. Biol. Chem. 249*, 5527.
Suttie, J. W. (1987) in *The New Dimensions of Warfarin Prophylaxis* (Wessler, S., Becker, C. G., & Nemerson, Y., Eds.) p 3, Plenum, New York.
Suttie, J. W. (1988) *BioFactors 1*, 55.
Suttie, J. W., & Jackson, C. M. (1977) *Physiol. Rev. 57*, 1.
Tarr, G. E. (1975) *Anal. Biochem. 63*, 361.
Tuhy, P. M., Bloom, J. W., & Mann, K. G. (1979) *Biochemistry 18*, 5842.
Walz, D. A., Hewett-Emmett, D., & Guillin, M.-C. (1986) in *Prothrombin and Other Vitamin K-Dependent Proteins* (Seegers, W. H., & Walz, D. A., Eds.) p 126, CRC, Boca Raton, FL.

Differential Accessibility of the Carbohydrate Moieties of $\bar{C}1s$–$\bar{C}1r$–$\bar{C}1r$–$\bar{C}1s$, the Catalytic Subunit of Human $\bar{C}1$†

Catherine A. Aude,‡ Monique B. Lacroix,‡ Gérard J. Arlaud,*,‡ Jean Gagnon,§,∥ and Maurice G. Colomb‡

Département de Recherches Fondamentales (Unité INSERM 238), Laboratoire d'Immunochimie, Centre d'Etudes Nucléaires de Grenoble, 85x, 38041 Grenoble Cédex, France, and Conseil National de Recherches du Canada, Institut de Recherches en Biotechnologie, 6100 Avenue Royalmount, Montréal, Québec, Canada H4P 2R2

Received May 13, 1988; Revised Manuscript Received July 6, 1988

ABSTRACT: The catalytic subunit of human $\bar{C}1$, $\bar{C}1s$–$\bar{C}1r$–$\bar{C}1r$–$\bar{C}1s$, is a Ca^{2+}-dependent tetrameric association of two serine proteases, $\bar{C}1r$ and $\bar{C}1s$, which are glycoproteins containing asparagine-linked carbohydrates. With a view to investigate the accessibility and the possible functional role of these carbohydrates, the isolated proteases and their Ca^{2+}-dependent complexes were submitted to deglycosylation by peptide:*N*-glycosidase F, an endoglycosidase that specifically hydrolyzes all classes of N-linked glycans. Treatment of isolated $\bar{C}1r$ and $\bar{C}1s$ led to the removal of the carbohydrate moieties attached to their N-terminal α region, whereas those located in the C-terminal γ-B catalytic domains were resistant to hydrolysis. Formation of the Ca^{2+}-dependent $\bar{C}1s$–$\bar{C}1s$ dimer and $\bar{C}1s$–$\bar{C}1r$–$\bar{C}1r$–$\bar{C}1s$ tetramer induced specific protection of the single carbohydrate attached to the α region of $\bar{C}1s$ and of one of the two carbohydrates located in the corresponding region of $\bar{C}1r$. Sequence studies indicated that the carbohydrates protected upon homologous ($\bar{C}1s$–$\bar{C}1s$) or heterologous ($\bar{C}1r$–$\bar{C}1s$) interactions are attached to asparagine residues 159 of $\bar{C}1s$ and 204 of $\bar{C}1r$, at the C-terminal end of the EGF-like domain of both proteases. These data bring further evidence that Ca^{2+}-dependent interactions between $\bar{C}1r$ and $\bar{C}1s$ are mediated by their N-terminal α regions and strongly suggest that, inside these regions, the EGF-like domains play an essential role in these interactions.

The first component of the classical complement pathway is a macromolecular protease assembled from C1q, the recognition unit, and from C1s–C1r–C1r–C1s, the catalytic unit, a Ca^{2+}-dependent tetrameric association of two serine proteases, C1r and C1s, that are sequentially activated upon C1 activation [reviewed by Cooper (1985), Arlaud et al. (1987a), and Schumaker et al. (1987)]. In both cases, activation occurs through cleavage of a single Arg–Ile bond (Arlaud et al., 1985; Spycher et al., 1986), converting the single-chain proenzymes (C1r, C1s) into active proteases ($\bar{C}1r$, $\bar{C}1s$)[1] comprising two disulfide-linked chains. Complete sequences of the N-terminal A chain and of the C-terminal B chain of each protein have been determined, indicating that the B chains are homologous to the catalytic chains of other mammalian serine proteases, whereas the A chains are subdivided into five structural units, referred to as I–V and including one EGF-like segment and two pairs of internal repeats (Arlaud & Gagnon, 1983; Journet & Tosi, 1986; Leytus et al., 1986; Arlaud et al., 1987b; Mackinnon et al., 1987; Tosi et al., 1987). Each monomeric protease is organized in at least two independent domains: a catalytic domain, which includes the B chain, associated to γ, the C-terminal portion of the A chain, and an interaction region α, derived from the N-terminal half of the A chain (Villiers et al., 1985; Weiss et al., 1986; Busby & Ingham, 1987). The former is considered a true globular domain (Arlaud et al., 1986), whereas the latter is more likely divided into a series of small subdomains responsible for specific interactions (Arlaud et al., 1987a; Tosi et al., 1987). These

† A preliminary report of this work was presented at the XIIth International Complement Workshop in Chamonix, France, September 1987.

* Author to whom correspondence should be addressed.
‡ Centre d'Etudes Nucléaires de Grenoble.
§ Institut de Recherches en Biotechnologie, Montréal.
∥ Present address: Département de Recherches Fondamentales, Laboratoire de Biologie Structurale, Centre d'Etudes Nucléaires de Grenoble, 85x, 38041 Grenoble Cédex, France.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; iPr_2P-F, diisopropyl phosphorofluoridate; PNGase F, peptide:*N*-glycosidase F; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-(L-tosylamino)butan-2-one; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGF, epidermal growth factor; the nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by a superscript bar, e.g., $\bar{C}1r$.

0006-2960/88/0427-8641$01.50/0 © 1988 American Chemical Society